Characterization of the Ipl1/Aurora protein kinase in chromosome segregation and the spindle checkpoint

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A dissertation submitted in partial fulfillment of the
requirements for the degree of

Doctor of Philosophy

University of Washington

2005

Program Authorized to Offer Degree:
Molecular and Cellular Biology

University of Washington
Graduate School

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Abstract

Characterization of the Ipl1/Aurora protein kinase in chromosome segregation and the spindle checkpoint

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The flawless execution of cell division is fundamental to the formation and survival of living organisms. It depends on the accurate and orderly partitioning of chromosomes, resulting in two daughter cells with the correct complement of genetic material. In my research, I sought to understand the role of the Ipl1/Aurora protein kinase in regulating the fidelity of chromosome segregation. This process requires that sister kinetochores bind spindle microtubules arising from opposite poles and come under tension. Furthermore, it requires the function of the spindle checkpoint, a monitoring system that delays cell cycle progression until all chromosomes make proper attachments. I describe here the identification and characterization of the Mtw1/Mis12 kinetochore complex, and show that impairing Ipl1/Aurora function in the *mtw1* kinetochore mutant both restores attachment and satisfies the spindle checkpoint. Analysis of a series of kinetochore mutants revealed similar results, suggesting a mechanism to explain how Ipl1/Aurora regulates chromosome

segregation. We propose that Ipl1/Aurora responds to the tension defects associated with most kinetochore mutants by destabilizing kinetochore-microtubule interactions and generating unattached kinetochores. These unoccupied microtubule-binding sites then lead to spindle checkpoint activation and eventual correction of the improper attachments. In addition, I examine the mitotic functions of the opposing phosphatase, Glc7/PP1. These studies indicate that Ipl1/Aurora and Glc7/PP1 work in parallel to ensure the accuracy of chromosome segregation by maintaining the balance of phosphorylation on a common set of important targets.

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ACKNOWLEDGEMENTS

I have been extremely fortunate to have Sue Biggins as my mentor. She allowed me the freedom to discover and learn on my own, yet always provided steady guidance and support when I needed it most. Thank you, Sue.

Significant contributions were made to this thesis by the Biggins Lab members Sean Tatsutani, Kim Collins, and Chitra Kotwaliwale, as well as our collaborators Charles Kung and Kevan Shokat.

I also wish to thank my family, and especially Thuy, for their unconditional love and support.

DEDICATION

In memory of Daniel Pinsky.

CHAPTER 1:

Introduction

Chromosome Segregation and the Spindle Checkpoint

The flawless execution of cell division is fundamental to the formation and survival of living organisms. It requires the accurate and orderly partitioning of chromosomes, resulting in two daughter cells with the correct complement of genetic material. Following genome replication, the replicated chromosomes (sister chromatids) are physically linked by cohesion. This allows the cell to identify the sister chromatids as chromosome copies and facilitates their orientation towards opposite sides of the cell. To pull the sister chromatids away from one another, the cell relies on the forces generated by the mitotic spindle. This bipolar array of microtubules is composed of dynamic tubulin polymers organized by a pair of organelles called spindle poles. The forces of the spindle are translated into chromosome movements primarily through the interaction of spindle microtubules with kinetochores, specialized protein complexes that assemble on centromeric DNA. In most organisms, a single kinetochore contains multiple microtubule binding sites that must interact with microtubules arising from the same spindle pole while its sister kinetochore binds microtubules originating from the opposite pole. At this point, a bipolar attachment is attained and the sister chromatids are poised for proper segregation.

Though bipolar kinetochore-microtubule interactions are essential for the high fidelity of chromosome segregation, the process of achieving proper attachment is complicated and somewhat random (Figure 1.1) (for reviews, see (Biggins and Walczak, 2003; Hauf and Watanabe, 2004)). Microtubules exhibit dynamic

instability, a property where polymerizing and depolymerizing microtubules interconvert and co-exist in the same population (Mitchison and Kirschner, 1984). Dynamic instability allows microtubules to probe for kinetochore attachments, stochastic events termed "search and capture" that occur during prometaphase. Initially, one kinetochore in a sister chromatid pair binds to the side of a probing spindle microtubule. This side-on attachment allows the rapid transport of the sister chromatids to the pole, where additional microtubules bind the captured kinetochore in an end-on fashion to create a microtubule fiber. The remaining unattached kinetochore then interacts with microtubules arising from the opposite pole, allowing the sister chromatids to eventually congress to the center of the cell. This characteristic alignment of the sister chromatids is defined as metaphase. Importantly, kinetochores that make bipolar attachments come under tension due to the forces generated by kinetochore microtubules that are opposed by the cohesion between sister chromatids. When all of the chromatid pairs make proper attachments and come under tension, the cell transitions into anaphase where cohesion is dissolved allowing the spindle to pull the sister chromatids to opposite poles.

The random nature of the attachment process does not always result in bipolar attachment and there are a number of kinetochore-microtubule arrangements that lead to chromosome missegregation if left uncorrected. The terminology describing these attachments accounts for both kinetochore orientation and the interacting microtubules' pole of origin (Figure 1.2). The bipolar, or bioriented, attachment described above where sister kinetochores face opposite poles and each

kinetochore binds only spindle microtubules emanating from the pole it is facing, is called an amphitelic attachment. Syntelic attachment indicates that both sister kinetochores face the same pole and attach to microtubules emanating from that pole. In contrast, monotelic attachment describes kinetochores facing opposite poles in which only one kinetochore in the pair is bound to microtubules. Though quite different, both syntelic and monotelic attachments are referred to in the literature as mono-oriented because they are bound to a single spindle pole. Finally, merotelic attachments occur when one (or both) sister kinetochores bind microtubules arising from both poles even though they orient toward opposite poles. Although merotelic attachments rarely cause chromosome missegregation because the kinetochores tend to make enough bipolar attachments to pull the sister chromatids to opposite poles (Cimini et al., 2004), chromosomes that make syntelic or monotelic attachments will be segregated improperly if left uncorrected. The resulting cells lack the normal chromosomal complement and this aneuploid state predisposes multicellular organisms towards the development of a variety of cancers and birth defects (for reviews, see (Hassold and Hunt, 2001; Rajagopalan and Lengauer, 2004)). Therefore, it is critical to determine how cells regulate the attachment process and correct improper attachments.

To ensure that segregation does not occur before all chromosomes are properly attached to the spindle, there exists a surveillance system called the spindle checkpoint that delays the metaphase to anaphase transition to allow the cell time to repair defective attachments (Figure 1.3) (for review, see (Lew and Burke, 2003)).

This signal transduction network, consisting of the Mad, Bub, and Mps1 proteins, prevents the premature segregation of improperly attached chromosomes by inhibiting the activity of the anaphase promoting complex (APC), a ubiquitin ligase that targets the anaphase inhibitor Pds1/Securin for destruction. Though both the lack of kinetochore-microtubule attachments and defects in the tension exerted by microtubule-generated forces on kinetochores result in activation of the spindle checkpoint, it is not clear whether these signals constitute separate pathways or are instead interdependent (for further discussion see Chapter 3). The identity of the primary signal recognized by the spindle checkpoint remains a basic, unresolved question in our understanding of checkpoint function.

The regulation and machinery of chromosome segregation are well conserved, making the genetically tractable budding yeast, *Saccharomyces cerevisiae*, a particularly powerful model organism for the study of kinetochore attachment and the spindle checkpoint. In addition, budding yeast have only a single microtubule-binding site on each kinetochore (Winey et al., 1995), thereby simplifying the analysis of kinetochore attachment. In this dissertation, I utilize the budding yeast model system to investigate the regulatory mechanisms that facilitate accurate chromosome segregation.

The Kinetochore

The budding yeast kinetochore is composed of a rapidly expanding list of proteins, many of which are conserved (Figure 1.4) (for review, see (Biggins and

Walczak, 2003)). The kinetochore is organized into distinct subcomplexes that have been placed into inner, central and outer domains based on two criteria: 1) *in vitro* centromere or microtubule binding activities and 2) *in vivo* dependency relationships for centromere association. The inner kinetochore CBF3 complex binds centromeric DNA and is required for the centromere association of all other kinetochore proteins (Lechner and Carbon, 1991). The inner kinetochore also contains Cse4/CENP-A, the conserved centromeric histone H3 variant, as well as the Cbf1 and Mif2/CENP-C proteins (Cai and Davis, 1990; Meluh and Koshland, 1995; Stoler et al., 1995). The central kinetochore includes the conserved Ndc80/HEC-1 complex (Cheeseman et al., 2002; Janke et al., 2001; McCleland et al., 2003; Wigge and Kilmartin, 2001), implicated in the stabilization of kinetochore-microtubule attachments, and the large Ctf19 complex (Cheeseman et al., 2002), which can be divided into at least three subcomplexes (Cheeseman et al., 2002; Measday et al., 2002; Ortiz et al., 1999; Pot et al., 2003). The outer kinetochore contains the Dam1/DASH/DDD complex (Cheeseman et al., 2002; Janke et al., 2002; Li et al., 2002), the only complex known to require microtubules for its kinetochore association, as well as several microtubule motors and non-motor microtubule-associated proteins. Though a large number of kinetochore components have been identified, the specific contributions of individual complexes to amphitelic attachment have not been described.

Ipl1/Aurora B protein kinase

A critical regulator of both kinetochore attachment and the spindle checkpoint is the Ipl1/Aurora B protein kinase, the catalytic component of the chromosomal passenger complex that also includes the inner centromere protein Sli15/INCENP, and Bir1/Survivin (for review, see (Vagnarelli and Earnshaw, 2004)) The characteristic localization pattern of the passenger complex; first associating with kinetochores, then moving onto the elongating spindle, and finally concentrating at the spindle midzone, appears to reflect Ipl1's kinetochore functions as well as its roles in spindle assembly and disassembly, anaphase chromosome condensation, and cytokinesis. Cells carrying temperature-sensitive, loss of function mutations in the *IPL1* gene undergo massive chromosome missegregation due to the formation of syntelic attachments (Biggins et al., 1999; Chan and Botstein, 1993; Tanaka et al., 2002). Despite the presence of improper attachments that should activate the spindle checkpoint, *ipl1* mutant cells proceed through the cell cycle without detectable spindle checkpoint delay (Biggins et al., 1999). Subsequent analysis indicated that Ipl1 function was required to activate the checkpoint in response to the conditional inhibition of either replication or sister chromatid cohesion, conditions that prevent the chromosome pairing necessary for bipolar force generation and kinetochore tension (Biggins and Murray, 2001). In contrast, the nearly complete loss of attachment induced by the microtubule depolymerizing drug nocodazole activated the checkpoint in an Ipl1-independent manner, suggesting a specific requirement for Ipl1 to allow the absence of tension to activate the spindle checkpoint. Experiments in many model systems are consistent with these yeast

experiments and suggest that Aurora B plays a conserved role ensuring amphitelic kinetochore attachments and mediating the spindle checkpoint response to tension defects (for review, see (Vagnarelli and Earnshaw, 2004)). However, the mechanisms by which Ipl1/Aurora regulates these processes are not well understood and it is not clear that the relevant substrates have been identified.

Glc7/PP1 protein phosphatase

Ipl1 activity is opposed by Glc7 (Francisco et al., 1994), the sole, essential protein phosphatase I (PP1) catalytic subunit in budding yeast (Cannon et al., 1994). Glc7 regulates numerous cellular processes including mitosis, meiosis, glycogen and sugar metabolism, transcription, translation, and mRNA processing through its interactions with specific regulatory subunits that target the phosphatase to appropriate substrates (for review, see (Ceulemans and Bollen, 2004)). Although the Glc7 mitotic regulatory subunit has not yet been identified, many *glc7* alleles cause cells to arrest in mitosis (Andrews and Stark, 2000; Baker et al., 1997; Black et al., 1995; Hisamoto et al., 1994; MacKelvie et al., 1995), suggesting that Glc7 substrates must be dephosphorylated to allow cell cycle progression. Furthermore, impairing Glc7 function suppresses the *ipl1* temperature sensitive growth defect and restores the phosphorylation of the Ipl1 target, histone H3, indicating that Glc7 likely has a specific role antagonizing Ipl1-mediated phosphorylation (Francisco et al., 1994; Hsu et al., 2000). Consistent with this, several *glc7* mutants have phenotypes that are the opposite of impaired Ipl1 function, including spindle checkpoint activation

and reduced kinetochore binding to microtubules *in vitro* (Bloecher and Tatchell, 1999; Sassoon et al., 1999). However, the precise relationship between the kinase and phosphatase is not known.

Description of Dissertation

In my research, I sought to understand how Ipl1 functions in chromosome segregation and the spindle checkpoint. It was proposed that Ipl1 regulates these processes by destabilizing kinetochore-microtubule interactions that do not generate tension (Tanaka et al., 2002). In Chapter 2, I detail the first evidence supporting this hypothesis, demonstrating that Ipl1 activity is required to detach microtubules from kinetochores defective in the newly identified Mtw1 kinetochore subcomplex. The identification of this complex and the phenotypic characterization of cells with *mtw1* mutant kinetochores are also described in Chapter 2. In Chapter 3 I introduce the debate over the hypothesis that defects in tension act as the primary spindle checkpoint signal and in Chapter 4, I address the role of Ipl1 in sensing tension defects and activating the checkpoint. In Chapters 5 and 6 I explore the relationship between Ipl1 and Glc7, and examine their regulation of Dam1 complex phosphorylation. Taken together, these studies suggest that Ipl1 both corrects attachment errors and regulates the spindle checkpoint by creating unattached kinetochores. This work provides the strongest evidence to date that the primary signal recognized by the spindle checkpoint is an unattached kinetochore. Furthermore, these experiments indicate that Ipl1 and Glc7 work in parallel to ensure

the fidelity of chromosome segregation by maintaining the balance of phosphorylation on a common set of important targets.

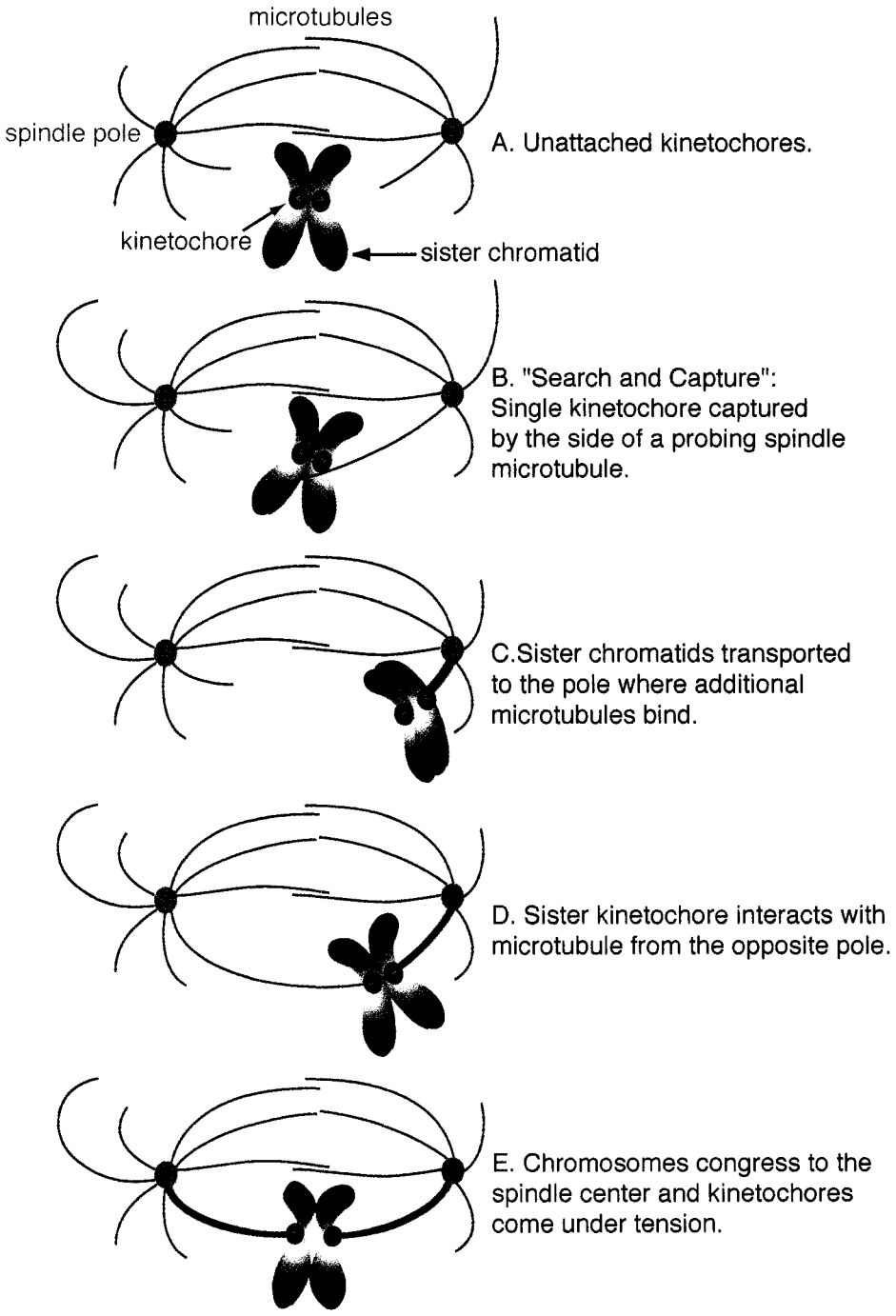


Figure 1.1 The process of making proper bipolar kinetochore-microtubule attachments proceeds through unattached and tensionless intermediates.

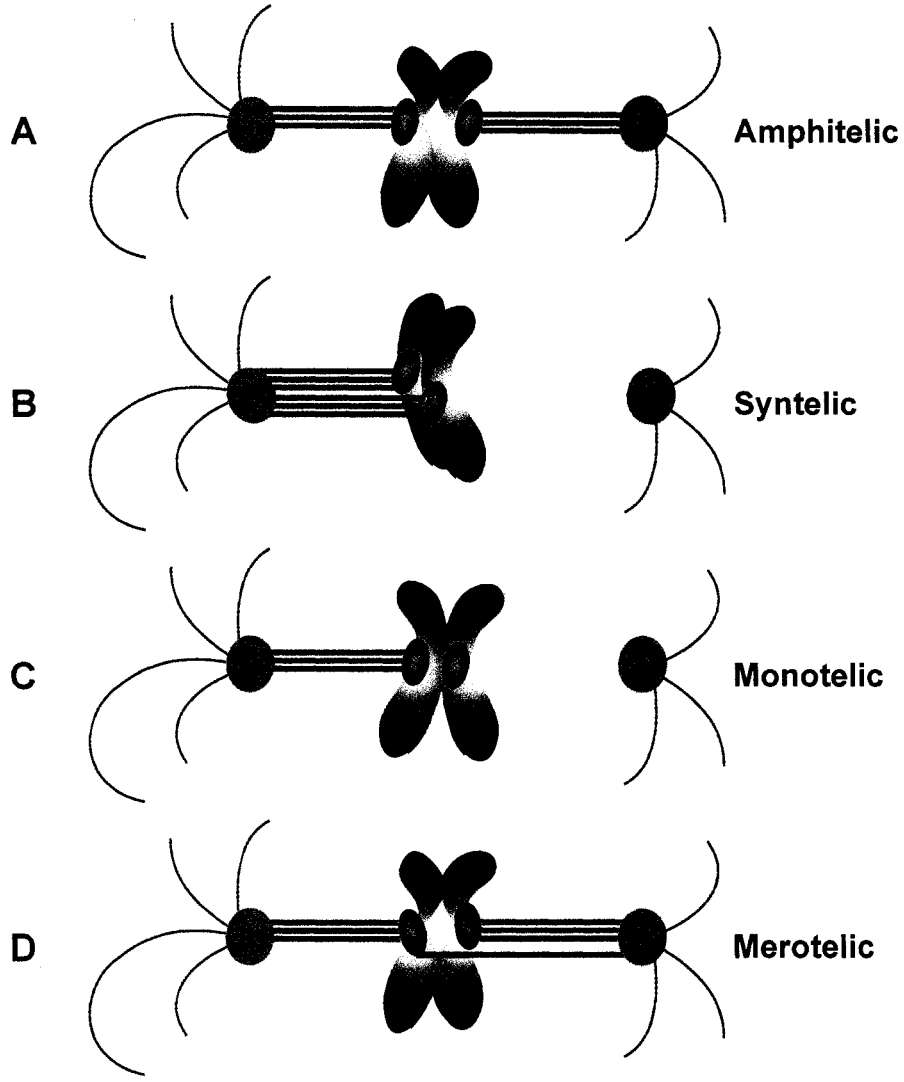


Figure 1.2 Types of kinetochore-microtubule attachments. (A) **Amphitelic:** A bipolar or bioriented attachment. Sister kinetochores face opposite poles and bind only microtubules arising from the adjacent pole. (B) **Syntelic:** Sister kinetochores face the same pole and attach to microtubules emanating from that pole. (C) **Monotelic:** Sister kinetochores face opposite poles but only one kinetochore binds microtubules leaving an unattached kinetochore. (D) **Merotelic:** Sister kinetochores face opposite poles but one (or both) kinetochore(s) interact with microtubules from both poles.

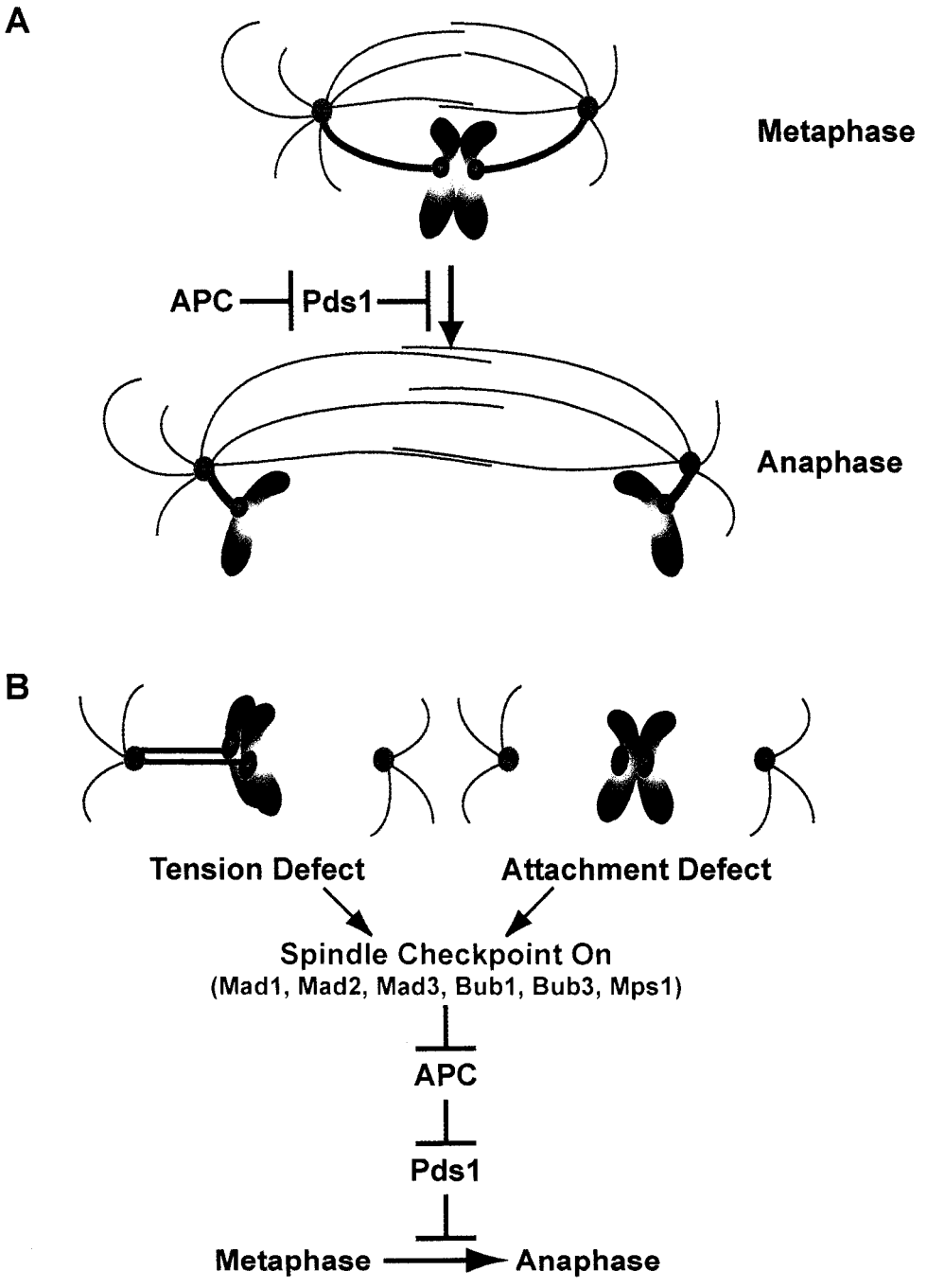


Figure 1.3 The spindle checkpoint monitors kinetochore-microtubule interactions and delays the metaphase to anaphase transition. (A) The Anaphase Promoting Complex (APC) regulates this transition by targeting the anaphase inhibitor Pds1 for destruction. (B) Activation of the spindle checkpoint by either attachment or tension defects inhibits the APC and delays anaphase onset.

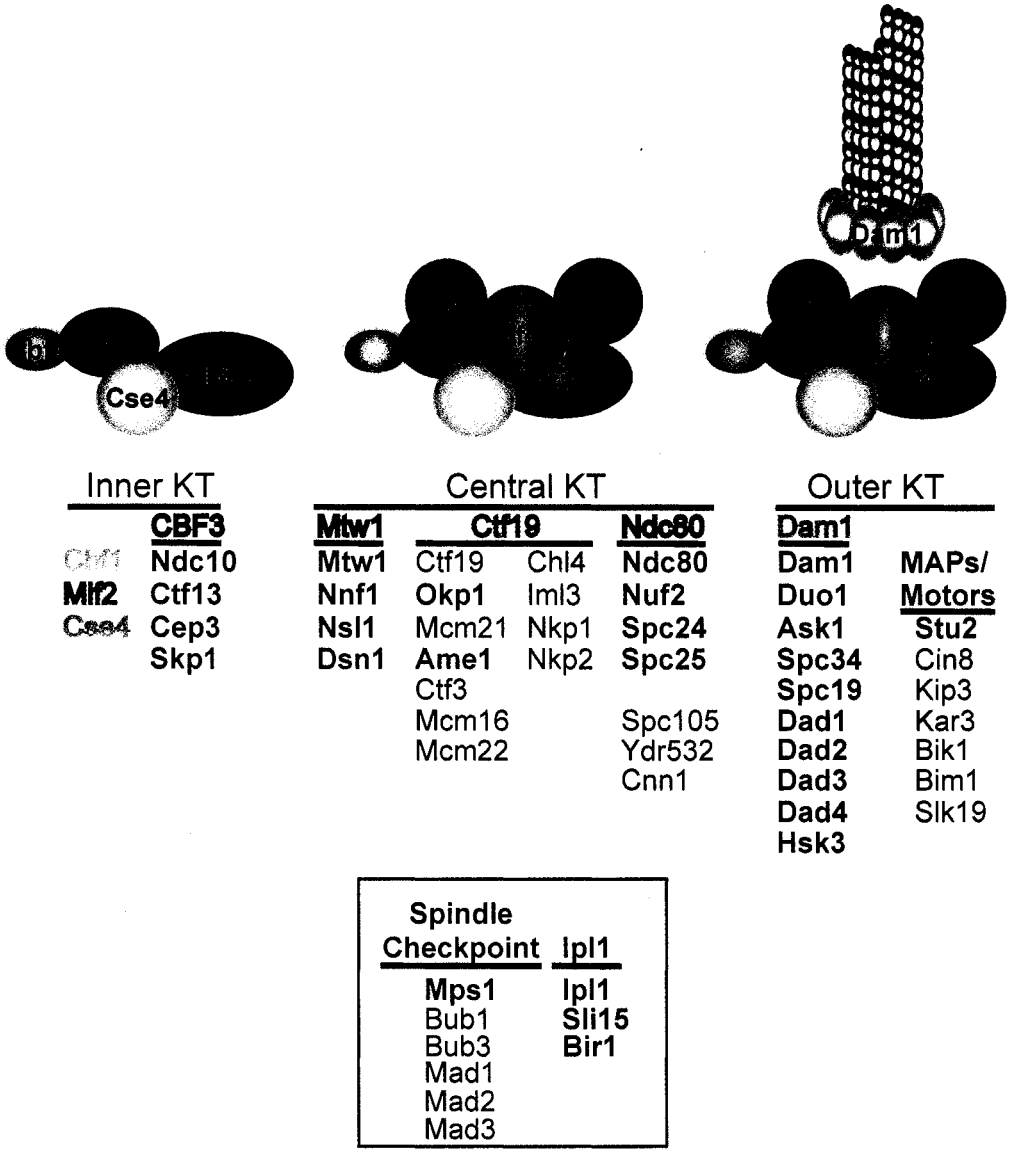


Figure 1.4 The budding yeast kinetochore (KT). The graphic represents the organization of kinetochore components into inner, central, and outer domains. The names of the kinetochore subcomplexes, as well as the Cbf1 and Mif2 proteins, are color coded to match the schematic. Listed below each named subcomplex are the proteins found in that subcomplex. Proteins encoded by essential genes are in bold. The microtubule associated proteins (MAPs) and microtubule motors are listed in the outer kinetochore section but are not depicted in the graphic. It is important to note that this schematic is based on *in vitro* centromere or microtubule binding activities and *in vivo* dependency relationships for centromere association. The true architecture of the budding yeast kinetochore awaits more sophisticated structural analyses and technological advances. The checkpoint proteins and the Ipl1 chromosomal passenger complex are shown in the box. These proteins are localized to kinetochores but it is not known whether they associate with a particular kinetochore domain.

CHAPTER 2:

An Mtw1 kinetochore complex promotes kinetochore biorientation that is monitored by the Ipl1/Aurora protein kinase

Summary

Chromosome segregation depends on kinetochore biorientation so that sister kinetochores attach to microtubules from opposite poles and come under tension. The budding yeast Ipl1/Aurora protein kinase allows the absence of tension to activate the spindle checkpoint. We found that checkpoint activation in the *mtw1-1* kinetochore mutant requires Ipl1, suggesting that Mtw1 promotes tension. We isolated *mtw1-1* dosage suppressors and identified Dsn1, a kinetochore protein that immunoprecipitates with the Mif2/CENP-C and Cse4/CENP-A proteins, as well as the Mtw1, Nnf1, and Nsl1 kinetochore proteins. *mtw1* and *dsn1* mutant strains exhibit similar phenotypes, suggesting that Mtw1 and Dsn1 act together. Although *mtw1* mutant cells contained unattached chromosomes, attachment was restored by impairing Ipl1 function. These results suggest that *mtw1* mutant kinetochores are competent to bind microtubules but Ipl1 generates unattached chromosomes. We therefore propose that an Mtw1 complex is required for kinetochore biorientation that is monitored by the Ipl1 kinase.

Introduction

The faithful transmission of genetic material relies upon the connection between chromosomes and the mitotic spindle. This essential interaction is mediated by the kinetochore, a specialized protein structure that assembles on centromeric DNA and facilitates the capture of spindle microtubules that emanate from opposite spindle poles. This bipolar attachment requires that sister kinetochores are physically bioriented. Tension is generated when microtubules bound to bioriented kinetochores exert poleward directed forces that are resisted by the physical linkage (cohesion) between sister chromatids. Once each chromatid pair achieves biorientation, the cell transitions into anaphase, allowing sister chromatids to rapidly segregate to opposite poles.

The interaction between kinetochores and microtubules is monitored by the spindle checkpoint, a conserved signal transduction system that prevents sister chromatid separation until biorientation is achieved (for review, see (Musacchio and Hardwick, 2002)). Defects in kinetochore-microtubule interactions result in checkpoint-mediated inhibition of the anaphase-promoting complex (APC), a multiprotein ubiquitin ligase that catalyzes the proteolysis of the anaphase inhibitor Pds1. Although the primary defect that signals the checkpoint is not known, lack of microtubule attachment to kinetochores or the tension that microtubule-generated forces exert on the kinetochore activates the spindle checkpoint. In budding yeast, the conserved kinetochore proteins Ipl1/Aurora and Skp1 differentiate between these two activators of the checkpoint (Biggins and Murray, 2001; Kitagawa et al., 2003).

Although the Ipl1/Aurora protein kinase is required to activate the spindle checkpoint in the absence of tension resulting from defects in DNA replication or sister chromatid cohesion, the requirement for Ipl1 in checkpoint arrests induced by kinetochore defects has not yet been explored.

Our understanding of kinetochore function has been greatly aided by studies in the budding yeast where over 65 kinetochore proteins have been identified, many of which are conserved (for review, see (Biggins and Walczak, 2003)). The kinetochore is organized into distinct subcomplexes that have been placed into inner, central and outer domains based on two criteria: 1) *in vitro* centromere or microtubule binding activities and 2) *in vivo* dependency relationships for centromere association. The inner kinetochore CBF3 complex binds centromeric DNA and is required for the centromere association of all other kinetochore proteins (Lechner and Carbon, 1991). The inner kinetochore also contains Cse4/CENP-A, the conserved centromeric histone H3 variant, as well as the Cbf1 and Mif2/CENP-C proteins (Cai and Davis, 1990; Meluh and Koshland, 1995; Stoler et al., 1995). The central kinetochore includes the conserved Ndc80/HEC-1 complex (Cheeseman et al., 2002; Janke et al., 2001; McCleland et al., 2003; Wigge and Kilmartin, 2001), implicated in the stabilization of kinetochore-microtubule attachments, and the large Ctf19 complex (Cheeseman et al., 2002), which can be divided into at least three subcomplexes (Cheeseman et al., 2002; Measday et al., 2002; Ortiz et al., 1999; Pot et al., 2003). The outer kinetochore contains the Dam1/DASH/DDD complex (Cheeseman et al., 2002; Janke et al., 2002; Li et al., 2002), the only one known to

require microtubules for its kinetochore association, as well as several microtubule motors and non-motor microtubule-associated proteins.

Although a number of kinetochore proteins have been identified, little is known about how their assembly and activity is regulated. The Ipl1/Aurora protein kinase is a key regulator of kinetochore function because it is required to generate bioriented kinetochore-microtubule attachments (Biggins et al., 1999; Tanaka et al., 2002). In *ipl1* mutant cells, kinetochores are competent to bind to microtubules, but 85% of the time sister chromatids are segregated to the same spindle pole. A similar increase in mono-oriented attachments is observed in cultured vertebrate cells when Aurora B function is compromised using inhibitory antibodies (Kallio et al., 2002) or small-molecule inhibitors (Ditchfield et al., 2003; Hauf et al., 2003), suggesting conservation of function. Because the biased segregation of chromosomes to the bud in *ipl1* mutants was eliminated by the transient disruption of microtubules, it was proposed that Ipl1 promotes the turnover of mono-oriented kinetochore-microtubule interactions (Tanaka et al., 2002). A crucial *in vivo* target of Ipl1 is the Dam1/DASH/DDD complex (Cheeseman et al., 2002). The phenotypes of a *dam1* mutant thought to mimic constitutive Ipl1 phosphorylation are consistent with this complex acting as a microtubule release factor.

It is not known whether there are kinetochore proteins in addition to Ipl1 and Dam1 that are required for biorientation. The essential Mtw1 (mis twelve like) protein, which copurifies with the largely non-essential Ctf19 complex (Cheeseman et al., 2002), is a particularly intriguing candidate for a mediator of kinetochore

biorientation. In *mtw1* mutants and cells depleted of Mtw1 protein, DNA segregates into two unequal masses and sister centromeres frequently lose biorientation (Goshima and Yanagida, 2000), suggesting that kinetochore-microtubule attachments are made but not properly regulated. Mutations in the fission yeast *mis12* result in sister chromatids that are initially pulled toward the same pole indicating mono-oriented attachments (Goshima et al., 1999). Furthermore, when protein levels of the human homologue, hMis12, are reduced by siRNA in cell culture, chromosomes are misaligned in a manner consistent with biorientation defects (Goshima et al., 2003). *MTW1 (DSN3, NSL2)* was also identified along with the essential genes *DSN1* (dosage suppressor of *nnf1-17)* and *NSL1* (*nnf1-17* synthetic lethal) based on genetic interactions with *NNF1* (necessary for nuclear function), an essential gene with a role in chromosome segregation (Euskirchen, 2002; Shan et al., 1997). The genetic interactions and similar localization patterns suggested that these proteins might physically interact at the kinetochore.

We show here that Mtw1 does associate with Dsn1, Nnf1 and Nsl1, as well as the Mif2 and Cse4 inner kinetochore proteins. Defects in Mtw1 require Ipl1 function to activate the spindle checkpoint, suggesting that Mtw1 is required for kinetochore tension. Mutations in *MTW1* and *DSN1* have similar phenotypes leading to spindle checkpoint activation with chromosomes unattached to the spindle. Strikingly, microtubule attachments to the spindle are completely restored in *mtw1* mutants when Ipl1 function is absent, suggesting that Mtw1 is required to generate kinetochore biorientation but not microtubule attachments. Taken together, these

data demonstrate that the Ipl1 kinase detects improper kinetochore-microtubule attachments and is required to generate unattached chromosomes in response to these defects.

Results

Spindle checkpoint activation in the mtw1-1 mutant requires Ipl1

Kinetochore components involved in generating and/or maintaining tension but not microtubule attachment should require Ipl1 for checkpoint activation. One mutant of particular interest was *mtw1-1* because its phenotype was consistent with a defect in biorientation. We first confirmed that the metaphase delay in the *mtw1-1* mutant is due to the spindle checkpoint by analyzing Pds1 protein levels over the cell cycle. Because the spindle checkpoint inhibits the APC, checkpoint activation leads to the stabilization of Pds1. Although Pds1 levels cycled in wild type cells, they were stabilized in *mtw1-1* mutant cells (Figure 2.1). Pds1 stabilization in the *mtw1-1* cells is due to the spindle checkpoint because *mtw1-1 mad2Δ* cells that lack checkpoint function no longer stabilized Pds1. In *mtw1-1 ipl1-321* cells, Pds1 levels cycled similar to wild type indicating that Ipl1 is required to engage the spindle checkpoint in response to defects in Mtw1 function. These results suggest that *mtw1-1* mutants are defective in tension.

Dsn1 is a kinetochore protein that suppresses mtw1-1

To identify additional genes that act with *MTW1*, we isolated *mtw1-1* dosage suppressors (Figure 2.2A). One of the suppressing plasmids contained the *DSN1* gene. At the restrictive temperature, high-copy *DSN1* restored *mtw1-1* growth to wild type levels. In addition, low-copy *DSN1* partially suppressed *mtw1-1* growth defects, indicating that *DSN1* is a strong suppressor of the *mtw1-1* mutant.

The strong genetic interaction between *DSN1* and *MTW1* suggested that Dsn1 might also be a kinetochore protein. We therefore monitored the association of Dsn1 protein with centromeric DNA by chromatin immunoprecipitation (ChIP) in wild type and *ndc10-1* mutant cells defective in CBF3 function. Dsn1 was immunoprecipitated from chromatin extracts and co-precipitating centromere III (CEN III) sequence was amplified by multiplex PCR in a reaction also containing control primers to a sequence 4 kb upstream (IIIL). In wild type cells, the CEN III signal was enriched over the background control (Figure 2.2B), confirming that Dsn1 is a centromere-associated protein. This association requires functional kinetochores because *ndc10-1* cells grown at the restrictive temperature showed a marked reduction in CEN III signal. We also localized an endogenous COOH-terminal fusion of Dsn1 to GFP by microscopy and found that it localized to kinetochores, consistent with previous data (data not shown and (Euskirchen, 2002)). Taken together, these data demonstrate that Dsn1 is a kinetochore protein.

Dsn1 immunoprecipitates with Mtw1, Nnf1, Nsl1, and the inner kinetochore proteins Mif2 and Cse4

We next tested whether the Dsn1 and Mtw1 kinetochore proteins associate by immunoprecipitation experiments. We generated strains co-expressing endogenous COOH-terminal fusions of Dsn1-HA3 and Mtw1-myc13 and found that Mtw1 immunoprecipitated Dsn1 (Figure 2.3A). In addition, Dsn1 immunoprecipitates contained Mtw1 (data not shown). Due to the genetic interactions between the

DSN1, MTW1, *NNF1,* and *NSL1* genes, we next tested whether Nnf1 and Nsl1 also bound to Dsn1. We found that both Nnf1 and Nsl1 immunoprecipitate Dsn1 to the same high level as Mtw1 (Figure 2.3A). In addition, Nnf1 and Nsl1 immunoprecipitate Mtw1 (data not shown). Therefore, Dsn1 and Mtw1 associate with each other, as well as Nnf1 and Nsl1.

Because Mtw1 had been shown by mass spectrometry to copurify with the central kinetochore Ctf19 complex, we examined the association of Mtw1 and Dsn1 with Ctf19. Using strains co-expressing either Dsn1-HA3 or Mtw1-HA3, and Ctf19-myc13, we found that Ctf19 immunoprecipitated low levels of Dsn1 and Mtw1 (Figure 2.3B and data not shown). This substoichiometric interaction with Dsn1 was also seen in experiments with other members of the Ctf19 complex, the Ctf3 and Chl4 proteins (data not shown). In contrast, higher levels of Dsn1 immunoprecipitated with a comparable amount of Mtw1. These experiments indicate that Mtw1 and Dsn1 are more commonly associated with each other than with the Ctf19 complex.

To further determine the context of physical interactions that define the position of Dsn1 and Mtw1 in the kinetochore, we carried out a series of immunoprecipitation assays using strains with Dsn1-HA3 or Mtw1-HA3 and test kinetochore proteins tagged with the myc13 epitope. We analyzed interactions with Ctf13, Ndc80, and Dam1, components of the inner CBF3 complex, the central Ndc80p complex, and the outer Dam1/DASH/DDD complex, respectively. In addition, we evaluated the interactions with the inner kinetochore protein Mif2.

Dsn1 and Mtw1 did not immunoprecipitate with Ctf13, Dam1, or Ndc80 suggesting that they do not associate with any of these known complexes (Figure 2.3C and data not shown). However, Mif2 interacted with Dsn1 and Mtw1, thereby linking Dsn1 and Mtw1 to the inner kinetochore.

This link to the inner kinetochore suggested that there might also be interactions with Cse4. To test this, we immunoprecipitated Dsn-myc13 and immunoblotted with anti-Cse4 antibody (Figure 2.4A). In a similar manner, we analyzed the interactions between Cse4 and Mtw1, Nsl1, Nnf1, and Mif2. All of these proteins immunoprecipitated Cse4, although the strongest interaction detected was between Mif2 and Cse4 (Figure 2.4A). Consistent with a previous report (Measday et al., 2002), we did not detect Cse4 association with Ctf19, indicating that the interactions described here are specific (data not shown).

Dsn1 centromere association requires Cse4/CENP-A

To determine the requirements for Dsn1 kinetochore assembly, we utilized ChIP to monitor the centromere association of Dsn1 in a series of temperature sensitive mutants representing the various kinetochore subcomplexes. Wild type, *dam1-11*, *ndc80-1, ipl1-321, mtw1-1, mif2-3*, and *cse4-323* cells were subjected to Dsn1 ChIP analysis (Figure 2.4B). Dsn1 was centromere associated in all strains except *cse4-323* at the restrictive temperature, indicating that Cse4 is required for Dsn1 kinetochore assembly.

mtw1-1 mutants missegregate the spindle and DNA when the spindle checkpoint is active

To further investigate the function of Mtw1 and Dsn1 in chromosome segregation, we generated temperature sensitive *dsn1* mutants and analyzed them with the previously identified *mtw1-1* mutant. Wild type cells and *mtw1-1* and *dsn1-1* mutant cells were arrested in G1 with α-factor and then released at the restrictive temperature and monitored for budding index and DNA segregation. Consistent with our *mtw1-1* spindle checkpoint analysis, *dsn1-1* activated the checkpoint (data not shown). Despite spindle checkpoint activation, greater than 40% of the *mtw1-1* and 69% of the *dsn1-1* large budded cells segregated their DNA into two masses, consistent with previous studies on *mtw1-1* (Figure 2.5A and (Goshima and Yanagida, 2000)). Since the spindle checkpoint inhibits DNA segregation by preventing the loss of cohesion, the *mtw1-1* and *dsn1-1* mutant phenotypes could be due to a defect in the linkage between sister chromatids. We therefore analyzed whether sister chromatids precociously separated during the metaphase arrest in the *mtw1-1* and *dsn1-1* mutant cells. To visualize sister chromatids, a GFP-lactose repressor fusion protein (GFP-lacI) was expressed in strains containing an array of lactose operators (lacO) integrated on the arm of chromosome IV. We found that only 5% of the chromosome IV sister chromatids had separated into two discrete dots in the *mtw1-1* mutant large budded cells compared to 93% in wild type cells 140 minutes after release from G1. The *dsn1-1* mutant cells revealed similar phenotypes, indicating that the mutants are not defective in sister chromatid cohesion.

Because sister chromatids remain linked in the *mtw1-1* and *dsn1-1* mutant cells, we considered the possibility that the aberrant DNA segregation was due to defective kinetochores. In wild type cells, sister chromatid cohesion opposes the pulling forces of the mitotic spindle, keeping the metaphase spindle short (Waters et al., 1996). However, kinetochore defects can lead to premature spindle elongation and DNA segregation in the presence of Pds1. Consistent with a kinetochore defect, *mtw1-1 apc* mutant cells held in metaphase by preventing Pds1 proteolysis have partially elongated spindles (Goshima and Yanagida, 2000). To determine whether this expansion of the metaphase spindle could account for the observed missegregation, we examined spindles in *mtw1-1* cells expressing GFP-Tub1. 140 minutes after release from G1 at the restrictive temperature, the majority of large budded *mtw1-1* cells had short spindles (92%) compared to only 6% of wild type cells. Therefore, inappropriate spindle elongation cannot explain the apparent DNA segregation in the *mtw1-1* mutant cells. However, we observed a striking spindle phenotype that could account for the *mtw1-1* and *dsn1-1* mutant phenotypes. We analyzed *mtw1-1* large budded cells with segregated DNA masses and found that 79% had a short metaphase spindle that had migrated into the bud (Figure 2.5B). This phenotype has never been reported for checkpoint-arrested cells that typically maintain the short spindle in the mother cell at the bud neck. Consistent with this spindle defect, the *mtw1-1* and *dsn1-1* mutant cells exhibited abnormally long cytoplasmic microtubules that sometimes stretched around the cell (data not shown). Similar astral microtubule phenotypes were previously reported in *nnf1-17* mutant

cells (Shan et al., 1997), providing further evidence that these proteins function in a complex. Although the short spindle was often misaligned, there were not apparent morphological defects in the nuclear microtubules (see Figures 2.5B and 2.6A).

mtw1-1 mutants contain chromosomes that are not attached to the spindle

The short spindle missegregation into the bud suggested a potential mechanism that could generate the asymmetric DNA segregation phenotype in the *mtw1* and *dsn1* mutant cells. DNA attached to the spindle in the mutant cells should segregate into the bud, while any unattached DNA would remain in the mother cell. To test this possibility, we co-localized spindles and fluorescently marked sister chromatids in the *mtw1-1* mutant cells. Wild type and *mtw1-1* mutant cells containing GFP-Tub1 and fluorescently marked chromosome IV were arrested in G1 and released to the restrictive temperature. Cells were analyzed 120 min after release for the distribution of the spindle and sister chromatids. When sister chromatids co-localized on the same axis with tubulin, we assumed that the chromosome was attached to the spindle (see Figure 2.6A). We characterized a sister chromatid as unattached to the spindle when it was at least 0.65 microns away from the spindle axis. Although this underestimates the percentage of unattached sister chromatids, this distance ensures that the chromosomal position of the lactose operators does not affect our analysis. We found that chromosome IV was unattached in 56% of the *mtw1-1* cells. We also analyzed fluorescently marked chromosomes III and VII in *mtw1-1* mutants with GFP-tubulin. In contrast to

chromosome IV, we found that chromosomes III and VII were not attached 13% and 17% of the time, respectively. Although we cannot account for the chromosomal difference in attachment, these data do explain why the bud often contains a significant fraction of DNA.

To confirm our hypothesis about the origin of *mtw1-1* mutant phenotypes, we performed live cell imaging of *mtw1-1* mutant cells containing GFP-tubulin and fluorescently marked chromosome IV. Because *mtw1-1* cells arrest in metaphase, we used a *cdc23-1* mutant that arrests in metaphase due to defects in Pds1 proteolysis as a control. Cells were released to the restrictive temperature and time-lapse images were captured every 30s. In control metaphase arrested cells, we found that the short spindle quickly became properly positioned at the bud neck where it wobbled slightly during the arrest (Figure 2.6B). We were unable to visualize chromosome IV in these cells, presumably because it is on the spindle axis and therefore obscured by tubulin fluorescence. When we analyzed the *mtw1-1* mutant cells, we found that the short spindles rotated 180° and moved entirely into and then out of the bud. In addition, chromosome IV was clearly unattached from the spindle in many frames (for example, see frame 33'). Taken together, this data is consistent with our fixed cell imaging and supports our proposal that the *mtw1* mutants appear to have segregated their DNA asymmetrically because the short spindle inappropriately migrates into the bud leaving unattached DNA in the mother cell.

Ipl1 activity generates unattached chromosomes in mtw1-1 mutant cells

The analysis of chromosome attachment to the spindle suggested that *mtw1-1* mutant cells are defective in making microtubule attachments. However, this was not consistent with our observation that the *mtw1-1* spindle checkpoint arrest requires Ipl1 function, which implicated Mtw1 in generating kinetochore tension. We therefore considered the possibility that Ipl1 activity promoted microtubule detachment in the *mtw1-1* mutant cells. If this were true, removing Ipl1 function in *mtw1-1* mutants should restore attachment. To analyze this, we co-localized spindles and fluorescently marked sister chromatids in the *mtw1-1 ipl1-321* mutant cells. Because the *mtw1-1 ipl1-321* mutants do not arrest in metaphase, we compared *mtw1-1 mad2Δ* mutant cells, which also fail to arrest, as well as the *mtw1-1* mutant. Wild type, *mtw1-1, mtw1-1 mad2Δ, ipl1-321,* and *mtw1-1 ipl1-321* mutant cells containing GFP-Tub1 and fluorescently marked chromosome IV were arrested in G1 and released to the restrictive temperature. We analyzed chromosome IV because it is the chromosome most often left in the mother cell in the *mtw1-1* mutants. 80 min after release, cells containing short spindles were analyzed. 29% of the chromosomes were unattached in *mtw1-1* mutant cells compared to less than 2% in the wild type, *ipl1-321,* and *mtw1-1 ipl1-321* cells (Figure 2.7A). Similar to the *mtw1-1* cells, 25% of the chromosomes were unattached in the *mtw1-1 mad2Δ* cells. The percentage of unattached chromosomes in both the *mtw1-1* and *mtw1-1 mad2Δ* mutant cells was lower than the *mtw1-1* mutant described in Figure 2.6 because the chromosomes do not have as much time to diffuse away from the

spindle. Therefore, we also analyzed chromosome segregation to the bud as a second assay for microtubule attachment since chromosomes can get to the daughter cell only by their attachment to the spindle. Wild type, *ipl1-321*, *mtw1-1 mad2Δ*, and *mtw1-1 ipl1-321* mutant cells were arrested in G1, released to the restrictive temperature, and monitored for the distribution of chromosome IV. 120 min after release, both copies of chromosome IV segregated to the bud in only 1% of wild type cells and 9% of the *mtw1-1 mad2Δ* mutant cells. Consistent with previous results (Tanaka et al., 2002), both copies of chromosome IV segregated to the daughter cell 40% of the time in *ipl1-321* mutant cells. Strikingly, chromosome segregation in the *mtw1-1 ipl1-321* double mutant cells was similar to the *ipl1-321* mutant cells: 45% of the time chromosome IV segregated to the bud (Figure 2.7B). We observed comparable results in the *mtw1-1 ipl1-321 mad2Δ* triple mutant (data not shown). Therefore, *mtw1-1* mutant kinetochores are competent to bind to microtubules, but the presence of Ipl1 activity generates unattached kinetochores. Taken together, these data suggest that one function of Mtw1 is to generate kinetochore biorientation that is sensed by the Ipl1 kinase.

Discussion

The biorientation of sister kinetochores ensures the capture of microtubules arising from opposite poles of the mitotic spindle and is fundamental to accurate segregation. We show here that the Mtw1 and Dsn1 proteins physically interact in an inner kinetochore complex. *mtw1-1* mutant cells activate the spindle checkpoint in an *IPL1* dependent manner, suggesting they are defective in achieving biorientation. In support of this, the generation of unattached chromosomes in *mtw1* mutants requires Ipl1 function. We therefore propose that the Mtw1 complex promotes sister kinetochore biorientation that is monitored by the Ipl1 kinase.

Protein Interactions at the Budding Yeast Inner Kinetochore.

We show here that Mtw1 physically associates with three previously unidentified kinetochore proteins, Dsn1, Nnf1 and Nsl1, as well as the Mif2 and Cse4 inner kinetochore proteins. These interactions place Mtw1 and the uncharacterized kinetochore proteins at the inner kinetochore proximal to the centromeric DNA. In multicellular eukaryotes, the Mtw1 homologue hMis12 co-localizes to the inner kinetochore plate with Mif2/CENP-C and Cse4/CENP-A (Goshima et al., 2003), suggesting that these interactions might be conserved. In addition, we found that the association of Mtw1 and Dsn1 with centromeric DNA depends on Cse4. This is in contrast to the CENP-A independent kinetochore localization of fission yeast and human Mis12 (Goshima et al., 2003; Takahashi et al., 2000) and we currently do not understand the basis for these differences.

Previously, Mtw1 was co-purified as a component of the central kinetochore Ctf19 complex (Cheeseman et al., 2002). We found that Mtw1 was able to immunoprecipitate more Dsn1 protein than Ctf19, suggesting that Dsn1 and Mtw1 may not be stoichiometric components of the Ctf19 complex. In addition, Ctf19 association with centromeric DNA is independent of Mtw1 function (data not shown), suggesting that Mtw1 does not recruit Ctf19 to the kinetochore. Mtw1 has also been implicated as an outer kinetochore protein because it was isolated in a highly enriched preparation of the spindle pole body (SPB) that also contained components of the peripheral Ndc80 and Dam1/DASH/DDD complexes (Wigge and Kilmartin, 2001). However, we were unable to detect association of Mtw1 with components of these complexes. Therefore, Mtw1 may function in multiple cellular contexts, or these co-purifications might represent an additional complexity in kinetochore structure.

We identified *DSN1* as a dosage suppressor of the *mtw1-1* mutant and showed that Dsn1, Nnf1 and Nsl1 are kinetochore proteins that interact to equal levels with one another and Mtw1 as previously proposed (Euskirchen, 2002). Though these proteins are conserved in a variety of fungi, we have not yet identified homologues in multicellular eukaryotes. Because mutant alleles of these genes have similar phenotypes and exhibit strong genetic interactions, we believe that Mtw1, Dsn1, Nnf1, and Nsl1 constitute a kinetochore subcomplex. Cse4 and Mif2 also immunoprecipitate with these proteins, suggesting they might be additional components of this putative subcomplex.

mtw1 mutants are defective in chromosome segregation and spindle position

The spindle checkpoint inhibits sister chromatid separation until cells achieve biorientation. Most defects in kinetochore function that activate the spindle checkpoint arrest cells in metaphase with a short spindle and a single DNA mass positioned at the bud neck. Because it was previously reported that *mtw1-1* mutant cells missegregate their DNA in two unequal masses (Goshima and Yanagida, 2000), it was unclear whether the spindle checkpoint was activated. However, we found that this missegregation occurs in the presence of an active spindle checkpoint. In contrast, *S. pombe mis12* mutants and hMis12 knockdown cells do not activate the spindle checkpoint despite severe defects in chromosome segregation that include lagging chromosomes in the knockdown cells (Goshima et al., 2003; Goshima et al., 1999). Because lagging chromosomes can result from biorientation defects that lead to kinetochores binding to microtubules from opposite poles (merotelic attachments), these data are consistent with Mis12 having a role in biorientation. In addition, merotelic attachments do not engage the spindle checkpoint (Cimini et al., 2002; Cimini et al., 2001), possibly because tension is generated when kinetochores inappropriately attach to microtubules from both poles. However, budding yeast kinetochores cannot make merotelic attachments because there is only one microtubule-binding site per kinetochore. Therefore, budding yeast may activate the spindle checkpoint in response to biorientation defects because tension cannot be generated.

We show that the DNA missegregation in the *mtw1-1* mutant is not due to either premature sister chromatid separation or spindle elongation. Rather, the short spindles in *mtw1-1* mutant cells often migrate into the bud, therefore moving all attached DNA into the bud. Because the mispositioned spindle leaves behind some unattached DNA, the cell appears to have attempted chromosome segregation. Two lines of evidence indicate that intact chromosomes and not chromosomal fragments are left in the mother cell. First, the *mtw1-1* metaphase arrest is alleviated by deletion of the spindle checkpoint, indicating that defects in Mtw1 function do not activate DNA damage checkpoints. Second, the attachment defect is completely suppressed by inactivation of Ipl1, a result that would not be possible if there were chromosomal breaks.

The spindle migration defect observed in the *mtw1-1* mutant has not been reported for other spindle checkpoint arrested cells and suggests that Mtw1 function contributes to SPB asymmetry or proper interactions between the astral microtubules and the cell cortex. It is important to note that both *mtw1-1* and *nnf1-17* mutants are also defective in spindle orientation, as the short spindles are often perpendicular to the mother-bud axis. Future work will be needed to determine whether these phenotypes are a consequence of defects in Mtw1's kinetochore function or instead reflect independent roles for the Mtw1 complex in spindle positioning. Because the budding yeast undergoes a closed mitosis, it will also be critical to evaluate the state of the nuclear envelope in *mtw1-1* mutant cells.

mtw1-1 mutants uncover chromosomal differences in segregation fidelity

In our analysis of the *mtw1-1* mutant, we found that the defect in attachment to the spindle consistently varied among chromosomes. Though this data explains why the bud often contains a significant fraction of DNA, it is unclear why these particular chromosomes differentially attach to the spindle. The differences could be due to variation in the ability of chromosomes to achieve biorientation, to signal defects in kinetochore tension, or to be detached by an Ipl1-mediated correction mechanism. One determinant might be chromosome size. We found that chromosome IV, the second largest budding yeast chromosome (1532 kb), was unattached more often than the smaller chromosomes III and VII. However, chromosome III (317 kb) was unattached nearly as often as chromosome VII (1091 kb), indicating that size is not the only contributing feature. Factors such as the position of the centromere or the distribution of centromeric cohesion might also result in chromosomal differences. In the future, it will be important to analyze additional chromosomes in both wild type and *mtw1-1* mutant cells to determine the mechanistic basis for these differences.

Mtw1 is required for kinetochore biorientation

We propose that Mtw1 is required for the physical tension that results from biorienting sister kinetochores, consistent with previous data. Although we were able to detect unattached chromosomes in the *mtw1-1* mutant, we provide two lines of evidence consistent with this being due to a biorientation defect that leads to Ipl1-

mediated release of microtubule attachments. First, the checkpoint arrest induced by the *mtw1-1* mutant requires Ipl1 function. Because Ipl1 is required to activate the spindle checkpoint in response to defects in kinetochore tension but not attachment, this suggests that *mtw1-1* mutant kinetochores are not under sufficient tension and that Ipl1 does directly monitor the state of kinetochores. This result is not necessarily specific to *mtw1-1* and future work will be required to determine if defects in other kinetochore components also require Ipl1 to engage the spindle checkpoint. We believe that this tension deficiency is a direct consequence of defects in Mtw1 function and not a failure in kinetochore assembly because kinetochore proteins we have tested remain centromere associated in *mtw1-1* mutant cells (data not shown). In addition, Mtw1 is required to maintain biorientation that is established at metaphase (Goshima and Yanagida, 2000). Second, we found that removing Ipl1 function in *mtw1-1* mutants restored the attachment of sister chromatids to the spindle. Because both chromosome attachment and segregation in *mtw1-1 ipl1-321* cells was identical to the *ipl1-321* cells, the *mtw1-1* attachment defect is completely suppressed by loss of Ipl1 function. It is therefore unlikely that the elimination of Ipl1 function simply strengthens weak kinetochore-microtubule interactions. In addition, this data strongly argues that *mtw1-1* mutants have kinetochores that are competent to attach to microtubules. In support of this, the lack of a cell cycle delay when Mis12 is depleted by siRNA in human cells or mutated in fission yeast is consistent with microtubules attaching to kinetochores, thus satisfying the spindle checkpoint (Goshima et al., 2003; Goshima et al., 1999). We

therefore favor the interpretation that *mtw1-1* mutant kinetochores bind to microtubules but are defective in biorientation so that Ipl1 generates unattached kinetochores.

There are several ways that Mtw1 could facilitate the biorientation of sister kinetochores. First, Mtw1 might contribute to the rigidity of the kinetochore and sterically inhibit mono-orientation. The placement of Mtw1 at the inner kinetochore due to interactions with the Mif2 and Cse4 proteins might reflect a role for centromeric chromatin structure in mediating proper kinetochore orientation. However, if Mtw1 facilitated sister kinetochore orientation, *mtw1-1 ipl1-321* double mutants would likely be more defective for biorientation than either single mutant. Since the double mutant phenotype is the same as the *ipl1-321* phenotype, it suggests that they act in the same pathway to ensure biorientation. Mtw1 cannot be required to signal Ipl1 that there is a tension defect because Ipl1 can detach chromosomes in the absence of Mtw1 function. Therefore, one possibility is that Mtw1 acts downstream of Ipl1 to stabilize bioriented attachments. For example, Mtw1 could convert lateral microtubule attachments into end on attachments that are more stable. Alternatively, Mtw1 might be important for generating tension on kinetochores that have already made bipolar attachments.

The data presented here suggest that Mtw1 is involved in biorientation and not in microtubule attachment. In addition, this work directly demonstrates that Ipl1 promotes the turnover of improper kinetochore-microtubule interactions as previously proposed (Tanaka et al., 2002). We have provided evidence that Mtw1 is

part of an inner kinetochore complex, and in the future it will be important to elucidate the mechanism that this complex uses to promote biorientation. We are actively pursuing the molecular basis for the difference between *mtw1* and *mtw1 ipl1* mutant kinetochores to understand how Ipl1 detects and responds to defects in Mtw1 function.

Experimental Procedures

Microbial Techniques. Media and microbial techniques were essentially as described (Rose et al., 1990; Sherman et al., 1974). All experiments in which cells were released from a G1 arrest were carried out by α-factor arrest and release as described (Biggins and Murray, 2001).

Yeast strain construction. Yeast strains are listed in Table 2.1 and were constructed by standard genetic techniques. All strains are isogenic with the W303 background and were generated for this study. Plasmids are indicated in parentheses. The *ndc10-1* (Goh and Kilmartin, 1993), *cse4-323* (Biggins et al., 2001), *mif2-3* (Brown et al., 1993), *ndc80-1* (Wigge et al., 1998), *dam1-11* (Cheeseman et al., 2001b), and *ipl1-321* (Biggins et al., 1999) alleles were crossed to make strains for this study. Strains containing *TUB1-GFP:URA3* were obtained by integrating plasmid pMAS27 (a gift from Marion Shonn, Tufts University, Boston, MA) digested with *StuI* at the *URA3* locus. Deletions in yeast genes, as well as the HA3 and myc13 epitope tags were made using a PCR-based integration system (Longtine et al., 1998). Specific primer sequences are available on request. Deletions and epitope tags were confirmed by PCR. All fusion proteins are fully functional except SBY2480 that is temperature sensitive.

Plasmid Construction. The *MTW1* genomic region was isolated by digesting pSB507 with *KpnI-BglII* and ligating the 2182 bp fragment into the *KpnI-BamHI*

sites of pRS306 (Sikorski and Hieter, 1989) to create pSB512. The *DSN1* gene was PCR amplified from pSB654 using primers SB569 and SB570. These primers engineer *EcoRI* and *XhoI* sites 500bp upstream and downstream of the *DSN1* gene, respectively. The resulting PCR product was digested with *EcoRI-XhoI* and ligated into the *EcoRI-XhoI* sites of pRS316, pRS314, and pRS326 (Sikorski and Hieter, 1989), to create pSB624, pSB631, and pSB630, respectively. To create a *dsn1-1* integrating plasmid, pSB655 was digested with *EcoRI-XhoI* and ligated into the *EcoRI-XhoI* sites of pRS306 to create pSB656. The GST-Cse4p clone was made by PCR amplification of the *CSE4* gene using primers SB248 and SB247 that have *EcoRI* and *BamHI* restriction sites engineered, respectively. The PCR product was digested with *EcoRI* and *BamHI* and ligated into the same sites in the pGEX-2T vector (Qiagen, Chatsworth, CA).

mtw1-1 dosage suppressor screen. pSB512 was used as a template for site-directed mutagenesis (Stratagene) with primers SB396 and SB397 to create pSB651 containing the *mtw1-1* mutation described previously (Goshima and Yanagida, 2000). The *mtw1-1* strain (SBY1646) was transformed with a high-copy *URA3*-marked genomic yeast library, plated on selective media at the permissive temperature (23 °C) for three days, and then replica printed to the restrictive temperature (37 °C) for 1 day. 40 temperature-resistant colonies were identified and subjected to plasmid rescue and retransformation. The 35 remaining positives were placed in 8 groups based on restriction mapping and a representative from each

group was sequenced to determine candidate suppressors. 6 groups (32 positives) contained the *MTW1* genomic region while 2 groups (3 positives) encoded the *DSN1* genomic region.

Generation of the temperature sensitive dsn1-1 allele. The *DSN1* locus was mutagenized by error-prone PCR as described (Castillo et al., 2002) using primers SB569 and SB570. The mutagenized PCR products were used to gap-repair *HincII-XcmI* digested pSB631 in the haploid *dsn1* deletion strain that was kept alive by plasmid pSB624 (SBY2318). The transformants were subjected to plasmid shuffle and subsequently tested for temperature sensitivity. 17 temperature sensitive alleles were isolated and only the *dsn1-1* allele was characterized in this study. pSB656 was digested with *Eco47III* to integrate *dsn1-1* upstream of the endogenous *DSN1* locus in the *DSN1/dsn1Δ* heterozygous diploid strain SBY2520 that was sporulated to generate the haploid *dsn1-1* strain SBY2582.

Microscopy. Analysis of sister chromatids and GFP-Tub1 in fixed cells was performed as described (Biggins et al., 1999). DAPI was obtained from molecular probes (Eugene, OR) and used at 1μg/ml final concentration. At least 100 cells were analyzed for all reported experiments. Live microscopy was performed as described (Buvelot et al., 2003).

Protein and immunological techniques. Protein extracts were made and immunoblotted as described (Minshull et al., 1996). 9E10 antibodies that recognize the myc tag and 12CA5 antibodies that recognize the hemagglutinin (HA) tag were obtained from Covance and used at a 1:10,000 dilution. To generate anti-Cse4 antibodies, denatured GST-Cse4 was injected into rabbits at R and R Research and Development (Stanwood, WA). The GST-Cse4 protein was purified as described (Kellogg et al., 1995). The antibodies were affinity purified by coupling GST-Cse4 to SulfoLink Coupling gel (Pierce Chemical Co.).

Immunoprecipitation Assays. 50 ml cultures of mid-log cells were collected and lysates were prepared as described (Buvelot et al., 2003). 400 ml supernatant was incubated with 5 ml protein G dynabeads (Dynal Biotech, Inc.) and 5 ml of A-14 anti-myc antibody (Santa Cruz Biotechnology) for 2 hrs at 4 °C. The beads were washed 5x with 500 ml lysis buffer and the immunoprecipitates were separated by SDS-PAGE and immunoblotted as above.

Chromatin Immunoprecipitation. ChIP was as described (Aparicio et al., 1997). The immunoprecipitations used A-14 anti-myc antibody (Santa Cruz Biotechnology). To detect centromeric sequences, DNA was analyzed by multiplex PCR using a primer pair specific for CENIII (SB399, SB400) and a control pair specific for a region 4kb to the left (SB225, SB226). PCR reactions were carried out for 28 cycles as described (Aparicio et al., 1997).

Table 2.1 *Yeast strains used in this study*

Strain	Genotype
SBY817	*MAT**a** ura3-1:GFP-TUB1:URA3 leu2,3-112 his3-11:pCUP1-GFP12-lacI12:HIS3 trp1-1:lacO:TRP1 lys2Δ ade2-1 can1-100 bar1Δ cdc23-1*
SBY818	*MAT**a** ura3-1 leu2,3-112 his3-11:pCUP1-GFP12-lacI12:HIS3 trp1-1:lacO:TRP1 lys2Δ ade2-1 can1-100 bar1Δ PDS1-myc18:LEU2*
SBY819	*MAT**a** ura3-1 leu2,3-112 his3-11:pCUP1-GFP12-lacI12:HIS3 trp1-1:lacO:TRP1 lys2Δ ade2-1 can1-100 bar1Δ PDS1-myc18:LEU2 ipl1-321*
SBY1575	*MAT**a** ura3-1 leu2,3-112 his3-11:pCUP1-GFP12-lacI12:HIS3 trp1-1:lacO:TRP1 lys2Δ ade2-1 can1-100 bar1Δ mtw1-1*
SBY1646	*MAT**a** ura3-1 leu2,3-112 his3-11:pCUP1-GFP12-lacI12:HIS3 trp1-1:lacO:TRP1 lys2Δ ade2-1 can1-100 bar1Δ PDS1-myc18:LEU2 mtw1-1*
SBY1713	*MAT**a** ura3-1 leu2,3-112 his3-11:pCUP1-GFP12-lacI12:HIS3 trp1-1:lacO:TRP1 ade2-1 can1-100 bar1Δ PDS1-myc18:LEU2 MAD2::URA3 mtw1-1*
SBY1724	*MAT**a** ura3-1 leu2,3-112 his3-11:pCUP1-GFP12-lacI12:HIS3 trp1-1:lacO:TRP1 lys2Δ ade2-1 can1-100 bar1Δ PDS1-myc18:LEU2 ipl1-321 mtw1-1*
SBY1998	*MAT**a** ura3-1:GFP-TUB1:URA3 leu2,3-112 his3-11:pCUP1-GFP12-lacI12:HIS3 trp1-1:lacO:TRP1 lys2Δ ade2-1 can1-100 bar1Δ PDS1-myc18:LEU2 ipl1-321*
SBY2153	*MAT**a** ura3-1 leu2,3-112 his3-11 trp1-1 ade2-1 can1-100 bar1Δ DSN1-HA3:HIS3*
SBY2155	*MAT**a** ura3-1 leu2,3-112 his3-11 trp1-1 ade2-1 can1-100 bar1Δ DSN1-GFP:HIS3*
SBY2191	*MAT**a** ura3-1 leu2,3-112 his3-11 trp1-1 ade2-1 can1-100 bar1Δ DSN1-HA3:HIS3 MTW1-myc13:KAN*
SBY2192	*MAT**a** ura3-1 leu2,3-112 his3-11 trp1-1 ade2-1 can1-100 bar1Δ DSN1-HA3:HIS3 CTF19-myc13:KAN*
SBY2223	*MAT**a** ura3-1 leu2,3-112 his3-11 trp1-1 ade2-1 can1-100 bar1Δ DSN1-GFP:HIS3 ndc10-1*
SBY2318	*MAT**a** ura3-1 leu2,3-112 his3-11 trp1-1 lys2Δ ade2-1 can1-100 bar1Δ dsn1::KAN (pSB624, CEN DSN1, URA3)*
SBY2337	*MAT**a** ura3-1 leu2,3-112 his3-11:pCUP1-GFP12-lacI12:HIS3 trp1-1:lacO:TRP1 lys2Δ ade2-1 can1-100 bar1Δ PDS1-myc18:LEU2 mtw1-1 (pRS316, 2μ, URA3)*
SBY2339	*MAT**a** ura3-1 leu2,3-112 his3-11:pCUP1-GFP12-lacI12:HIS3 trp1-1:lacO:TRP1 lys2Δ ade2-1 can1-100 bar1Δ PDS1-myc18:LEU2 mtw1-1(pSB630, 2μ DSN1, URA3)*
SBY2340	*MAT**a** ura3-1 leu2,3-112 his3-11:pCUP1-GFP12-lacI12:HIS3 trp1-1:lacO:TRP1 lys2Δ ade2-1 can1-100 bar1Δ PDS1-myc18:LEU2 mtw1-1(pSB624, CEN DSN1, URA3)*

Table 2.1 *continued*

Strain	Genotype
SBY2341	*MAT**a** ura3-1 leu2,3-112 his3-11:pCUP1-GFP12-lacI12:HIS3 trp1-1:lacO:TRP1 lys2Δ ade2-1 can1-100 bar1Δ PDS1-myc18:LEU2 (pRS316, 2μ, URA3)*
SBY2350	*MAT**a** ura3-1:GFP-TUB1:URA3 leu2,3-112 his3-11:pCUP1-GFP12-lacI12:HIS3 trp1-1:lacO:TRP1 lys2Δ ade2-1 can1-100 bar1Δ mtw1-1*
SBY2359	*MAT**a** ura3-1 leu2,3-112 his3-11 trp1-1 ade2-1 can1-100 bar1Δ DSN1-myc13:HIS3*
SBY2360	*MAT**a** ura3-1 leu2,3-112 his3-11 trp1-1 ade2-1 can1-100 bar1Δ DSN1-myc13:HIS3 ndc10-1*
SBY2361	*MAT**a** ura3-1 leu2,3-112 his3-11 trp1-1 ade2-1 can1-100 bar1Δ DSN1-myc13:HIS3 dam1-11::KAN*
SBY2362	*MAT**a** ura3-1 leu2,3-112 his3-11 trp1-1 ade2-1 can1-100 bar1Δ DSN1-myc13:HIS3 ndc80-1*
SBY2367	*MAT**a** ura3-1 leu2,3-112 his3-11 trp1-1 ade2-1 can1-100 bar1Δ DSN1-myc13:HIS3 mtw1-1*
SBY2370	*MAT**a** ura3-1:GFP-TUB1:URA3 leu2,3-112 his3-11:pCUP1-GFP12-lacI12:HIS3 trp1-1 lys2Δ ade2-1 can1-100 bar1Δ ChrIIICEN:lacO:TRP1*
SBY2371	*MAT**a** ura3-1:GFP-TUB1:URA3 leu2,3-112 his3-11:pCUP1-GFP12-lacI12:HIS3 trp1-1 lys2Δ ade2-1 can1-100 bar1Δ ChrIIICEN:lacO:TRP1 mtw1-1*
SBY2466	*MAT**a** ura3-1:GFP-TUB1:URA3 leu2,3-112 his3-11:pCUP1-GFP12-lacI12:HIS3 trp1-1:lacO:TRP1 lys2Δ ade2-1 can1-100 bar1Δ PDS1-myc18:LEU2 mtw1-1 ipl1-321*
SBY2467	*MAT**a** ura3-1:GFP-TUB1:URA3 leu2,3-112 his3-11:pCUP1-GFP12-lacI12:HIS3 trp1-1:lacO:TRP1 lys2Δ:pGAL-Δ176-CLB2:LYS2 ade2-1 can1-100 bar1Δ*
SBY2469	*MAT**a** ura3-1 leu2,3-112 his3-11 trp1-1 ade2-1 can1-100 bar1Δ DSN1-myc13:HIS3 cse4-323*
SBY2477	*MAT**a** ura3-1 leu2,3-112 his3-11 trp1-1 ade2-1 can1-100 bar1Δ DSN1-HA3:HIS3 DAM1-myc9:TRP1*
SBY2478	*MAT**a** ura3-1 leu2,3-112 his3-11 trp1-1 ade2-1 can1-100 bar1Δ DSN1-HA3:HIS3 MIF2-myc13:KAN*
SBY2480	*MAT**a** ura3-1 leu2,3-112 his3-11 trp1-1 ade2-1 can1-100 bar1Δ DSN1-HA3:HIS3 CTF13-myc13:KAN*
SBY2501	*MAT**a** ura3-1 leu2,3-112 his3-11 trp1-1 ade2-1 can1-100 bar1Δ DSN1-myc13:HIS3 mif2-3*
SBY2513	*MAT**a** ura3-1 leu2,3-112 his3-11 trp1-1 ade2-1 can1-100 bar1Δ DSN1-HA3:HIS3 NSL1-myc13:KAN*
SBY2517	*MAT**a** ura3-1 leu2,3-112 his3-11 trp1-1 ade2-1 can1-100 bar1Δ DSN1-HA3:HIS3 NNF1-myc13:KAN*

Table 2.1 *continued*

Strain	Genotype
SBY2520	*MAT**a**/MATa ura3-1/ ura3-1 leu2,3-112/ leu2,3-112 his3-11/his3-11:pCUP1-GFP12-lacI12:HIS3 trp1-1/trp1-1:lacO:TRP1 lys2Δ/LYS2 ade2-1/ade2-1 can1-100/can1-100 bar1Δ/bar1Δ PDS1/PDS1-myc18:LEU2 dsn1::KAN/DSN1*
SBY2534	*MAT**a** ura3-1:GFP-TUB1:URA3 leu2,3-112 his3-11:pCUP1-GFP12-lacI12:HIS3 trp1-1 lys2Δ ade2-1 can1-100 bar1Δ ChrIIICEN:lacO:TRP1 mtw1-1*
SBY2535	*MAT**a** ura3-1 leu2,3-112:GFP-TUB1:LEU2 his3-11 trp1-1 ade2-1 can1-100 bar1Δ DSN1-myc13:HIS ipl1-321*
SBY2539	*MAT**a** ura3-1:GFP-TUB1:URA3 leu2,3-112 his3-11:pCUP1-GFP12-lacI12:HIS3 trp1-1 lys2Δ ade2-1 can1-100 bar1Δ ChrVIICEN:lacO:TRP1 mtw1-1*
SBY2582	*MAT**a** ura3-1 leu2,3-112 his3-11:pCUP1-GFP12-lacI12:HIS3 trp1-1:lacO:TRP1 ade2-1 can1-100 bar1Δ PDS1-myc18:LEU2 dsn1::KAN:dsn1-1:URA3*
SBY2611	*MAT**a** ura3-1:GFP-TUB1:URA3 leu2,3-112 his3-11:pCUP1-GFP12-lacI12:HIS3 trp1-1:lacO:TRP1 lys2Δ:pGAL-Δ176-CLB2:LYS2 MAD2::URA3 ade2-1 can1-100 bar1Δ PDS1-myc18:LEU2 mtw1-1*

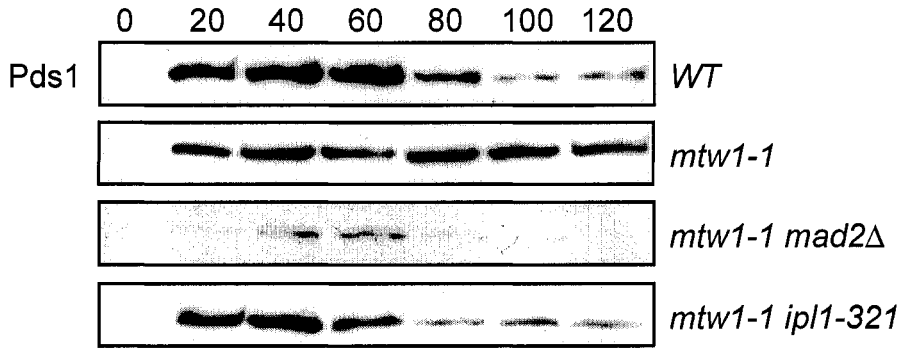


Figure 2.1 *mtw1-1* mutants activate the spindle checkpoint in an *IPL1*-dependent manner. Wild type (SBY818), *mtw1-1* (SBY1646), *mtw1-1 mad2*Δ (SBY1713), and *mtw1-1 ipl1-321* (SBY1724) cells containing Pds1-myc18 were arrested in G1 and released to 37 °C. Lysates were prepared at the indicated time points (min) and immunoblotted with anti-myc antibodies.

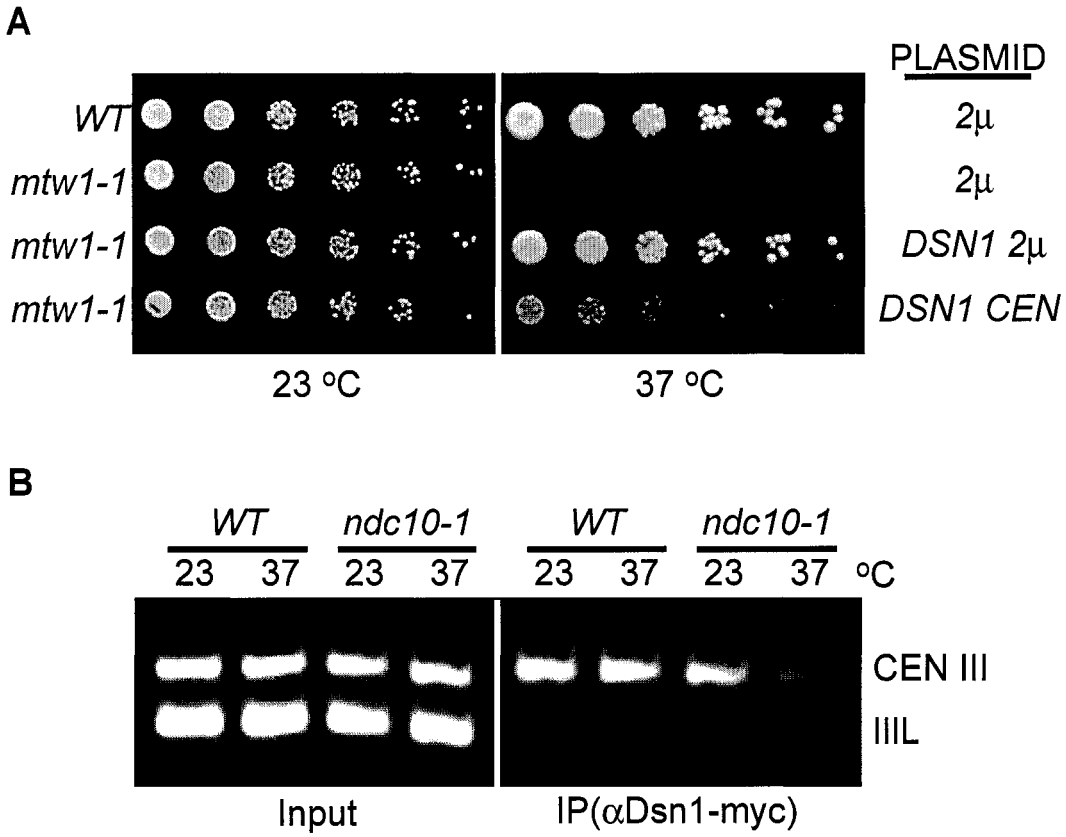


Figure 2.2 Dsn1 is a kinetochore protein that can suppress the *mtw1-1* mutant. (A) *DSN1* is a dosage suppressor of *mtw1-1*. Five-fold serial dilutions of wild type cells with 2μ (SBY2341) or *mtw1-1* cells with 2μ (SBY2337), *DSN1* 2μ (SBY2339) and *DSN1* CEN (SBY2340) were plated at 23 °C and 37 °C. (B) Dsn1 associates with centromeric DNA in an Ndc10-dependent manner. Wild type (SBY2359) and *ndc10-1* (SBY2360) cells expressing Dsn1-myc13 were incubated at either 23 °C or 37 °C for 3 hrs and analyzed by ChIP.

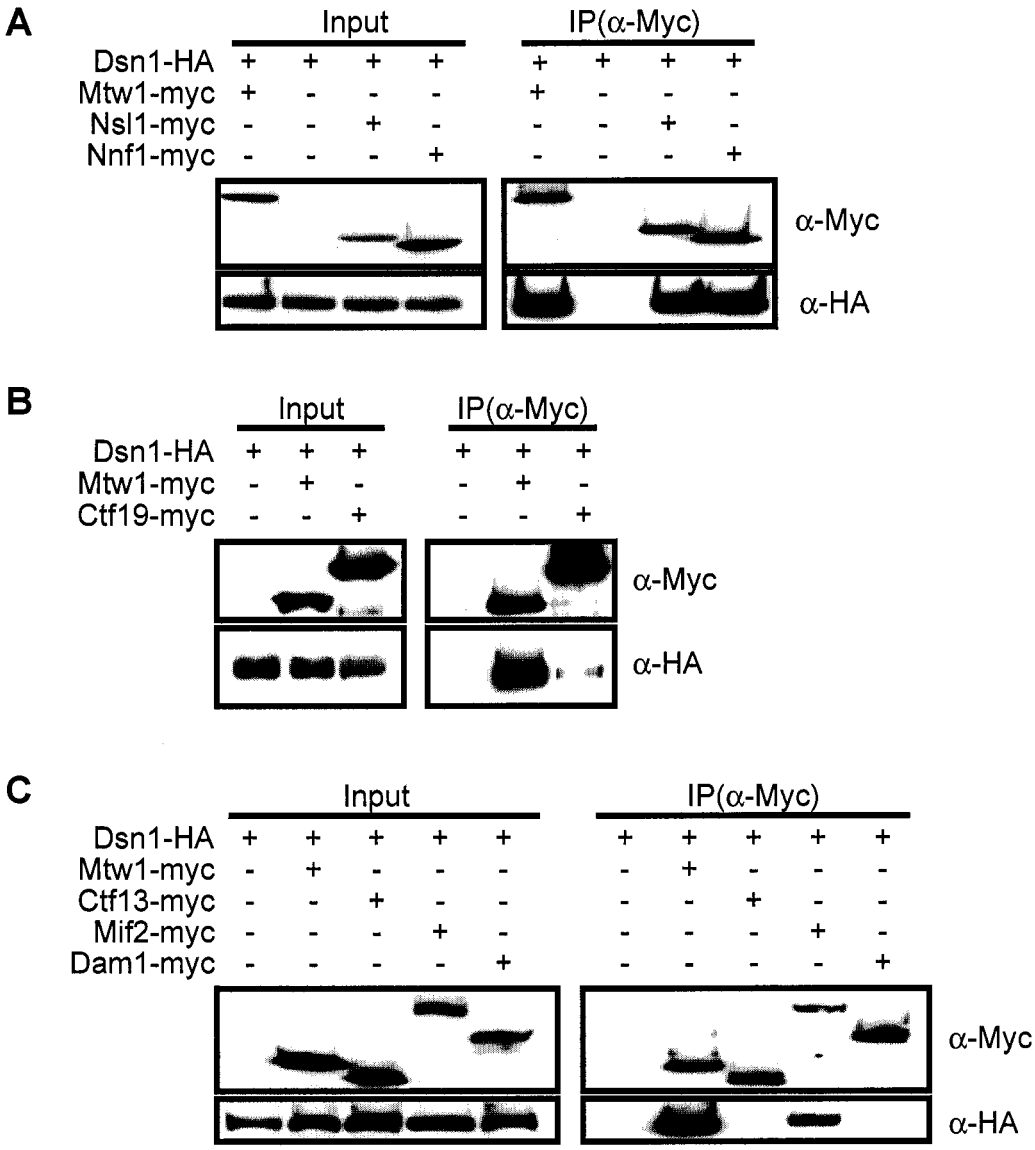


Figure 2.3 Dsn1 physically associates with the Mtw1, Nsl1, Nnf1, and Mif2 proteins. (A) Dsn1 immunoprecipitates with Mtw1, Nsl1 and Nnf1. Extracts from cells expressing Dsn1-HA3 alone (SBY2153) or in combination with Mtw1-myc13 (SBY2191), Nsl1-myc13 (SBY2513), or Nnf1-myc13 (SBY2517) were immunoprecipitated with anti-myc antibody. Extracts (Input) and immunoprecipitates (IP) were analyzed by anti-HA and anti-myc immunoblotting. (B) The association of Dsn1 with Ctf19 is not stoichiometric. Extracts from cells expressing Dsn1-HA3 alone (SBY2153) and in combination with either Mtw1-myc13 (SBY2191) or Ctf19-myc13 (SBY2192) were immunoprecipitated as above. (C) Dsn1 immunoprecipitates with Mif2 but not Ctf13 or Dam1. Extracts from cells expressing Dsn1-HA3 alone (SBY2153) or in combination with Mtw1-myc13 (SBY2191), Ctf13-myc13 (SBY2480), Mif2-myc13 (SBY2478), or Dam1-myc9 (SBY2477) were immunoprecipitated as above.

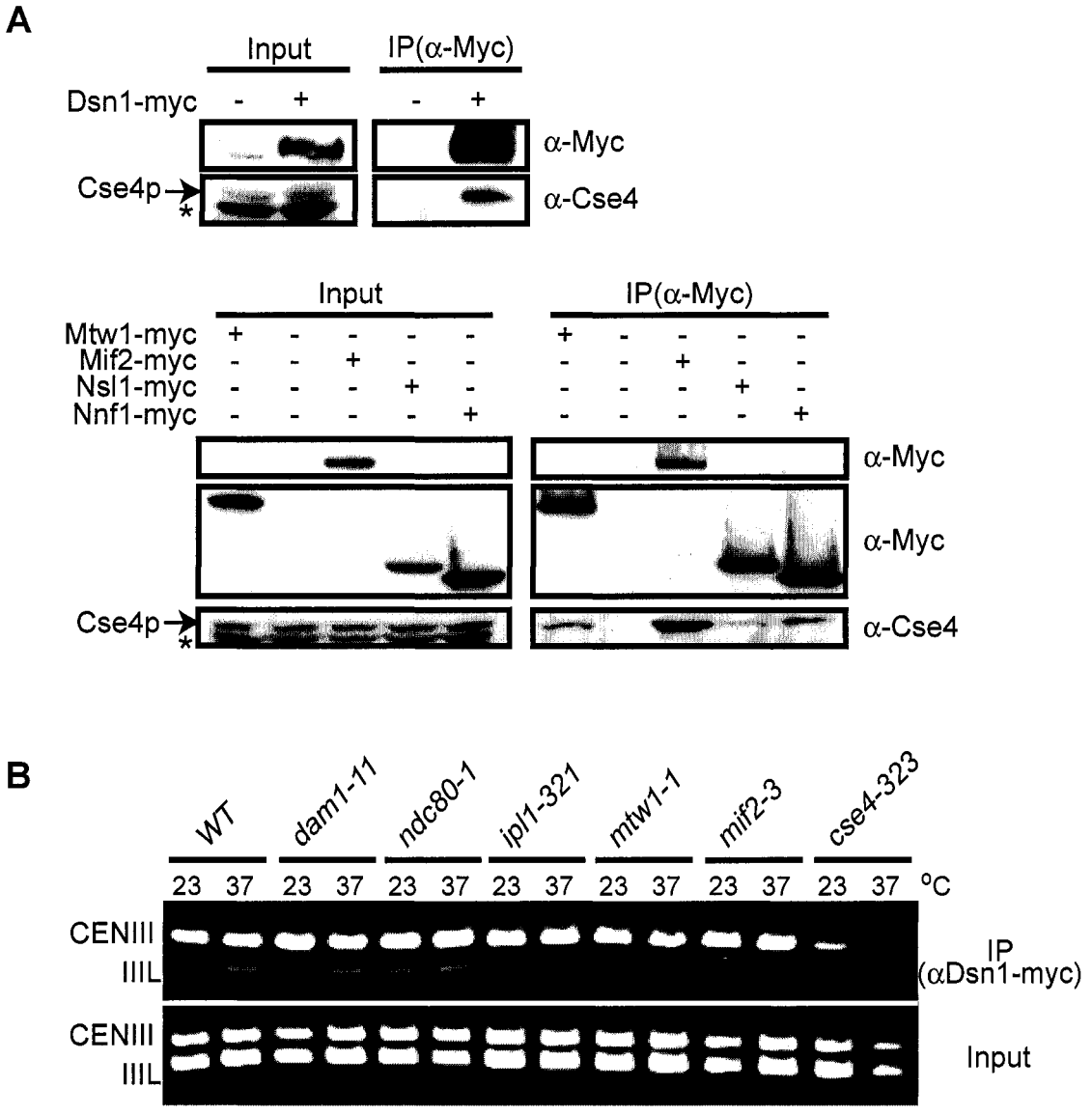


Figure 2.4 Cse4 interacts with Dsn1, Mtw1, Mif2, Nsl1 and Nnf1 and is required for Dsn1 kinetochore assembly. (A) Extracts from wild type cells (SBY2153) and cells expressing Dsn1-myc13, Mtw1-myc13 (SBY2191), Mif2-myc13 (SBY2478), Nsl1-myc13 (SBY2513), and Nnf1-myc13 (SBY2517) were immunoprecipitated with anti-myc antibody. Extracts (Input) and immunoprecipitates (IP) were analyzed by anti-Cse4 and anti-myc immunoblotting. Cse4 (arrow) migrates just above a cross-reacting band (asterisk). (B) Dsn1 associates with centromeric DNA in a Cse4-dependent manner. Wild type (SBY2359) *dam1-11* (SBY2361), *ndc80-1* (SBY2362), *ipl1-321* (SBY2535), *mtw1-1* (SBY2367), *mif2-3* (SBY2501), and *cse4-323* (SBY2469) cells expressing Dsn1-myc13 were incubated at either 23 °C or 37 °C for 3 hrs and analyzed by ChIP.

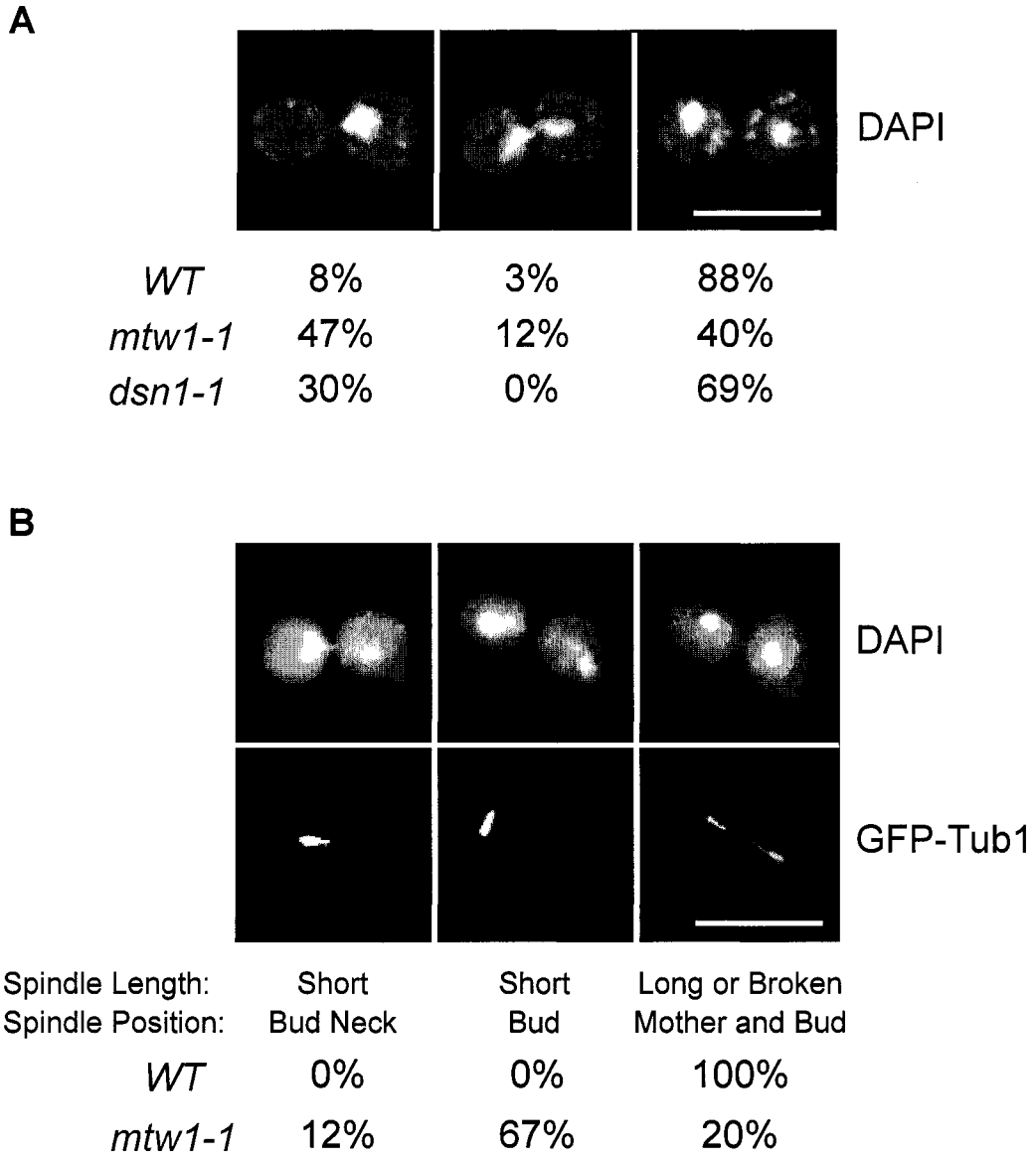


Figure 2.5 *mtw1-1* mutants missegregate DNA and spindles. (A) *mtw1-1* and *dsn1-1* mutant cells missegregate DNA. Wild type (SBY818), *mtw1-1* (SBY1646), and *dsn1-1* (SBY2582) strains were released to 37 °C, and cells were monitored for budding and DNA segregation. 140 min after release, large budded cells stained with DAPI were scored for DNA distribution. The two masses of DNA category contained both equal and unequal segregation. (B) *mtw1-1* mutants have short spindles that migrate into the bud. Wild type (SBY2370) and *mtw1-1* (SBY2371) cells expressing GFP-Tub1 were arrested in G1 and released to 37 °C. 140 min after release, large budded cells with two masses of DNA were analyzed for spindle length and position. Bar, 10μm.

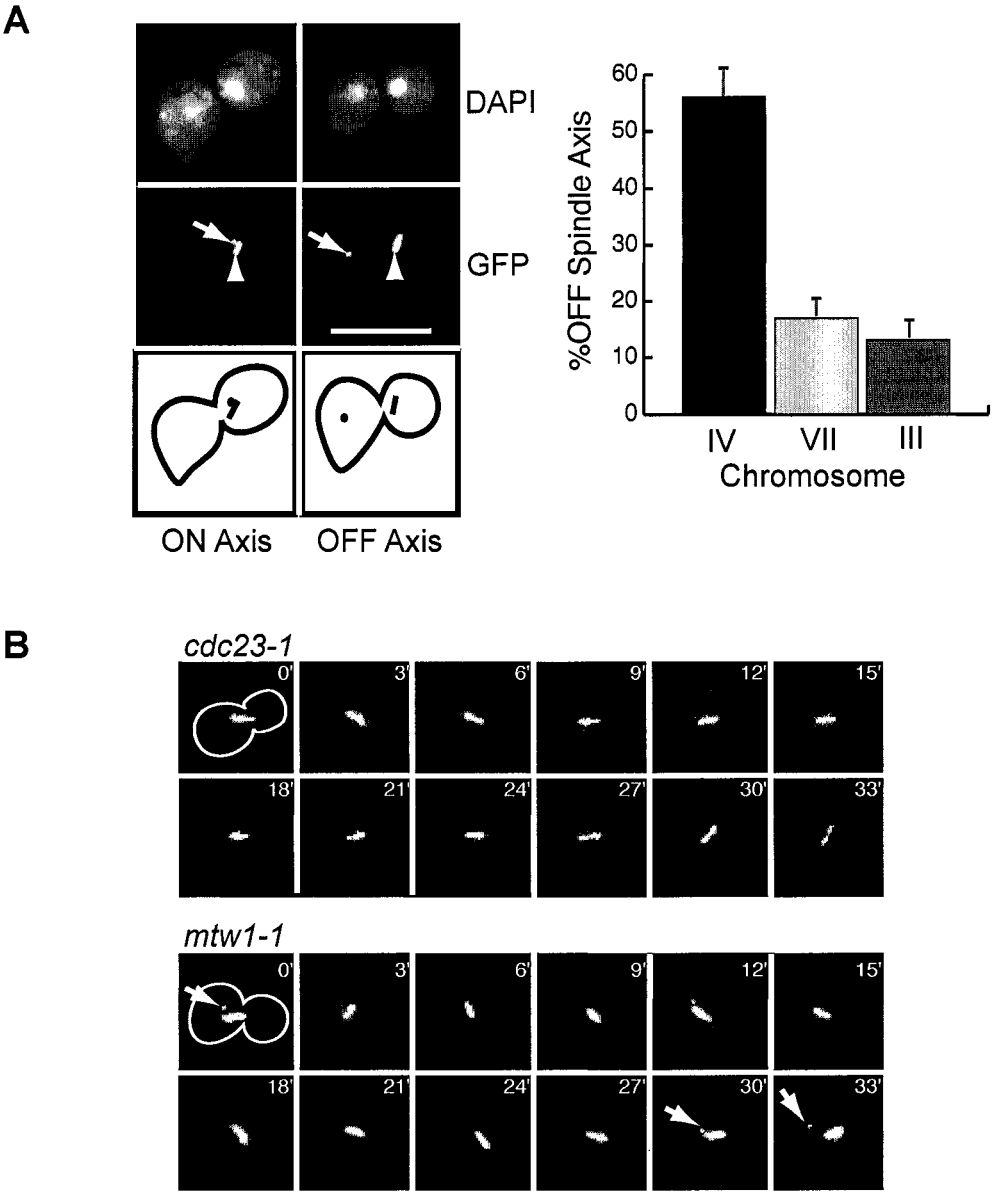


Figure 2.6 *mtw1-1* mutants contain unattached chromosomes. (A) *mtw1-1* mutant cells expressing GFP-Tub1 and fluorescently marked chromosome IV (SBY2350, 12 kb to the right of CEN IV), VII (SBY2539, 18kb to the left of CEN VII), or III (SBY2534, 2 kb to the right of CEN III) were arrested in G1 and released to 37 °C. 120 min after release, cells with short spindles (arrowheads) were scored for whether their sister chromatids (arrows) were on (left panels) or off the spindle axis (right panels). Sister chromatids were always found on the spindle axis in the wild type cells (data not shown). The bars indicate the 95% confidence interval. Bar, 10μm. (B) Live microscopy was performed on *cdc23-1* (SBY817) control and *mtw1-1* (SBY2350) mutant cells expressing GFP-Tub1 and fluorescently marked chromosome IV. Cells were arrested in G1, released to 36 °C and eight z-sections at 0.5 mm intervals were acquired every 30s. The stacked images of single cells are shown every 3 min. An outline of the cell is shown at time 0', when the spindle is properly aligned and positioned, and at time 33'. The arrows show the unattached sister chromatids in the *mtw1-1* mutant cell. Bar, 10μm.

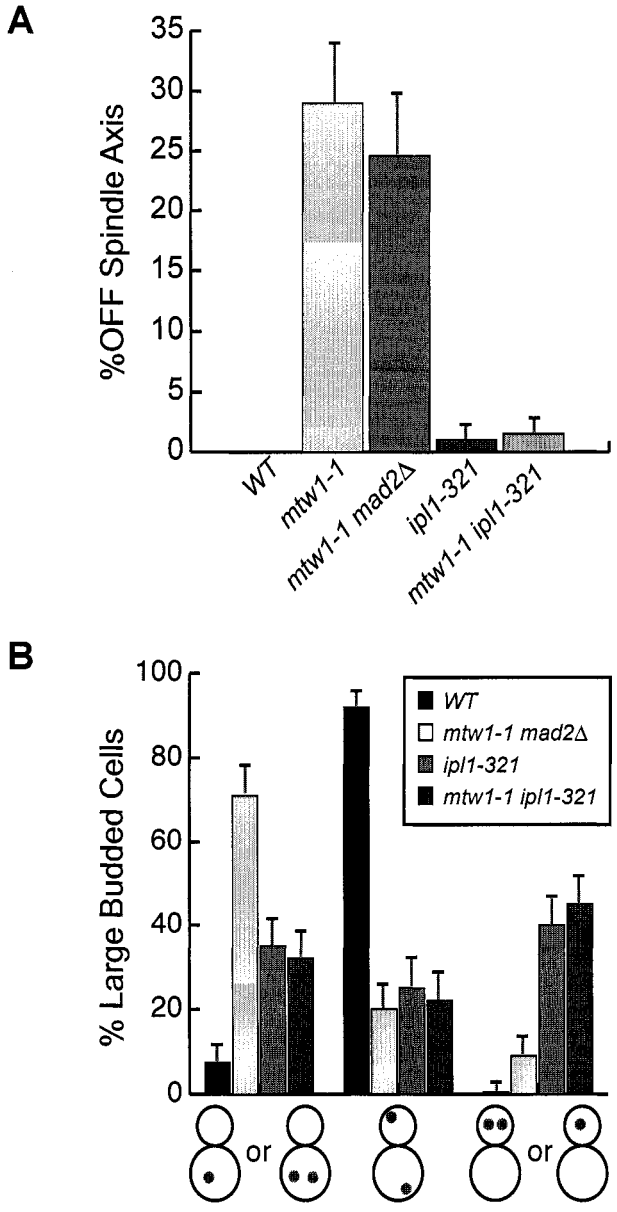


Figure 2.7 The *mtw1-1* mutant requires Ipl1 function to generate unattached chromosomes. (A) The removal of Ipl1 restores attachment in the *mtw1-1* mutant. Wild type (SBY2349), *mtw1-1* (SBY2350), *mtw1-1 mad2*Δ (SBY2611), *ipl1-321* (SBY1998), and *mtw1-1 ipl1-321* (SBY2466) cells expressing GFP-Tub1 and fluorescently marked chromosome IV were released to 37 °C. 80 min after release, cells with short spindles were scored for whether their sister chromatids were on or off the spindle axis. (B) Impairing Ipl1 alters *mtw1-1* chromosome segregation. Wild type (SBY818), *ipl1-321* (SBY819), *mtw1-1 mad2*Δ (SBY1713), and *mtw1-1 ipl1-321* (SBY1724) cells containing fluorescently marked chromosome IV were arrested in G1 and released to 37 °C for 120 min. Large budded cells were monitored for the distribution of chromosome IV. The bars indicate the 95% confidence interval.

CHAPTER 3:

The Spindle Checkpoint: Attachment versus Tension

Summary

The spindle checkpoint ensures the fidelity of chromosome segregation by preventing cell cycle progression until all chromosomes make proper bipolar attachments to the mitotic spindle and come under tension. Despite significant advances in our understanding of spindle checkpoint function, the primary signal that activates the spindle checkpoint remains unclear. While some experiments suggest that the checkpoint recognizes the lack of microtubule attachment to the kinetochore, others indicate that the checkpoint senses the absence of tension generated on the kinetochore by microtubules. The interdependence between tension and microtubule attachment make it difficult to determine whether these signals are separable. Here we consider the recent evidence supporting and opposing the hypothesis that defects in tension act as the primary checkpoint signal.

Tension and the Spindle Checkpoint

The spindle checkpoint prevents the premature segregation of improperly attached chromosomes by delaying the metaphase to anaphase transition to allow the cell time to correct any defects (for review, see (Lew and Burke, 2003)). Though it is clear that the spindle checkpoint causes metaphase arrest through the inhibition of the anaphase-promoting complex (APC), a ubiquitin ligase that targets an anaphase inhibitor for destruction, the primary defect that activates the spindle checkpoint remains controversial. The simplest hypothesis is that the checkpoint monitors some aspect of kinetochore-spindle interactions. Experiments in several organisms suggest that the spindle checkpoint can be activated by the lack of kinetochore-microtubule attachments or defects in the tension exerted by microtubule-generated forces on kinetochores (for review, see (Zhou et al., 2002)). However, it is unclear whether these signals are separate or actually interdependent. Because defects in the spindle checkpoint are associated with many cancers (for reviews, see (Bharadwaj and Yu, 2004; Draviam et al., 2004)), distinguishing between these potential checkpoint activators may have implications for our understanding and treatment of human disease. Here we examine recent developments in identifying the primary defect that is sensed by the spindle checkpoint.

The Origins of the Tension vs. Attachment Debate

Pioneering experiments in mitotic rat PtK cells and meiotic mantid spermatocytes laid the foundation for the attachment versus tension question by

providing strong evidence that the spindle checkpoint responds to both the lack of attachment and the absence of tension.

The tension hypothesis states that the absence of mechanical tension on chromosomes activates the spindle checkpoint. If the cell only responded to the absolute lack of microtubule attachment, syntelic attachments (see Figure 1.2) would fail to engage the spindle checkpoint and would lead to aneuploidy. Experimental evidence for the tension hypothesis was provided via the micromanipulation of chromosomes in praying mantid spermatocytes undergoing meiosis I (Li and Nicklas, 1995). In these cells, the formation of an attached, but tension defective syntelic chromosome pair resulted in spindle checkpoint activation. However, when tension was applied across these kinetochores using a force-calibrated microneedle, the spindle checkpoint was satisfied and the cells entered anaphase. Although the absence of tension appears to act as a checkpoint signal in most organisms in both mitosis and meiosis, tension micromanipulation experiments have only been performed on meiotic insect cells, leaving open the possibility that these results are system specific. Consistent with this possibility, a tension signal is actually required for *Drosophila* oocytes to induce a metaphase arrest, though it is not known if the arrest is spindle checkpoint dependent (Jang et al., 1995).

Evidence to suggest that it is the lack of kinetochore attachment and not the absence of tension that activates the spindle checkpoint is based on the careful analysis of chromosome behavior in mitotic rat PtK cells (Rieder et al., 1995). Live cell imaging coupled with electron microscopy revealed that a single unattached

kinetochore could inhibit anaphase onset. Ablation of the unattached kinetochore by laser microsurgery relieved the arrest, indicating that the unattached kinetochore is the source of the checkpoint signal. However, the unattached kinetochore was neither attached nor under tension so it could not distinguish between the two possible checkpoint activators. Rather, the conclusion that attachment is the checkpoint signal was based on the behavior of the intact, attached kinetochore in this monotelic chromosome pair. This mono-oriented kinetochore was not under tension, yet the checkpoint was inactive. Though it is possible that the attached sister kinetochore was under some tension due to anti-poleward forces on the chromosome arms, it is unlikely that this tension could approach the levels that occur on bioriented sister kinetochores. Therefore, the simplest explanation for the lack of spindle checkpoint activation in this experiment is that the absence of tension on the remaining attached kinetochore is not a sufficient checkpoint signal.

The relationship between tension and attachment

Although these experiments can be interpreted to mean that defects in tension or attachment specifically activate the spindle checkpoint, they are complicated by the intimate relationship between these two potential checkpoint signals. Because unattached kinetochores are not under tension, distinguishing between potential activators requires the analysis of attached but tension defective kinetochores. Yet, microtubule attachments are also affected by tension. The application of tension both stabilizes and increases the number of kinetochore microtubule attachments

(King and Nicklas, 2000; Nicklas and Ward, 1994). This was shown in an elegant set of micromanipulation experiments where the number of microtubule attachments on an attached kinetochore was halved when tension was relieved by detaching its sister kinetochore. The subsequent re-application of tension to the relaxed kinetochore restored attachment to its original level. Therefore, microtubule attachment and tension are coupled by an unknown mechanism. This leads to the question of whether the absence of tension activates the spindle checkpoint directly by regulating a tension-sensitive checkpoint component, or indirectly by altering kinetochore-microtubule occupancy (Figure 3.1).

The best evidence for the interdependence of the tension and attachment signals comes from the grasshopper spermatocyte system, where kinetochores with "weak" attachments were created using micromanipulation (Nicklas et al., 2001). These kinetochores completely lacked tension and had only a few attached microtubules. In this situation, markers for checkpoint activation decreased despite the lack of tension, indicating that the weak attachments were sufficient to regulate the checkpoint. However, the checkpoint was not completely silenced until the weakly attached kinetochores obtained full occupancy and came under tension. Therefore, in this system, the checkpoint appears to monitor attachment and the role of tension is to promote the stabilization of these microtubule attachments.

In the budding yeast model system, the relationship between tension and attachment is simplified. Unlike PtK and grasshopper spermatocyte metaphase kinetochores, which bind an average of 24 and 32 microtubules, respectively,

budding yeast kinetochores attach to a single microtubule (King and Nicklas, 2000; McEwen et al., 1997; Winey et al., 1995). The budding yeast kinetochore is either attached or unattached and cannot generate a "weak" attachment due to partial microtubule occupancy. Based on this reasoning, experiments performed in the budding yeast provide the most compelling evidence that the spindle checkpoint recognizes the absence of tension. To test the role of tension in both mitotic and meiotic progression, tension defects were manufactured by preventing the chromosome pairing necessary for bipolar force generation (Shonn et al., 2000; Stern and Murray, 2001). This was achieved in mitosis by conditionally inhibiting replication or sister chromatid cohesion, and in meiosis by preventing the recombination that holds homologous chromosomes together during meiosis I. The absence of tension in these situations caused a spindle checkpoint-dependent delay in cell cycle progression. In these experiments, the chromosomes were pulled to the poles indicating that the tensionless kinetochores make microtubule attachments, although it is possible there was an undetected delay in attachment. Because budding yeast kinetochores cannot be visualized by electron microscopy, it is extremely difficult to determine the precise status of attachments in this organism. Although these experiments are not complicated by questions of partial microtubule occupancy, it is not clear if these kinetochore-microtubule interactions differ from the amphitelic state. For instance, the tensionless kinetochore might bind to the side of the microtubule instead of properly interacting with the microtubule end. Therefore, it is possible that the absence of tension on budding yeast kinetochores

also affects microtubule attachment. It will be important to determine what type of yeast attachments occur in the absence of tension and how these budding yeast experiments translate to more complex kinetochores with multiple microtubule binding sites.

Molecular markers for the lack of attachment and the absence of tension

The continuing confusion over the primary defect that activates the spindle checkpoint has been fueled, in part, by the development of molecular markers thought to recognize either unattached or tensionless kinetochores. However, instead of clarifying the question, analysis of these markers has served to highlight just how difficult it is to distinguish between attachment and tension defects.

While evaluating these markers, it is important to consider the techniques used to create unattached or tension defective kinetochores. Although the use of chromosome micromanipulation to control the status of kinetochore attachment and tension is powerful, this approach is technically challenging and is not amenable to most cell types. More commonly, attachment and tension are manipulated in cultured cells via chemical inhibition of spindle function. To generate unattached kinetochores, cells are exposed to nocodazole or benomyl; compounds that cause microtubule depolymerization and therefore deprive kinetochores of their attachment partners. In contrast, tension defects are typically produced via treatment with the microtubule-stabilizing drug taxol. The sister kinetochores in these taxol-treated cells are closer together indicating the loss of kinetochore tension, and electron

microscopy confirmed that these tensionless kinetochores remain bound to microtubules. Though the average number of kinetochore bound microtubules was not different from control cells, the variation in the number of microtubules bound to each kinetochore was significantly greater, suggesting that taxol treatment may alter microtubule occupancy (McEwen et al., 1997). This may be due to the instability of microtubule attachments that are not under proper tension (Nicklas and Ward, 1994). Since a single unattached kinetochore is sufficient to engage the checkpoint (Rieder et al., 1995), one interpretation of these experiments is that taxol activates the checkpoint because it creates unoccupied microtubule binding sites and not because the checkpoint responds to the absence of tension. It is important to keep this caveat in mind when considering the specificity of molecular markers to unattached or tension defective kinetochores given the standard use of taxol to create tension defects.

The Mad2 checkpoint protein

The conserved checkpoint protein Mad2 has been proposed to be a marker for unattached kinetochores. Localization studies show that Mad2 binds to unattached kinetochores in prometaphase of the unperturbed cell cycle and is lost from kinetochores in metaphase when amphitelic attachments are made (Chen et al., 1996; Li and Benezra, 1996). In addition, Mad2 is recruited to kinetochores with reduced microtubule occupancy. Because Mad2 does not accumulate on the tension defective kinetochores generated via treatment with taxol or by micromanipulation, it

has been proposed that Mad2 specifically marks unattached kinetochores (Waters et al., 1998). However, cells treated with taxol always contained at least one kinetochore that stained with Mad2, consistent with the possibility that taxol alters microtubule occupancy. In addition, cells treated with the kinesin inhibitor monastrol, which blocks spindle pole separation and therefore results in the formation of syntelic attachments, accumulate Mad2 on the majority of attached but tension defective kinetochores (Kapoor et al., 2000). This suggests either that syntelic attachments generated by monastrol are not at their full microtubule occupancy, or that Mad2 kinetochore localization is not specific to the lack of attachment.

Another observation that suggests that Mad2's role in the checkpoint may not be specific to unattached kinetochores comes from the finding that Mad2 function is required for all known attachment and tension defects to activate the spindle checkpoint, including cells treated with taxol or monastrol. In addition, it is important to realize that while Mad2 localization to the kinetochore correlates with spindle checkpoint activity, it has not yet been demonstrated to be required for the checkpoint. Recent cell culture work indicates that unattached kinetochores with low or undetectable levels of Mad2 are capable of spindle checkpoint arrest (DeLuca et al., 2002; Martin-Lluesma et al., 2002). In addition, budding yeast Mad2 is not kinetochore localized during the checkpoint delay induced by mutations that create tension defects (Gillett et al., 2004). Therefore, Mad2 kinetochore localization may

not represent its role in the checkpoint, or the field may have reached the experimental limits of Mad2 detection.

The 3F3/2 phosphoepitope

The 3F3/2 antibody was originally developed against thiophosphorylated substrates in *Xenopus* egg extracts (Cyert et al., 1988) and subsequently shown to recognize phosphoepitopes on kinetochores and spindle poles in a wide variety of cell types (Gorbsky and Ricketts, 1993). Micromanipulation experiments such as those described above demonstrated that the intensity of the 3F3/2 kinetochore signal was sensitive to the application of tension. When one kinetochore in a syntelic attachment was put under tension, its 3F3/2 signal was significantly reduced compared to the unmanipulated control (Nicklas et al., 1995). Conversely, the tension defects induced by taxol treatment resulted in strong 3F3/2 kinetochore staining that appeared to be proportionally more intense than Mad2 staining (Waters et al., 1998). Of course, these experiments are subject to the important caveat that tension affects the stability and number of microtubule attachments. Nevertheless, the 3F3/2 labeling of kinetochores in the absence of tension has led to speculation that the phosphorylation recognized by 3F3/2 is important for cells to signal defects in tension to the spindle checkpoint. In support of this idea, microinjection of 3F3/2 antibody into mitotic cells protects the phosphoepitope and results in a metaphase delay (Campbell and Gorbsky, 1994). However, it is also possible that this phenotype is due to indirect effects of the injected antibody on kinetochore function.

The potential role of 3F3/2 phosphorylation in signaling tension defects has led to interest in identifying the 3F3/2 phospho-substrate(s) as well as the kinases and phosphatases that regulate kinetochore phosphorylation. The 3F3/2 antibody recognizes DNA topoisomerase IIα (TIIα) and reacts with a short TIIα peptide phosphorylated *in vitro* on threonine 1342 by casein kinase II (CKII) (Daum and Gorbsky, 1998). The effect of this phosphorylation on spindle checkpoint function has not been studied and there is currently no evidence that either TIIα or CKII is involved in the spindle checkpoint.

Recent work has shown that the Polo-like Kinase 1 (Plk1) is required to generate the 3F3/2 epitope on vertebrate kinetochores (Ahonen et al., 2005). Plk1 regulates numerous mitotic functions including mitotic entry, spindle assembly, cohesin dissociation, and cytokinesis, but had not previously been implicated in spindle checkpoint signaling (for review, see (van Vugt and Medema, 2005)). Plk1 can create 3F3/2 reactivity on kinetochores *in vitro*, and depletion of Plk1 by siRNA leads to a decrease in 3F3/2 kinetochore staining *in vivo*. In addition, cells with depleted Plk1 display a reduction in the kinetochore levels of many proteins involved in the spindle checkpoint, such as Mad2, Cenp-E, Ndc80 and Cdc20. However, instead of the expected defect in checkpoint function, Plk1 depletion leads to a spindle checkpoint-dependent metaphase delay. Though it is possible that Plk1-mediated generation of the 3F3/2 phosphoepitope is not required for tension-dependent checkpoint signaling, it may also be that complete depletion of Plk1 has not been achieved or that additional proteins are involved in the checkpoint response

to tension defects. Alternatively, Plk1 depletion may interfere with one of Plk1's other mitotic functions and activate the checkpoint due to unrelated attachment defects.

An intriguing possibility is that Plk1 links checkpoint signals to 3F3/2 through phosphorylation of the APC, a Plk1 substrate *in vitro* (for review, see (van Vugt and Medema, 2005)). The kinetochore localization of the APC depends on the spindle checkpoint and the 3F3/2 antibody recognizes several APC components specifically from mitotic extracts (Acquaviva et al., 2004; Daum et al., 2000). In the future it will be important to map these 3F3/2 phosphoepitopes and determine the *in vivo* consequences of this phosphorylation on checkpoint activity.

Other Checkpoint Proteins

In addition to 3F3/2 phosphoepitopes, reports suggest that several checkpoint proteins are specifically recruited to tension defective but attached kinetochores. In cultured cells, the conserved checkpoint component BubR1 accumulates on kinetochores in the absence of tension produced by treatment with taxol, incubation at low temperatures, or treatment with the microtubule inhibitor vinblastine (Logarinho et al., 2004; Shannon et al., 2002; Skoufias et al., 2001). However, as discussed above, these treatments may also affect microtubule occupancy. In addition, BubR1 function is required to activate the checkpoint in response to the lack of attachment, suggesting that BubR1 does not play a tension-specific role in the spindle checkpoint (Chan et al., 1999; Chen, 2002; Meraldi et al., 2004). In contrast,

the budding yeast BubR1 homolog Mad3 appears to be required for the checkpoint arrest signaled by the lack of attachment due to spindle depolymerization but dispensable for the checkpoint delay generated by the tension defects caused by replication inhibition (Lee and Spencer, 2004). Mad3 is the only checkpoint protein reported to behave in this manner and additional experiments should determine if this observation is truly specific to attachment. However, in our hands Mad3 is required for both attachment and tension defects to activate the checkpoint (S. Biggins, unpublished observations).

The conserved checkpoint component Bub1 is also reported to accumulate on kinetochores in the absence of tension (Shannon et al., 2002; Skoufias et al., 2001; Taylor et al., 2001). However, Bub1's kinetochore localization also appears to be sensitive to defects in attachment (Logarinho et al., 2004; Sharp-Baker and Chen, 2001; Taylor et al., 2001). Further complicating the situation is the observation that Bub1 is required for the kinetochore localization of a subset of checkpoint proteins that varies depending on the experimental system and conditions. These differences may explain why loss of Bub1 function results in varying degrees of checkpoint impairment (Johnson et al., 2004; Kitajima et al., 2005; Sharp-Baker and Chen, 2001; Tang et al., 2004a; Tang et al., 2004b). Additionally, Bub1 has a function regulating centromeric cohesion in meiosis and mitosis (Bernard et al., 2001; Kitajima et al., 2005; Tang et al., 2004b). Because centromeric cohesion is important for the generation of kinetochore tension, it is tempting to speculate that this regulation could allow Bub1 to monitor tension signals.

Aurora B kinases may regulate the spindle checkpoint response to the absence of tension

In addition to proteins that might mark unattached or tension defective kinetochores, there are those that appear to be able to distinguish between these two checkpoint activators. One of these proteins is Aurora B, the catalytic component of a complex that also includes the inner centromere protein (INCENP), Survivin, and Dasra B/Borealin/Csc1, as wells as the related protein Dasra A (for review, see (Vagnarelli and Earnshaw, 2004)). These proteins comprise the chromosomal passenger complex, named because of its dynamic localization pattern where it first appears in the inner centromere region between sister kinetochores, moves onto the elongating spindle, and finally concentrates at the spindle midzone. Aurora B regulates numerous mitotic events that include amphitelic kinetochore attachment and spindle checkpoint function, as well as spindle assembly and disassembly, anaphase chromosome condensation, and cytokinesis.

Amphitelic kinetochore attachment

Analysis of temperature-sensitive, loss of function mutations in the budding yeast Aurora B homolog *IPL1* gene revealed cells with massive chromosome missegregation due to the formation of syntelic attachments (Biggins et al., 1999; Chan and Botstein, 1993; Tanaka et al., 2002). Experiments in many model systems are consistent with the yeast experiments and suggest that Aurora B plays a

conserved role ensuring amphitelic kinetochore attachments (for review, see (Vagnarelli and Earnshaw, 2004)). It was proposed that Aurora B/Ipl1 promotes the turnover of kinetochore microtubule interactions that do not generate tension (Tanaka et al., 2002). Consistent with this hypothesis, Ipl1 activity was required to detach microtubules from tension defective kinetochores due to a mutation in the conserved Mtw1/Mis12 protein or a defect in replication (Dewar et al., 2004; Pinsky et al., 2003; Tanaka et al., 2002). These observations are similar to subsequent data from cell culture studies indicating that Aurora B selectively disassembles syntelically attached kinetochore microtubules (Lampson et al., 2004). Furthermore, impairing Aurora B function stabilizes kinetochore-microtubule attachments at defective kinetochores (Lampson and Kapoor, 2005; Pinsky et al., 2003). Taken together, these experiments indicate that Aurora B/Ipl1 alters kinetochore microtubules in response to the absence of tension and may be responsible for the instability of attachments at kinetochores that lack tension, as described above. These studies also suggest that Aurora B/Ipl1 is regulated by the absence of tension and therefore may also function to allow the spindle checkpoint to detect tension defects.

Spindle checkpoint

A role for Aurora B/Ipl1 in the spindle checkpoint was first suggested through the analysis of temperature-sensitive *ipl1* mutant cells, which despite syntelic attachments, proceed through the cell cycle without detectable spindle

checkpoint delay (Biggins et al., 1999). It was later shown that Ipl1 function was required to activate the checkpoint in response to the conditional inhibition of either replication or sister chromatid cohesion, conditions that prevent the chromosome pairing necessary for bipolar force generation and kinetochore tension (Biggins and Murray, 2001). In contrast, the nearly complete loss of attachment induced by the microtubule depolymerizing drug nocodazole activates the checkpoint in an Ipl1-independent manner, suggesting a specific requirement for Ipl1 to allow the absence of tension to activate the spindle checkpoint. Similarly, in cell culture systems, Aurora B and the chromosomal passenger complex are required for defects in tension but not attachment to activate the spindle checkpoint (Carvalho et al., 2003; Ditchfield et al., 2003; Hauf et al., 2003; Lens et al., 2003).

The mechanism by which Aurora B/Ipl1 activates the spindle checkpoint in response to the absence of tension is not known. The simplest explanation is that Aurora B/Ipl1 facilitates both amphitelic attachment and spindle checkpoint activation by promoting the turnover of kinetochore-microtubule interactions. This hypothesis suggests that Aurora B/Ipl1 regulates checkpoint activation indirectly by creating unattached kinetochores and essentially amplifying a marginal checkpoint signal. Consistent with this hypothesis, impairing Ipl1 function in a budding yeast kinetochore mutant both restores attachment and satisfies the spindle checkpoint (Pinsky et al., 2003). It is also possible that Aurora B/Ipl1 plays a direct role in checkpoint activation that is independent of its role in kinetochore detachment. For example, in fission yeast and *Xenopus* egg extracts, Aurora B is required for

attachment defects to activate the spindle checkpoint suggesting a role separable from creating unattached kinetochores (Kallio et al., 2002; Petersen and Hagan, 2003). The differences in spindle checkpoint regulation and response to spindle depolymerization that makes these organisms completely dependent on Aurora B/Ipl1 for checkpoint activity is not well understood. One possibility is that the kinetochore structure in these organisms is disrupted in the absence of Aurora B/Ipl1 function in a manner that prevents the spindle checkpoint from ever being activated. Consistent with this hypothesis, Aurora B is required for the kinetochore localization of all other checkpoint proteins in *Xenopus* egg extracts (Vigneron et al., 2004).

Additional proteins that distinguish between a tension and attachment defect

There are several other gene products in budding yeast that are specifically required for tension defects to activate the spindle checkpoint. One such protein is Shugoshin or Sgo1 that was originally identified as a protector of centromeric cohesion during meiosis I (Katis et al., 2004; Kitajima et al., 2004; Marston et al., 2004; Rabitsch et al., 2004). A mitotic checkpoint role for Sgo1 was revealed in a genetic screen for mutants unable to respond to the absence of tension generated by the presence of short linear chromosomes (Indjeian et al., 2005). Subsequent experiments showed that *sgo1* mutant cells fail to activate the checkpoint due to the loss of cohesion but respond normally to spindle depolymerization, similar to *ipl1* mutants. It is particularly intriguing that Sgo1 appears to have a role in both tension-specific checkpoint activation and sister chromatid cohesion, leading to the

possibility that Sgo1 couples tension-sensing to cohesion maintenance, which is itself essential to generate tension. However, similar to Aurora B/Ipl1, Sgo1 also has roles in both microtubule and kinetochore function that may make it difficult to distinguish whether it truly has a separable role in the checkpoint response to tension defects.

In contrast to *sgo1* mutant budding yeast cells, siRNA depletion of human Sgo1 in cell culture results in severe mitotic defects that include premature sister separation, destabilization of kinetochore microtubules, loss of kinetochore tension, and spindle checkpoint arrest that depends on Mad2 and Aurora B (Kitajima et al., 2005; Salic et al., 2004; Tang et al., 2004b). It is therefore possible that the budding yeast mutants have separated Sgo1's role in the spindle checkpoint from its other mitotic functions. Alternatively, Sgo1's defect in the checkpoint may be an indirect result of the kinetochore and microtubule defects that result from a lack of Sgo1 function.

A specific defect in engaging the spindle checkpoint in response to the absence of tension is also observed in budding yeast cells with mutations in *SKP1*, a gene that encodes a critical component of both the kinetochore and the SCF (Skp1-Cullin-Fbox), an E3 ubiquitin ligase important for the cell cycle transition from G1 to S phase. The checkpoint deficient *skp1* alleles specifically abolish Skp1's physical interaction with the checkpoint protein Bub1 but do not affect SCF function (Kitagawa et al., 2003). How the Skp1-Bub1 interaction modulates tension-dependent checkpoint functions remains to be determined. Because Bub1 function is

required for Sgo1 kinetochore localization in some organisms and Bub1 binds to Skp1 in budding yeast, there may also be a link between Sgo1 and Skp1 that may help to explain their roles in mediating the checkpoint response to the absence of tension (Kitajima et al., 2005; Kitajima et al., 2004; Tang et al., 2004b).

Concluding Remarks

The primary defect sensed by the spindle checkpoint continues to be an unresolved question, and both the lack of attachment and the absence of tension remain reasonable possibilities. Distinguishing between these potential models has been complicated by the clear inter-relationship between attachment and tension. In organisms with kinetochores that bind multiple microtubules, it has been difficult to create tension defects that do not reduce microtubule occupancy. Because of this, the budding yeast, with a single microtubule per kinetochore, has provided the best evidence that the checkpoint directly monitors tension. However, micromanipulation experiments in grasshopper spermatocytes have demonstrated that microtubule attachment is more important for regulating the spindle checkpoint and tension facilitates occupancy. One possibility is that the difference in the number of microtubule binding sites on kinetochores between budding yeast and other organisms reflects a fundamental difference in the checkpoint. In yeast, it may be more important to monitor tension because mono-oriented attachments will always lead to aneuploidy. In addition, yeast are less likely to have unattached kinetochores since microtubules remain bound to kinetochores throughout the cell cycle. In

organisms with larger kinetochores, however, a single misattached microtubule on a kinetochore that has several properly bound microtubules is not likely to generate enough force to cause chromosome missegregation. Furthermore, kinetochores with multiple microtubule binding sites proceed through a completely unattached intermediate, suggesting that monitoring attachment may be a more effective way for these cells to ensure proper spindle assembly.

It is clear that uncovering the true nature of the spindle checkpoint signal is difficult with the current information. The isolation of specific checkpoint alleles defective only in their response to the absence of tension, or the identification of a protein whose activity *in vitro* is regulated specifically by tension, may provide the evidence that tension is a direct activator of the spindle checkpoint. As we learn more, it will be interesting to determine whether the checkpoint signal differs depending on the organism and its kinetochore structure as well as determine whether the activating signal is the same in mitosis and meiosis.

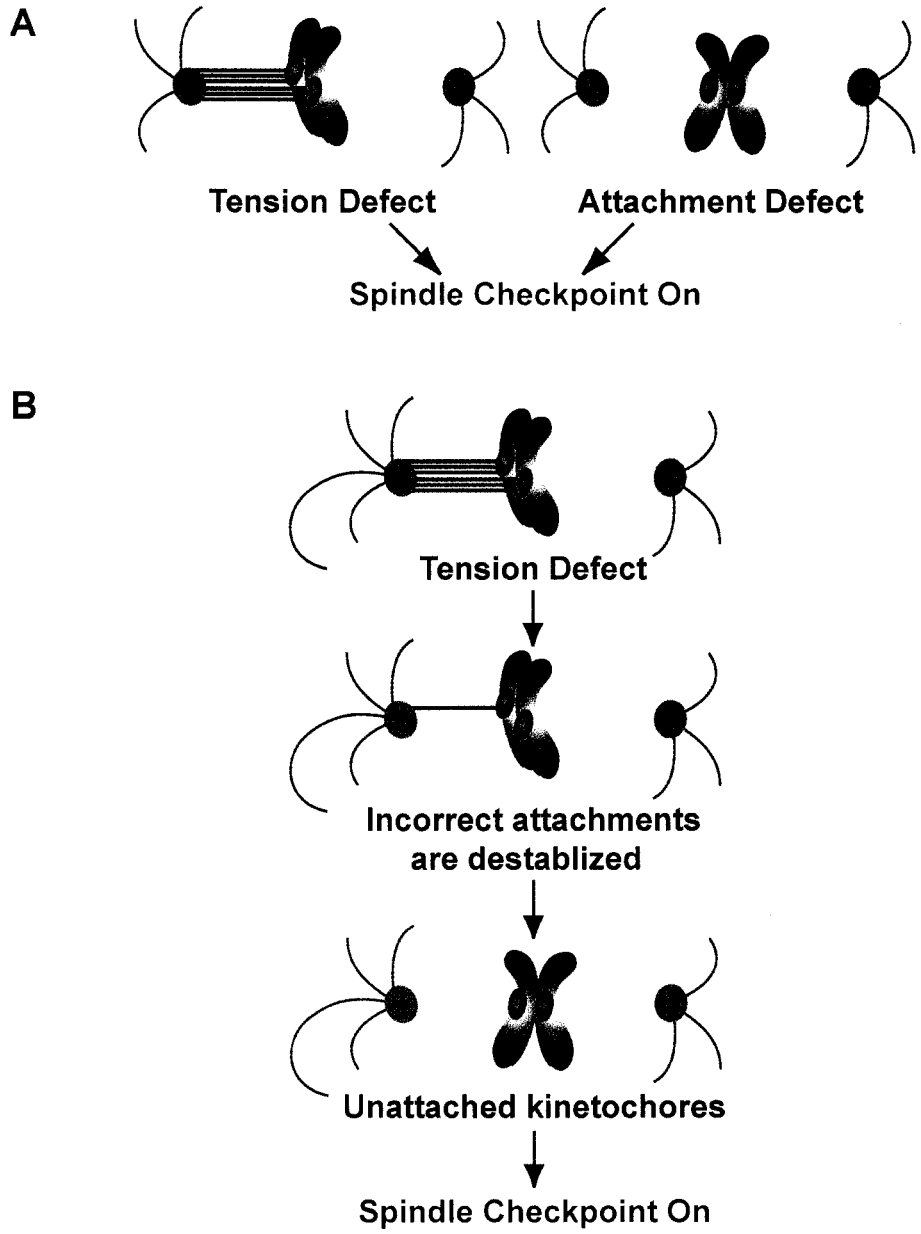


Figure 3.1 Two possible models for spindle checkpoint activation. (A) In one model, defects in tension and attachment are separable signals that are sensed by the cell leading to activation of the spindle checkpoint. (B) In the second model, defects in tension are sensed by the cell and result in the destabilization of the inappropriate microtubule attachments. The destabilization of these attachments creates unattached kinetochores that are monitored by the spindle checkpoint and lead to its activation.

CHAPTER 4:

The Ipl1/Aurora protein kinase activates the spindle checkpoint by creating unattached kinetochores

Summary

The spindle checkpoint ensures accurate chromosome segregation by delaying cell cycle progression until all sister kinetochores capture spindle microtubules arising from opposite poles and come under tension. Although the checkpoint is activated by either the lack of kinetochore-microtubule attachments or defects in the tension exerted by microtubule-generated forces on kinetochores, it is not clear whether these signals constitute separate pathways or are instead linked. To investigate the connection between tension and attachment, we took advantage of a property of the budding yeast Ipl1/Aurora protein kinase to activate the spindle checkpoint in the absence of kinetochore tension but not attachment. We show here that impairing Ipl1 function in a series of kinetochore mutants that are defective in microtubule attachment both satisfied the checkpoint and restored attachments, indicating that Ipl1-mediated microtubule detachment is coupled to checkpoint activation. Consistent with this, kinetochore mutants were able to activate the checkpoint in the absence of Ipl1 if the kinetochores were detached by spindle depolymerization. We also found that Ipl1 does not regulate the spindle checkpoint entirely through phosphorylation of the Dam1 kinetochore complex, indicating that additional Ipl1 substrates important for destabilizing kinetochore-microtubule interactions likely exist. We therefore propose that Ipl1 responds to the tension defects associated with most kinetochore mutants by creating unattached kinetochores that lead to spindle checkpoint activation and eventual correction of the

improper attachments. In addition, these data suggest that the primary signal for spindle checkpoint activation is an unattached kinetochore.

Results and Discussion

The spindle checkpoint monitors kinetochore-microtubule interactions and prevents the premature segregation of chromosomes that have not made proper bipolar attachments to the mitotic spindle (for review, see (Lew and Burke, 2003)). This conserved signal transduction system allows cells time to correct improper attachments by inhibiting the activity of the anaphase promoting complex (APC), a ubiquitin ligase that targets the anaphase inhibitor Pds1/Securin for destruction. Though the precise signal recognized by the checkpoint is not known, both the lack of kinetochore-microtubule attachments and defects in the tension exerted by microtubule-generated forces on kinetochores result in activation of the spindle checkpoint (for review see, (Pinsky and Biggins, 2005)). Although it is possible that tension and attachment defects activate the spindle checkpoint by separate mechanisms, kinetochore-microtubule attachments are unstable in the absence of tension leading to the possibility that tension defects activate the checkpoint through the generation of unattached kinetochores (Figure 4.1A) (King and Nicklas, 2000; Nicklas and Ward, 1994; Nicklas et al., 1995; Rieder et al., 1995).

An essential regulator of both kinetochore-microtubule attachments and the spindle checkpoint response to tension defects is the Ipl1/Aurora B protein kinase, the conserved catalytic component of the chromosomal passenger complex (for review, see (Vagnarelli and Earnshaw, 2004)). Though Ipl1/Aurora is thought to facilitate proper attachment by destabilizing kinetochore-microtubule interactions that do not generate tension, the mechanism by which it activates the spindle

checkpoint is not known. The simplest explanation is that Ipl1/Aurora responds to tension defects by creating unattached kinetochores that serve as the signal for checkpoint activation. Consistent with this, impairing Ipl1 function in the budding yeast *mtw1-1* kinetochore mutant both restores attachment and satisfies the spindle checkpoint (Pinsky et al., 2003).

To determine whether Ipl1's role in the checkpoint is coupled to the generation of unattached kinetochores, we analyzed a series of temperature sensitive kinetochore mutants in the presence and absence of Ipl1 function for both spindle checkpoint activity and chromosome attachment. We carried out this study in budding yeast where there is a single microtubule-binding site on each kinetochore (Winey et al., 1995), which therefore allows a precise determination of the attachment state. The yeast kinetochore contains distinct subcomplexes that have been organized into inner, central and outer domains based on various criteria (for reviews, see (Biggins and Walczak, 2003; McAinsh et al., 2003)). Because the previously characterized *mtw1-1* mutant has defects in the central kinetochore Mtw1/hMis12 complex, we analyzed mutations that affect the other central kinetochore subcomplexes: *ndc80-1* and *nuf2-61* of the Ndc80/Hec1 complex and *okp1-5* of the Ctf19 complex. Central kinetochore mutations cause severe attachment defects that suggest these complexes are important for microtubule capture (Tanaka et al., 2005). In addition, we analyzed mutations that impair the inner kinetochore Mif2/Cenp-C protein, *mif2-3*, and the outer kinetochore Dam1 complex, *ask1-2*. To establish whether these mutants require Ipl1 to activate the

checkpoint, *mif2-3*, *okp1-5*, *nuf2-61*, *ndc80-1*, and *ask1-2* mutant cells, and each *ipl1-321* double mutant were arrested in G1 with alpha factor (αF), released to the restrictive temperature (37 °C), and monitored by immunoblotting for Pds1 levels (Figure 4.1B). As expected, Pds1 levels cycled in wild type cells and were stabilized in all kinetochore single mutants, indicating a metaphase arrest. In contrast, Pds1 levels cycled similar to wild type in the *ipl1* double mutants, demonstrating that these mutants arrest in metaphase due to Ipl1-dependent spindle checkpoint activation.

We next tested whether the lack of checkpoint arrest in the absence of Ipl1 function correlates with restored attachment in the kinetochore mutants. In *ipl1* mutant cells, sister kinetochores attach to microtubules that arise from the same pole (mono-oriented attachments) resulting the segregation of sister chromatid pairs into the bud (Biggins et al., 1999; Kim et al., 1999; Pinsky et al., 2003; Tanaka et al., 2002). This segregation pattern represents the basal state of kinetochore attachment in the absence of the Ipl1-mediated correction mechanism. To assess attachment in the various kinetochore mutants, we therefore determined whether impairing Ipl1 function allowed the kinetochore mutants to segregate sister chromatids to the bud. This would indicate that the basal state of kinetochore attachment had been restored even though chromosome segregation was still defective. In addition, analyzing segregation to the bud provides an unambiguous determination of the state of attachment because the only way chromosomes can segregate to the bud is via attachment to the spindle.

To analyze chromosome segregation in the mutants, we used a fluorescently labeled chromosome IV (Chr IV). Because the kinetochore mutants activate the spindle checkpoint, we eliminated the spindle checkpoint gene *MAD2* to allow cell cycle progression and comparison with the *ipl1* double mutants. In addition, this control allows us to confirm that the checkpoint itself does not have a role in kinetochore detachment.

Ipl1-321 cells, and each kinetochore mutant combined with either *mad2Δ* or *ipl1-321* were arrested in G1 with αF to mark the mother cell, released to the restrictive temperature, and assayed for the distribution of Chr IV at anaphase. We found two classes of mutants based on scoring chromosome segregation to the bud. In the first class of mutants, unattached chromosomes were generated entirely by Ipl1 activity. A representative of this class, *mif2-3*, is shown in Figure 4.1C. 120 min after release, Chr IV sister chromatids mis-segregated to the bud in 20% of the *mif2-3 mad2Δ* cells. However, chromosome segregation to the bud in *mif2-3 ipl1-321* double mutant cells was nearly identical to *ipl1-321* single mutants, 47% and 46% of the time, respectively. *Nuf2-61, ask1-2*, and the inner kinetochore CBF3 complex mutant *ctf13-30* behaved similarly (Figures 4.1C and 4.2A), as did *mtw1-1* (Pinsky et al., 2003). These data suggest that these mutant kinetochores are competent to make attachments to microtubules (albeit inappropriate ones) and only contained unattached chromosomes due to Ipl1 activity. Because impairing Ipl1 function in all of these mutants both restored attachment and prevented spindle checkpoint arrest, Ipl1's role in the checkpoint appears linked to the generation of

unattached kinetochores. If this were true, kinetochore mutants with unattached chromosomes should not depend on Ipl1 to activate the checkpoint.

However, the second class of mutants appeared to have unattached chromosomes yet still required Ipl1 to activate the checkpoint. This class includes *ndc80-1* and *okp1-5* (Figure 4.1D). The difference between the Ndc80 complex mutants *ndc80-1* and *nuf2-61* likely reflects allele specific differences in the level of residual complex function. In *ndc80-1 mad2Δ* cells, both Chr IV sisters segregated to the bud only 1% of the time. While 44% of *ipl1-321* cells segregated both Chr IV sisters to the bud, this occurred in just 13% of *ndc80-1 ipl1-321* cells. This segregation pattern was not due to a defect in spindle elongation because we observed similar Chr IV distribution when only cells with long or broken down anaphase spindles were counted (Figure 4.2B). In contrast to the first class of mutants, impairing Ipl1 function in *ndc80-1* mutant cells was unable to completely restore segregation to the bud and suggested that there were unattached chromosomes. We therefore did additional attachment analysis of the *ndc80-1 ipl1-321* cells to determine if Ipl1's function in generating unattached chromosomes was separable from its role in the spindle checkpoint.

The segregation to the bud assay analyzes chromosome attachment indirectly by determining the final position of chromosomes late in the cell cycle. In addition, it requires that chromosomes undergo the forces of spindle elongation and movement across the bud neck, two processes that could potentially displace weak attachments. To address these concerns, we monitored attachment in metaphase cells with short

spindles prior to segregation by localizing fluorescently labeled spindles and kinetochores. Wild type, *ipl1-321*, *ndc80-1*, and *ndc80-1 ipl1-321* cells containing Tub1-CFP and Mtw1-GFP3 were arrested in G1 and released to the restrictive temperature. 70 min after release, cells with short spindles were analyzed for Mtw1-GFP3 kinetochore foci. When the foci localized on the same axis as tubulin, we assumed that the kinetochores were attached to the spindle. Kinetochore foci that did not localize with tubulin were considered unattached. Wild type cells displayed the characteristic bilobed kinetochore localization pattern that was always associated with the spindle and indicates complete attachment (Figure 4.3A). Similarly, the kinetochores in *ipl1-321* cells were found on the spindle axis in a bilobed configuration, although the bud proximal focus often appeared brighter than the bud distal focus. In contrast, the kinetochores in *ndc80-1* cells were completely disorganized with most cells containing >2 foci with at least one focus off the spindle axis, as previously described (De Wulf et al., 2003). Strikingly, the kinetochores in *ndc80-1 ipl1-321* cells appeared as organized as in *ipl-321* and wild type cells, suggesting that impairing Ipl1 function had restored attachments enough to silence the checkpoint but not mediate chromosome segregation to the bud. Consistent with this, *ndc80-1 ipl1-321* mutant cells with long spindles had multiple Mtw1 kinetochore foci in the mother cell that were not pulled to the poles (Figure 4.3B). Therefore, it is likely that weak attachments are made in the double mutant that cannot withstand the forces of spindle elongation and the transit necessary to deliver chromosomes to the bud. In support of this idea, *ndc80-1 ipl1-321* cells

contained unattached chromosomes if the nucleus was forced through the bud neck due to a defect in spindle pole duplication (Figure 4.4). Taken together, these results suggest that kinetochores in *ndc80-1 ipl1-321* cells are at least weakly attached to microtubules until the spindle elongates and moves through the bud neck. Therefore, our analysis of kinetochore mutants is consistent with the hypothesis that Ipl1 activates the spindle checkpoint by creating unattached kinetochores.

If this were true, regenerating unattached kinetochores in cells where loss of Ipl1 function had prevented kinetochore detachment should activate the spindle checkpoint. To examine this, we monitored Pds1 levels in wild type, *ndc80-1 ipl1-321*, and *ndc80-1 ipl1-321 mad2Δ* cells treated with nocodazole to depolymerize microtubules and create unattached kinetochores (Figure 4.5). As predicted, Pds1 levels were stabilized in wild type and *ndc80-1 ipl1-321* cells, and cycled in *ndc80-1 ipl1-321 mad2Δ* cells, indicating that spindle checkpoint activity could be restored in the absence of Ipl1 if kinetochore-microtubule interactions were disrupted by another method. This experiment demonstrates that the *ndc80-1 ipl1-321* mutant cells do indeed have kinetochore-microtubule attachments and therefore strongly suggests that Ipl1's role in the spindle checkpoint is linked to the generation of unattached kinetochores.

Ipl1 is thought to regulate kinetochore attachment through the phosphorylation of the outer kinetochore Dam1/DASH/DDD complex, a critical mediator of kinetochore-microtubule interactions and microtubule function. We therefore tested whether this complex is also the Ipl1 target of the spindle checkpoint

as expected if Ipl1's role in the checkpoint is through the generation of unattached kinetochores. We used a special *dam1* phospho-deficient mutant (*dam1 (S257A S265A S292A) spc34 (T199A)*) that has three out of the four Ipl1 consensus sites mutated to alanine in addition to the single site on Spc34, another essential Dam1 complex component (Cheeseman et al., 2002). The *dam1* mutant with all of the consensus Ipl1 sites changed to alanine is inviable and therefore has not been studied. Previous phenotypic analysis of the *dam1* phospho-deficient allele indicated that it made mono-oriented attachments similar to ipl1 mutants (Cheeseman et al., 2002). To determine whether this *dam1* phospho-deficient mutant is also defective in the spindle checkpoint as predicted, wild type, *dam1 (S257A S265A S292A) spc34 (T199A)*, and *dam1 (S257A S265A S292A) spc34 (T199A) mad2Δ* cells were arrested in G1, released to the restrictive temperature (37 °C), and monitored for Pds1 levels over the cell cycle (Figure 4.6A). Strikingly, the *dam1* phospho-deficient cells stabilized Pds1levels in a *MAD2*-dependent manner, indicating checkpoint activation. Therefore, unlike *ipl1* mutant cells, the *dam1* phospho-deficient mutant cells activate the spindle checkpoint.

We next determined whether this checkpoint arrest is Ipl1 dependent. The *dam1* mutant is synthetically lethal with *ipl1-321* (data not shown), so to analyze the consequences of Ipl1 loss of function in the phospho-deficient mutant background we used an analogue-sensitive *ipl1* allele (*ipl1-as5*) that has an enlarged ATP binding pocket to allow specific inhibition by bulky ATP analogue inhibitors (for review, see (Bishop et al., 2001)). Kinase assays *in vitro* verified that Ipl1-as5

protein was completely inhibited by the ATP analogue 1-NA-PP1 whereas wild type Ipl1 activity was unaffected (data not shown). Similar to analog-sensitive alleles of other kinases (Jones et al., 2005; Weiss et al., 2000), the Ipl1-as5 mutant protein also had reduced basal kinase activity *in vitro* compared to wild type Ipl1. To increase overall kinase activity *in vivo* and obtain conditional *ipl1 dam1* phospho-deficient mutant cells for analysis, we expressed *ipl1-as5* using the galactose inducible promoter (*pGAL*). This promoter is highly transciptionally active when cells are grown in galactose (inducing conditions) and is repressed when cells are grown in glucose (non-inducing conditions).

To determine whether Ipl1 function is required for the *dam1* phospho-deficient checkpoint arrest, duplicate cultures of *dam1 (S257A S265A S292A) spc34 (T199A)* and *dam1 (S257A S265A S292A) spc34 (T199A) pGAL-ipl1-as5* cells were arrested in G1 under inducing conditions, released into non-inducing media at the restrictive temperature, and monitored for Pds1 levels by immunoblotting (Figure 4.6B). 80 min after release, we added either DMSO as a control or 1-NA-PP1 to inactivate any remaining ipl1-as5. Pds1 levels remained high in both strains treated with DMSO, as well as inhibitor treated *dam1* phospho-deficient cells. However, Pds1 was degraded within 10 min of inhibitor addition in the *dam1 (S257A S265A S292A) spc34 (T199A) pGAL-ipl1-as5* cells indicating that the *dam1* phospho-deficient mutant depends on Ipl1 to activate the spindle checkpoint. Therefore, it is likely that there are additional phosphorylation sites on the Dam1 complex or other

targets that must be phosphorylated by Ipl1 to properly regulate both kinetochore attachment and the spindle checkpoint.

Our analyses indicate that the attachment defects in many kinetochore mutants are the indirect consequence of Ipl1-mediated detachment and are not due to the direct defect of a particular kinetochore protein. Instead, it appears that most mutant kinetochores actually attach to microtubules, making it critical that the cell has a mechanism to sense and correct these improper, tension defective attachments. This work is consistent with the hypothesis that Ipl1 is the kinetochore's tension sensor and is responsible for the instability of kinetochore attachments that are not under tension. In addition, our data suggests that conclusions about the role of individual kinetochore complexes in microtubule attachment based on the analysis of kinetochore mutants in the presence of Ipl1 function may need to be re-evaluated.

These data strongly suggest that Ipl1 potentiates spindle checkpoint activation by creating unattached kinetochores. We were unable to identify a situation where Ipl1's function in kinetochore attachment was uncoupled from its role in the spindle checkpoint and even the severely compromised *ndc80* mutant kinetochores were able to make weak attachments in the absence of Ipl1 function. Consistent with this, generating unattached kinetochores in the *ndc80 ipl1* mutant cells by spindle depolymerization restored the spindle checkpoint, arguing that these cells must have weakly attached kinetochores. Though it is possible that there exists a special *IPL1* separation of function allele that would allow cells to bypass the checkpoint in the presence of unattached kinetochores, we favor the model (Figure

4.7) in which Ipl1 both corrects attachment errors and engages the spindle checkpoint by destabilizing improper kinetochore-microtubule interactions. In addition, our data provide the strongest demonstration so far that the primary signal that is recognized by the spindle checkpoint is an unattached kinetochore, and that tension defects must be converted to attachment defects to engage the checkpoint. Therefore, tension and attachment appear to be intimately linked, as previously suggested (King and Nicklas, 2000; Nicklas et al., 2001).

It is likely that the role of Ipl1 in generating unattached kinetochores to allow spindle checkpoint activation is conserved because Aurora B has also been shown to be required for tension defects to activate the checkpoint (Carvalho et al., 2003; Ditchfield et al., 2003; Hauf et al., 2003; Lens et al., 2003). However, it will be interesting to determine whether organismal differences in the kinetochore's microtubule binding capacity affect the requirement for Aurora activity in spindle checkpoint activation. Because both spindle checkpoint and Aurora B dysfunction are associated with many cancers and remain potential targets for cancer therapy (for reviews, see (Bharadwaj and Yu, 2004; Draviam et al., 2004)), our findings may have important implications for our understanding and treatment of human disease.

Experimental Procedures

Microbial Techniques. Media and microbial techniques were essentially as described (Rose et al., 1990; Sherman et al., 1974). All experiments in which cells were released from a G1 arrest were carried out by αF arrest and release, using αF at 1 μg/ml for *bar1Δ* strains and 10 μg/ml for *BAR1* strains. 1-naphthyl-pyrazolo[3,4-d]pyrimidine (1-NA-PP1) was used at 50 μM and Nocodazole was used at 10 μg/ml.

Yeast strain construction. Yeast strains are listed in Table 4.1 and were constructed by standard genetic techniques. All strains were generated for this study and are isogenic with the W303 background except SBY4172 and SBY4199, which are in the S288c background. The *dam1 (S257A S265A S292A) spc34 (T199A)* strains (Cheeseman et al., 2002) (a gift from David Drubin and Georjana Barnes, University of California, Berkeley, CA) were backcrossed at least 5 times into the W303 background. As reported for the S288c background (Cheeseman et al., 2002), the *dam1 (S257A S265A S292A) spc34 (T199A)* combination was temperature sensitive, while the individual alleles, *dam1 (S257A S265A S292A) and spc34 (T199A)*, were not (Figure 4.8A). In addition, the *dam1 (S257A S265A S292A) spc34 (T199A)* mutant activated the spindle checkpoint in the original S288c background (Figure 4.8B). The *mif2-3* strains (Brown et al., 1993) (a gift from Anthony Hyman, Max-Planck-Institute of Molecular Cell Biology and Genetics, Dresden, Germany) were also backcrossed at least 5 times into the W303 background. The *nuf2-61* (Osborne et al., 1994) (a gift from Pamela Silver, Harvard Medical School, Boston, MA),

ndc80-1 (Wigge et al., 1998) (a gift from John Kilmartin, MRC Laboratory of Molecular Biology, Cambridge, England), *ask1-2* (Li et al., 2002) (a gift from Stephen Elledge, Harvard Medical School, Boston, MA), *spc29-3* (Elliott et al., 1999) (a gift from Mark Winey, University of Colorado, Boulder, CO), and *ipl1-321* (Biggins et al., 1999) alleles were crossed to make strains for this study. Strains containing *PDS1-myc18:LEU2* were made by integrating plasmid pSB205 (a gift from Kim Nasmyth, Research Institute of Molecular Pathology, Vienna, Austria) digested with *HindIII* at the *PDS1* locus. Strains containing *SPC42-GFP* were made by integrating plasmid pSB208 (a gift from John Kilmartin, MRC Laboratory of Molecular Biology, Cambridge, England) digested with *Bsu36I* at the *TRP1* locus. Strains containing *MTW1-GFP3* were generated by integrating plasmid pSB818 digested with *SnaBI* at the *MTW1* locus. Strains containing *TUB1-CFP:URA3* were obtained by integrating plasmid pSB375 (a gift from Kerry Bloom, University of North Carolina, Chapel Hill, NC) digested with *StuI* at the *URA3* locus. Strains containing *mad2::URA3* were generated by integration of plasmid pSB99 digested with *EcoRI-HindIII*. Other deletions in yeast genes were made using a PCR-based integration system (Longtine et al., 1998) and were confirmed by PCR. Specific primer sequences are available upon request. All fusion proteins are fully functional.

Generation of the ipl1-as5 strains. The *ipl1-as5* allele has the mutations M181G and T244A, which confer inhibitor sensitivity, and were generated as described (Kung et al., 2005). The *ipl1-as5:LEU2* integrating plasmid was made by digesting pSB338

with *BamHI-HindIII* and ligating the fragment containing *ipl1-as5* into the *BamHI-HindIII* sites of plasmid pSB425, to create pSB428. To create the *pGAL-ipl1-as5* strain, *MluI* digested pSB425 was integrated upstream of the *IPL1* locus in an *ipl1* deletion strain covered by the *IPL1, CEN, URA3* plasmid, pSB148. Loss of pSB148 was selected for on 5-FOA and the *pGAL* promoter was introduced using a PCR-based integration system (Longtine et al., 1998).

Plasmid Construction. The *MTW1-GFP3* integrating plasmid was made by PCR amplification of the C-terminal 600 base pairs of *MTW1* using primers SB844 and SB845, that have *EcoRI* and *BamHI* restriction sites engineered, respectively. The resulting PCR product was digested with *EcoRI-BamHI* and ligated into the *EcoRI-BamHI* sites of PB1585 (a gift from David Pellman, Harvard Medical School, Boston, MA), to create pSB818.

Microscopy. Analysis of fixed cells was performed as described (Biggins et al., 1999). DAPI (Molecular Probes) was used at 1μg/ml final concentration. At least 100 cells were analyzed for all reported experiments. The error bars represent the 95% confidence interval.

Protein and immunological techniques. Protein extracts were made and immunoblotted as described (Minshull et al., 1996). 9E10 antibodies that recognize the myc epitope were obtained from Covance and used at a 1:10,000 dilution. Equal

protein loading was confirmed in all experiments by anti-tubulin immunoblotting (data not shown).

Table 4.1 *Yeast strains used in this study*

Strain	Genotype
SBY322	*MAT**a** ura3-1 leu2,3-112 his3-11:pCUP1-GFP12-lacI12:HIS3 trp1-1:256lacO:TRP1 lys2Δ ade2-1 can1-100 bar1Δ ipl1-321*
SBY818	*MAT**a** ura3-1 leu2,3-112 his3-11:pCUP1-GFP12-lacI12:HIS3 trp1-1:256lacO:TRP1 lys2Δ ade2-1 can1-100 bar1Δ PDS1-myc18:LEU2*
SBY1433	*MAT**a** ura3-1 leu2,3-112 his3-11:pCUP1-GFP12-lacI12:HIS3 trp1-1:256lacO:TRP1 ade2-1 can1-100 bar1Δ PDS1-myc18:LEU2 ask1-2 ipl1-321*
SBY1440	*MAT**a** ura3-1 leu2,3-112 his3-11:pCUP1-GFP12-lacI12:HIS3 trp1-1:256lacO:TRP1 ade2-1 can1-100 bar1Δ ask1-2 ipl1-321*
SBY1444	*MAT**a** ura3-1 leu2,3-112 his3-11:pCUP1-GFP12-lacI12:HIS3 trp1-1:256lacO:TRP1 lys2Δ ade2-1 can1-100 bar1Δ PDS1-myc18:LEU2 ask1-2*
SBY2063	*MAT**a** ura3-1 leu2,3-112 his3-11:pCUP1-GFP12-lacI12:HIS3 trp1-1:256lacO:TRP1 ade2-1 can1-100 bar1::KAN PDS1-myc18:LEU2 nuf2-61 ipl1-321*
SBY2064	*MAT**a** ura3-1 leu2,3-112 his3-11:pCUP1-GFP12-lacI12:HIS3 trp1-1:256lacO:TRP1 ade2-1 can1-100 bar1::KAN PDS1-myc18:LEU2 nuf2-61*
SBY2497	*MAT**a** ura3-1 leu2,3-112 his3-11:pCUP1-GFP12-lacI12:HIS3 trp1-1:256lacO:TRP1 ade2-1 can1-100 bar1Δ PDS1-myc18:LEU2 mif2-3*
SBY2810	*MAT**a** ura3-1 leu2,3-112 his3-11:pCUP1-GFP12-lacI12:HIS3 trp1-1 lys2Δ ade2-1 can1-100 bar1Δ PDS1-myc18:LEU2 mif2-3 ipl1-321*
SBY2857	*MAT**a** ura3-1 leu2,3-112 his3-11:pCUP1-GFP12-lacI12:HIS3 trp1-1:256lacO:TRP1 lys2Δ ade2-1 can1-100 bar1Δ PDS1-myc18:LEU2 ndc80-1 ipl1-321*
SBY2877	*MAT**a** ura3-1:TUB1-CFP:URA3 leu2,3-112 his3-11:pCUP1-GFP12-lacI12:HIS3 trp1-1:256lacO:TRP1 lys2Δ ade2-1 can1-100 bar1Δ*
SBY2910	*MAT**a** ura3-1 leu2,3-112 his3-11:pCUP1-GFP12-lacI12:HIS3 trp1-1:256lacO:TRP1 lys2D ade2-1 can1-100 bar1Δ PDS1-myc18:LEU2 ctf13-30 ipl1-321*
SBY2929	*MAT**a** ura3-1 leu2,3-112 his3-11:pCUP1-GFP12-lacI12:HIS3 trp1-1:256lacO:TRP1 ade2-1 can1-100 bar1Δ mif2-3 ipl1-321*
SBY2956	*MAT**a** ura3-1 leu2,3-112 his3-11:pCUP1-GFP12-lacI12:HIS3 trp1-1 lys2Δ ade2-1 can1-100 bar1Δ PDS1-myc18:LEU2 okp1-5:TRP1*
SBY2957	*MAT**a** ura3-1 leu2,3-112 his3-11:pCUP1-GFP12-lacI12:HIS3 trp1-1 lys2Δ ade2-1 can1-100 bar1Δ PDS1-myc18:LEU2 okp1-5:TRP1 ipl1-321*
SBY2987	*MAT**a** ura3-1:TUB1-CFP:URA3 leu2,3-112 his3-11:pCUP1-GFP12-lacI12:HIS3 trp1-1:256lacO:TRP1 lys2Δ ade2-1 can1-100 bar1Δ PDS1-myc18:LEU2 ndc80-1 ipl1-321*
SBY3000	*MAT**a** ura3-1 leu2,3-112 his3-11:pCUP1-GFP12-lacI12:HIS3 trp1-1:256lacO:TRP1 lys2Δ ade2-1 can1-100 bar1Δ ndc80-1 mad2::URA3*

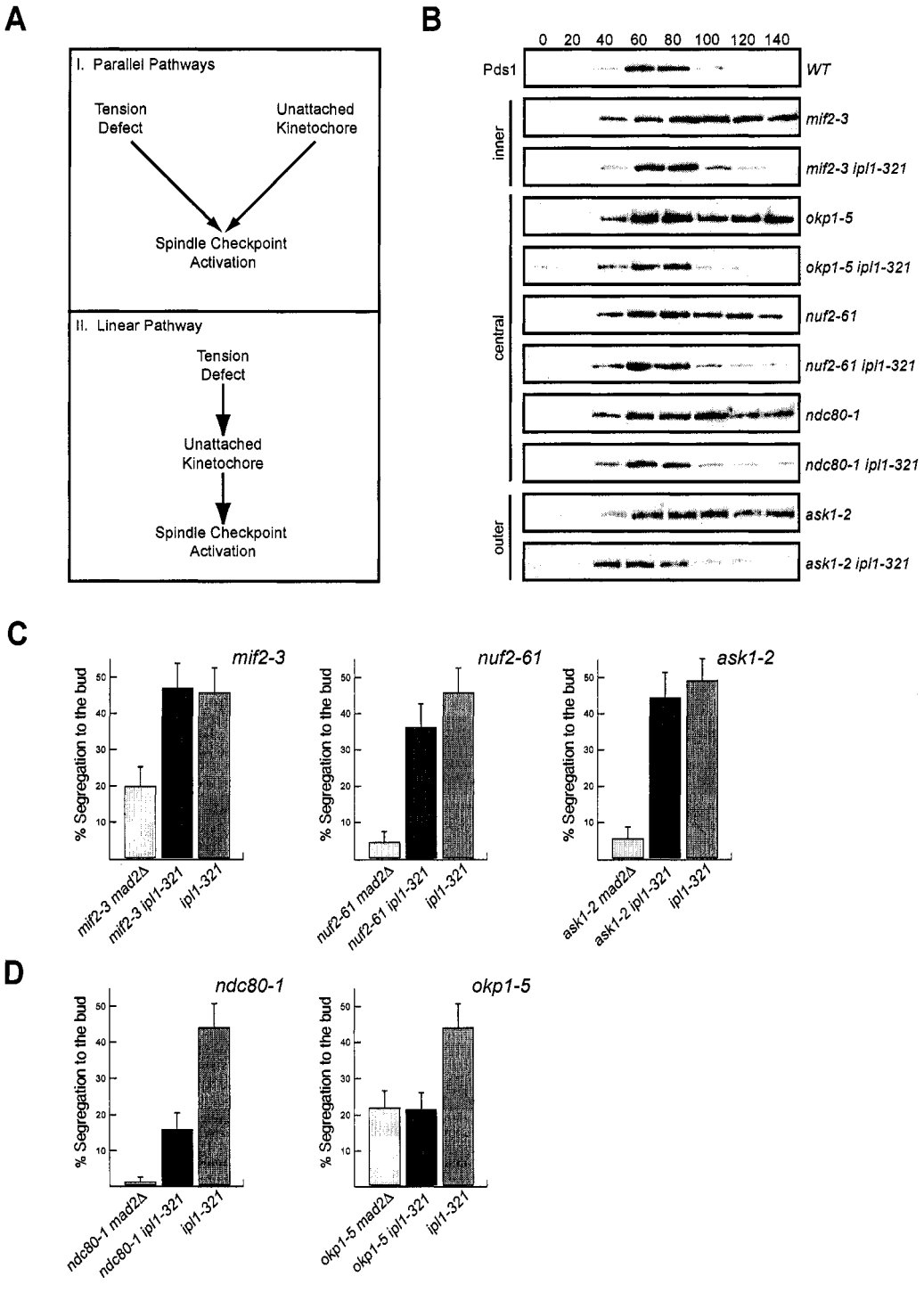
Table 4.1 *continued*

Strain	Genotype
SBY3015	*MATa ura3-1 leu2,3-112 his3-11:pCUP1-GFP12-lacI12:HIS3 trp1-1:256lacO:TRP1 lys2Δ ade2-1 can1-100 bar1Δ ctf13-30 mad2::URA3*
SBY3022	*MATa ura3-1 leu2,3-112 his3-11:pCUP1-GFP12-lacI12:HIS3 trp1-1:256lacO:TRP1 lys2Δ ade2-1 can1-100 bar1Δ mif2-3 mad2::URA3*
SBY3023	*MATa ura3-1 leu2,3-112 his3-11:pCUP1-GFP12-lacI12:HIS3 trp1-1:256lacO:TRP1 ade2-1 can1-100 bar1Δ okp1-5:TRP1 mad2::URA3*
SBY3025	*MATa ura3-1:TUB1-CFP:URA3 leu2,3-112 his3-11:pCUP1-GFP12-lacI12:HIS3 trp1-1:256lacO:TRP1 lys2Δ ade2-1 can1-100 bar1Δ ipl1-321*
SBY3148	*MATa ura3-1 leu2,3-112 his3-11:pCUP1-GFP12-lacI12:HIS3 trp1-1:256lacO:TRP1 ade2-1 can1-100 bar1Δ nuf2-61 mad2::URA3*
SBY3186	*MATa ura3-1 leu2,3-112 his3-11:pCUP1-GFP12-lacI12:HIS3 trp1-1:256lacO:TRP1 lys2Δ ade2-1 can1-100 bar1Δ nuf2-61 ipl1-321*
SBY3702	*MATa ura3-52:spc34(T199A):URA3 leu2,3-112 his3-11 trp1-1 lys2Δ ade2-1 can1-100 bar1Δ dam1(S257A S265A S292A):KAN spc34::HIS3 PDS1-myc18:LEU2*
SBY3801	*MATa ura3-52:spc34(T199A):URA3 leu2,3-112 his3-11 trp1-1 lys2Δ ade2-1 can1-100 bar1Δ dam1(S257A S265A S292A):KAN spc34::HIS3 mad2::URA3 PDS1-myc18:LEU2*
SBY4006	*MATa ura3-1 leu2,3-112 his3-11 trp1-1:SPC42-GFP:TRP1 ade2-1 can1-100 bar1Δ spc29-3 ipl1-321*
SBY4007	*MATa ura3-1 leu2,3-112 his3-11 trp1-1:SPC42-GFP:TRP1 ade2-1 can1-100 bar1Δ PDS1-myc18:LEU2 spc29-3 ndc80-1 ipl1-321*
SBY4042	*MATa ura3-1 leu2,3-112 his3-11 trp1-1:SPC42-GFP:TRP1 ade2-1 can1-100 bar1Δ spc29-3 ndc80-1 mad2::URA3*
SBY4172	*MATa ura3-52 leu2Δ1 his3Δ200 trp1Δ63 lys2-801 ade2-101 PDS1-myc18:LEU2*
SBY4199	*MATa ura3-52:spc34(T199A):URA3 leu2Δ1 his3Δ200 trp1Δ63 lys2-801 ade2-101 dam1(S257A S265A S292A):KAN spc34::HIS3 PDS1-myc18:LEU2*
SBY4326	*MATa ura3-52:spc34(T199A):URA3 leu2,3-112 his3-11:pCUP1-GFP12-lacI12:HIS3 trp1-1:256lacO:TRP1 lys2Δ ade2-1 can1-100 bar1Δ dam1(S257A S265A S292A):KAN spc34::HIS3 mad2::URA3*
SBY4329	*MATa ura3-52:spc34(T199A):URA3 leu2,3-112 his3-11 trp1-1:256lacO:TRP1 lys2Δ ade2-1 can1-100 bar1Δ dam1(S257A S265A S292A):KAN spc34::HIS3 PDS1-myc18:LEU2 pGAL-ipl1-as5:LEU2:HIS3:ipl1::KAN*
SBY4340	*MATa ura3-1:TUB1-CFP:URA3 leu2,3-112 his3-11 trp1-1 ade2-1 can1-100 bar1Δ MTW1-GFP3:HIS3*
SBY4341	*MATa ura3-1:TUB1-CFP:URA3 leu2,3-112 his3-11 trp1-1 ade2-1 can1-100 bar1Δ MTW1-GFP3:HIS3 ndc80-1*

Table 4.1 *continued*

Strain	Genotype
SBY4343	*MAT**a** ura3-1:TUB1-CFP:URA3 leu2,3-112 his3-11 trp1-1 ade2-1 can1-100 bar1Δ MTW1-GFP3:HIS3 ipl1-321*
SBY4393	*MAT**a** ura3-1 leu2,3-112 his3-11 trp1-1:SPC42-GFP:TRP1 ade2-1 can1-100 bar1Δ spc29-3 mad2::URA3*
SBY4631	*MAT**a** ura3-1:TUB1-CFP:URA3 leu2,3-112 his3-11:pCUP1-GFP12-lacI12:HIS3 trp1-1:256lacO:TRP1 lys2Δ ade2-1 can1-100 bar1Δ ndc80-1 mad2::KAN*
SBY5005	*MAT**a** ura3-1 leu2,3-112 his3-11 trp1-1 lys2Δ ade2-1 can1-100 bar1Δ PDS1-myc18:LEU2 ndc80-1 ipl1-321 mad2::URA3*

Figure 4.1 Analysis of kinetochore mutants in the presence and absence of Ipl1 function reveals two classes of mutants. (A) Models for the role of kinetochore tension defects in spindle checkpoint activation. I. Tension defects signal checkpoint activation independent of an unattached kinetochore. II. Tension defects cause attachment instability and it is the unattached kinetochore that is recognized by the checkpoint. (B) Inner, central, and outer kinetochore mutants activate the spindle checkpoint in an *IPL1*-dependent manner. Wild type (SBY818), *mif2-3* (SBY2497), *mif2-3 ipl1-321* (SBY2810), *okp1-5* (SBY2956), *okp1-5 ipl1-321* (SBY2957), *nuf2-61* (SBY2064), *nuf2-61 ipl1-321* (SBY2063), *ndc80-1* (SBY2250), *ndc80-1 ipl1-321* (SBY2857), *ask1-2* (SBY1444) and *ask1-2 ipl1-321* (SBY1433) cells containing Pds1-myc18 were arrested in G1 and released to 37 °C. Lysates were prepared at the indicated time points and immunoblotted with anti-myc antibodies. (C) Impairing Ipl1 function restores *mif2-3*, *nuf2-61*, and *ask1-2* kinetochore attachment. *ipl1-321* (SBY322), *mif2-3 mad2Δ* (SBY3022), *mif2-3 ipl1-321* (SBY2929), *nuf2-61 mad2Δ* (SBY3148), *nuf2-61 ipl1-321* (SBY3186), *ask1-2 mad2Δ* (SBY3001), and *ask1-2 ipl1-321* (SBY1440), cells containing fluorescently marked Chr IV were arrested in G1 and released to 37 °C for 120 min. Large budded cells were monitored for the segregation of both Chr IV sister chromatids to the bud. (D) Impairing Ipl1 function does not fully restore *ndc80-1* and *okp1-5* kinetochore attachment. *ipl1-321* (SBY322), *ndc80-1 mad2Δ* (SBY3000), *ndc80-1 ipl1-321* (SBY2857), *okp1-5 mad2Δ* (SBY3023), and *okp1-5 ipl1-321* (SBY3083) cells were processed as in (C).


A
I. Parallel Pathways
Tension Defect
Unattached Kinetochore
Spindle Checkpoint Activation
II. Linear Pathway
Tension Defect
Unattached Kinetochore
Spindle Checkpoint Activation
B
0 20 40 60 80 100 120 140
Pds1
WT
inner
mif2-3
mif2-3 ipl1-321
central
okp1-5
okp1-5 ipl1-321
nuf2-61
nuf2-61 ipl1-321
ndc80-1
ndc80-1 ipl1-321
outer
ask1-2
ask1-2 ipl1-321
C
mif2-3
nuf2-61
ask1-2
% Segregation to the bud
mif2-3 mad2Δ
mif2-3 ipl1-321
ipl1-321
nuf2-61 mad2Δ
nuf2-61 ipl1-321
ipl1-321
ask1-2 mad2Δ
ask1-2 ipl1-321
ipl1-321
D
ndc80-1
okp1-5
% Segregation to the bud
ndc80-1 mad2Δ
ndc80-1 ipl1-321
ipl1-321
okp1-5 mad2Δ
okp1-5 ipl1-321
ipl1-321

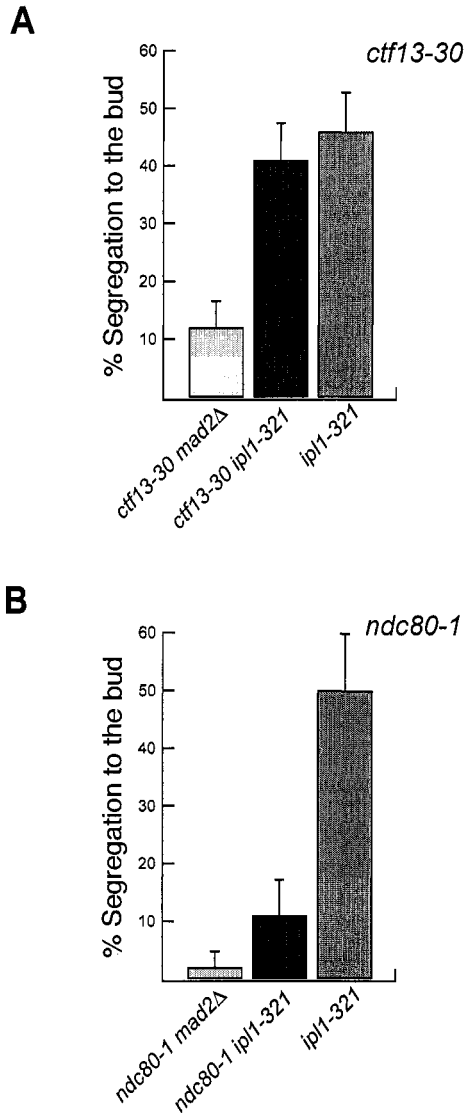


Figure 4.2 (A) Impairing Ipl1 function restores chromosome segregation to the bud in *ctf13-30* mutant cells. *ipl1-321* (SBY322), *ctf13-30 mad2*Δ (SBY3015) and *ctf13-30 ipl1-321* (SBY2910) cells containing fluorescently marked Chr IV were arrested in G1 and released to 37 °C for 120 min. Large budded cells were monitored for the segregation of both Chr IV sister chromatids to the bud. (B) The failure to restore segregation to the bud in *ndc80-1 ipl1-321* mutants is not due to defective spindle elongation. *ipl1-321* (SBY3025), *ndc80-1 mad2*Δ (SBY4631) and *ndc80-1 ipl1-321* (SBY2987) cells expressing Tub1-CFP and fluorescently marked Chr IV were released from G1 to 37 °C. 100 minutes after release, large budded cells with long or broken down spindles were analyzed as in (A).

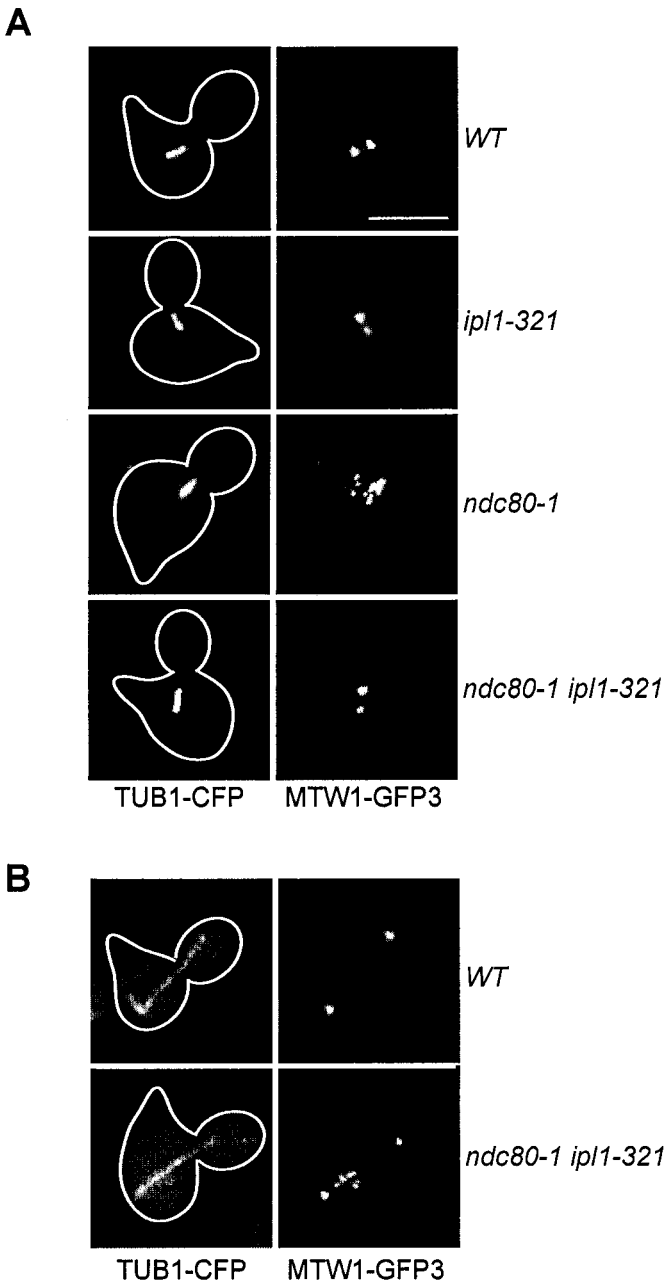
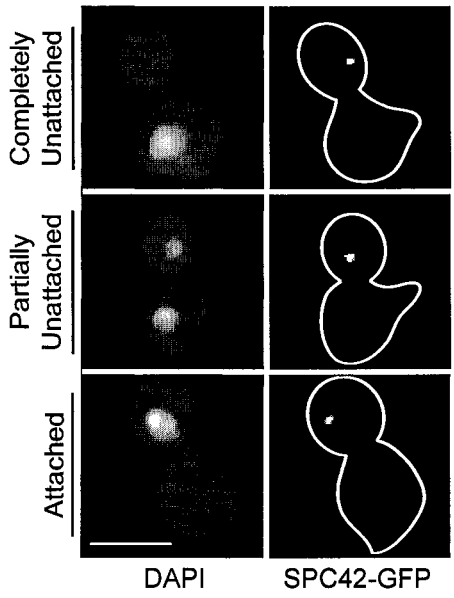


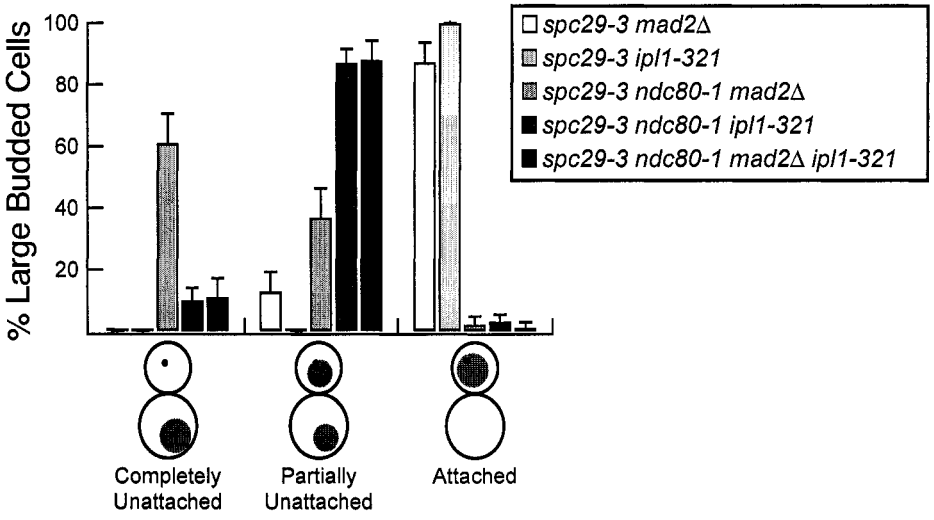
Figure 4.3 Ipl1 is responsible for generating unattached kinetochores in metaphase *ndc80-1* mutant cells. (A) Wild type (SBY4340), *ipl1-321* (SBY4343), *ndc80-1* (SBY4341), and *ndc80-1 ipl1-321* (SBY4342) cells expressing Tub1-CFP and Mtw1-GFP3 were released from G1 to 37 °C. 70 min after release, cells with short spindles were monitored for Mtw1-GFP3 kinetochore foci. (B) Anaphase *ndc80-1 ipl1-321* cells have Ipl1-independent attachment defects. Wild type (SBY4340) and *ndc80-1 ipl1-321* (SBY4342) cells were grown as in (A) and cells with long spindles were analyzed for Mtw1-GFP3 localization. Bar, 5μm.

Figure 4.4 Movement across the bud neck displaces weak attachments. (A, B) *ndc80-1* mutant kinetochores cannot support DNA movement across the bud neck in the absence of spindle elongation. Spc29 is an essential component of the spindle pole body (SPB). At the restrictive temperature, *spc29-3* mutant cells fail SPB duplication and therefore cannot undergo spindle elongation (Elliott et al., 1999). However, the sole functional SPB is pulled into the bud during anaphase, allowing us to visualize unattached chromosomes by determining whether any non-SPB associated DNA remained in the mother cell. *spc29-3 mad2Δ* (SBY4393), *spc29-3 ipl1-321* (SBY4006), *spc29-3 ndc80-1 mad2Δ* (SBY4042), *spc29-3 ndc80-1 ipl1-321* (SBY4007), and *spc29-3 ndc80-1 ipl1-321 mad2Δ* (SBY4574) cells expressing Spc42-GFP to mark the SPB were released from G1 to 37 °C. 100 minutes after release, large budded cells with a single SPB in the bud were scored for whether their DNA (DAPI) was completely unattached, partially unattached, or fully attached to the SPB. (C) The number of cells with a single SPB in the bud is correlated with the level of unattached DNA, suggesting that weak attachments might be displaced when chromosomes are pulled across the bud neck. 100 minutes after release, large budded cells with a single SPB were scored for whether the SPB was in the bud.

A


Completely Unattached
Partially Unattached
Attached
DAPI
SPC42-GFP

B


% Large Budded Cells
100
80
60
40
20
spc29-3 mad2Δ
spc29-3 ipl1-321
spc29-3 ndc80-1 mad2Δ
spc29-3 ndc80-1 ipl1-321
spc29-3 ndc80-1 mad2Δ ipl1-321
Completely Unattached
Partially Unattached
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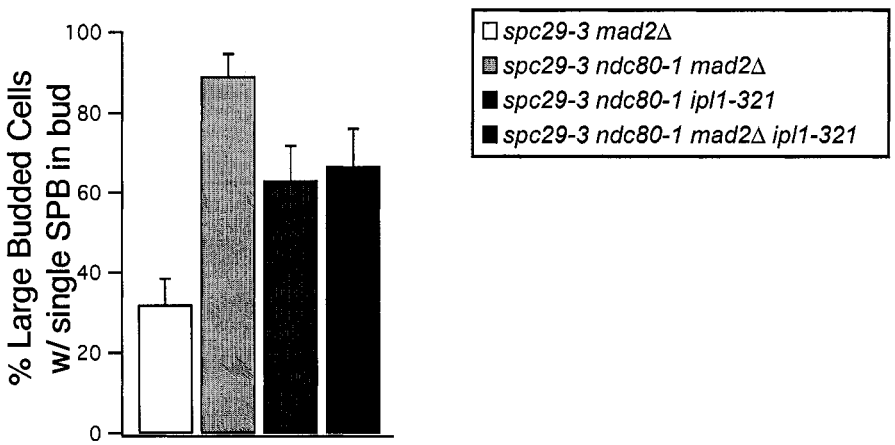
C


% Large Budded Cells w/ single SPB in bud
100
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spc29-3 mad2Δ
spc29-3 ndc80-1 mad2Δ
spc29-3 ndc80-1 ipl1-321
spc29-3 ndc80-1 mad2Δ ipl1-321

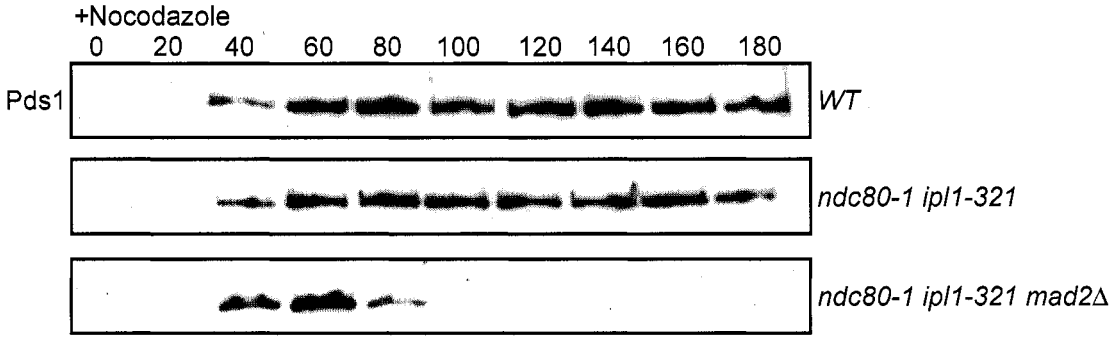


Figure 4.5 Spindle checkpoint activity is restored in *ndc80-1 ipl1-321* mutant cells by regenerating unattached kinetochores. Wild type (SBY818), *ndc80-1 ipl1-321* (SBY2857), and *ndc80-1 ipl1-321 mad2*Δ (SBY5005) cells containing Pds1-myc18 were arrested in G1 and released into nocodazole at 37 °C. Lysates were prepared at the indicated time points and immunoblotted with anti-myc antibodies.

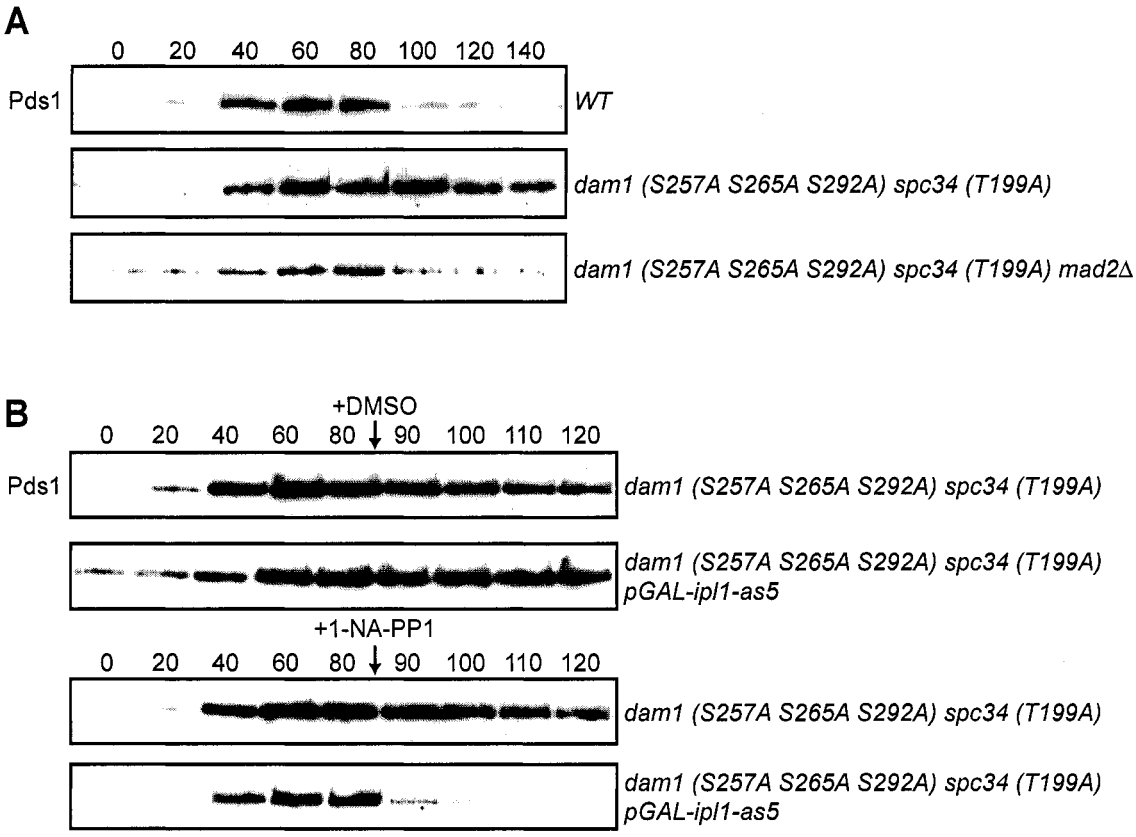


Figure 4.6 The Ipl1-consensus phosphorylation sites on Dam1 are not required for spindle checkpoint activation. (A) The *dam1* phospho-deficient mutant activates the spindle checkpoint. Wild type (SBY 818), *dam1 (S257A S265A S292A) spc34 (T199A)* (SBY 3702), and *dam1 (S257A S265A S292A) spc34 (T199A) mad2Δ* mutant cells (SBY3801) containing Pds1-myc18 were arrested in G1 and released to 37 °C. Lysates were prepared at the indicated time points and immunoblotted with anti-myc antibodies. (B) Ipl1 is required for the *dam1* phospho-deficient mutant to activate the checkpoint. Duplicate cultures of *dam1 (S257A S265A S292A) spc34 (T199A)* (SBY3702) and *dam1 (S257A S265A S292A) spc34 (T199A) pGAL-ipl1-as5* (SBY4329) cells containing Pds1-myc18 were grown in inducing media, arrested in G1, and released to 37 °C in non-inducing media. Control DMSO or1-NA-PP1 inhibitor was added at 80 min. Lysates were prepared as in (A).

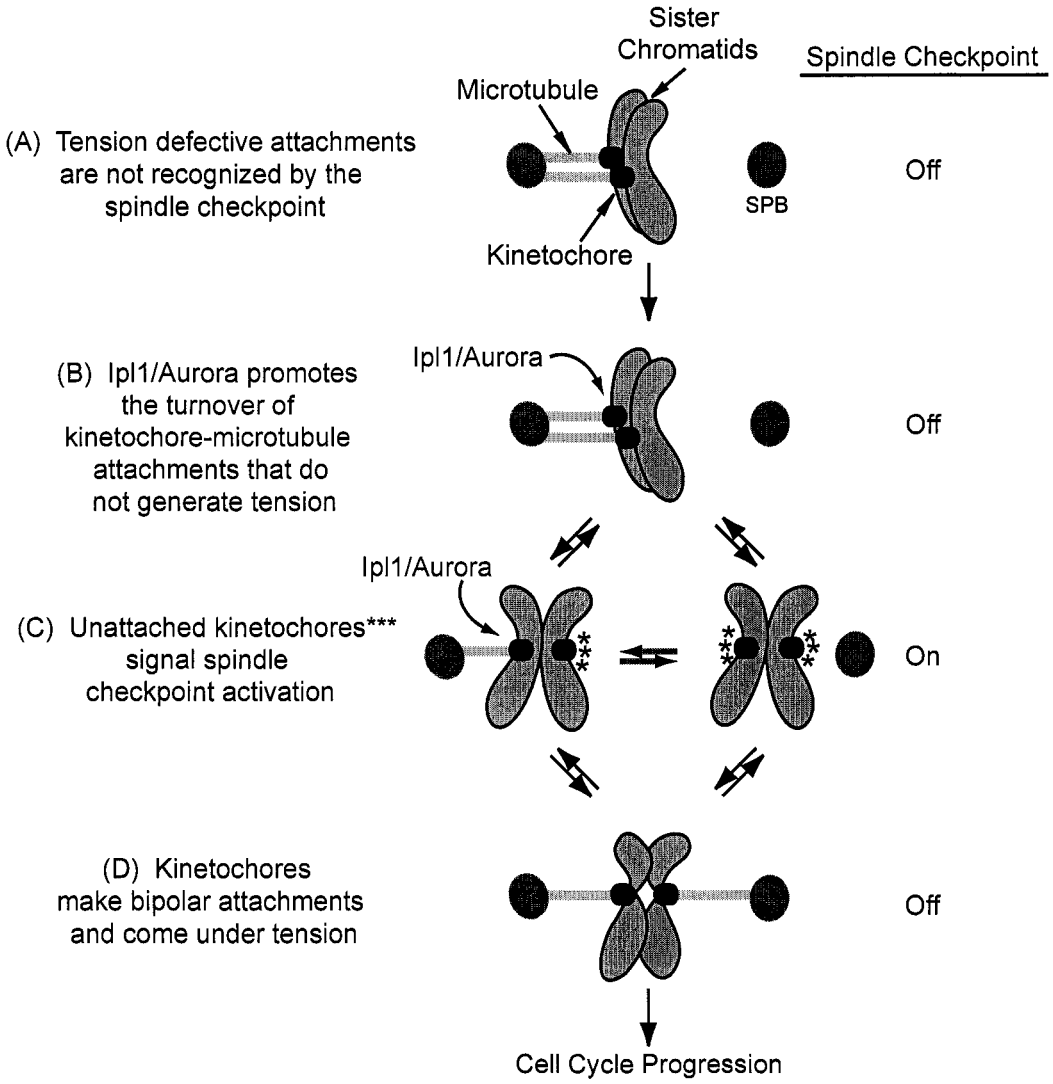


Figure 4.7 Model for Ipl1/Aurora in mitotic repair: Ipl1/Aurora corrects improper attachments and activates the spindle checkpoint by creating unattached kinetochores. (A) Mono-oriented attachments and other kinetochore-microtubule interactions that do not generate tension are not directly sensed by the spindle checkpoint. (B) Ipl1/Aurora recognizes tension defects and promotes the instability of improper attachments. (C) Ipl1/Aurora-mediated kinetochore detachment creates unoccupied microtubule binding sites that are the primary signal for spindle checkpoint activation. (D) The checkpoint halts the cell cycle until kinetochores make bipolar attachments and come under tension.

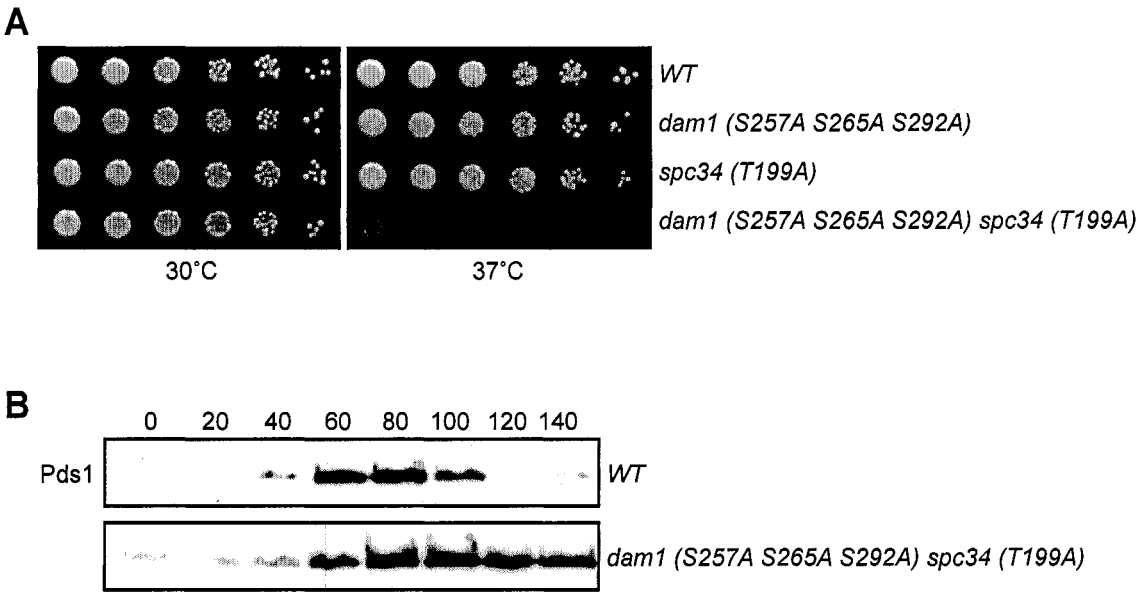


Figure 4.8 Strain background difference does not account for the *dam1* phospho-deficient mutant phenotype. (A) The Ipl1 consensus sites on Dam1 and Spc34 must be mutated to alanine in combination to create the temperature sensitive *dam1* phospho-deficient mutant. 5-fold serial dilutions of wild type (SBY214), *dam1 (S257A S265A S292A)* (SBY3783), *spc34 (T199A)* (SBY4185), and *dam1 (S257A S265A S292A) spc34 (T199A)* (SBY3702) W303 strains, were incubated for 1 day at 30 °C and 37 °C. S288c *dam1* phospho-deficient strains show the same requirement for temperature sensitivity (Cheeseman et al., 2002). (B) The *dam1* phospho-deficient mutant activates the spindle checkpoint in the S288c background. Wild type (SBY4172) and *dam1 (S257A S265A S292A) spc34 (T199A)* (SBY4199) cells containing Pds1-myc18 were arrested in G1 and released to 37 °C. Lysates were prepared at the indicated time points and immunoblotted with anti-myc antibodies.

CHAPTER 5:

The phosphoregulation of chromosome segregation requires the balance of Glc7/Protein Phosphatase-1 and Ipl1/Aurora protein kinase activities

Summary

Faithful chromosome segregation depends on the opposing activities of the budding yeast Glc7/PP1 protein phosphatase and Ipl1/Aurora protein kinase. We isolated seven newly identified *ipl1-321* dosage suppressors (*SDS22, BUD14, YPL137c, FUN21, SOL1, SOL2, PEX31*), found that they all encode proteins that physically interact with Glc7, and showed that they likely restore the balance of kinase/phosphatase activity by redistributing Glc7 away from the targets relevant to Ipl1's essential functions. The phosphorylation of the Ipl1 substrate, Dam1, was affected by decreased Glc7 activity, whereas Ipl1 levels, localization, and kinase activity were not. We therefore propose that Ipl1 and Glc7 ensure accurate chromosome segregation by regulating the phosphorylation of common targets.

Introduction

The accurate and orderly partitioning of the genome during mitosis requires the precise regulation of the connection between chromosomes and the mitotic spindle. This fundamental interaction is mediated by the kinetochore, a specialized protein complex that assembles on centromeric DNA and facilitates the capture of dynamic spindle microtubules that arise from opposite poles. These bipolar attachments (also called amphitelic or bioriented attachments) promote high fidelity chromosome segregation by ensuring that the spindle forces on the replicated chromosomes (sister chromatids) are directed away from one another. Furthermore, these poleward directed spindle forces generate tension on the sister kinetochores because the sister chromatids are physically linked by cohesion. Once all chromosomes make bipolar attachments and come under tension during metaphase, the cell transitions to anaphase where cohesion is dissolved allowing sister chromatids to be pulled to opposite poles. Failure to achieve bipolar attachments results in chromosome missegregation if left uncorrected and this aneuploid state predisposes multicellular organisms to the development of a variety of cancers and birth defects. To prevent the premature segregation of improperly attached chromosomes, the spindle checkpoint monitors kinetochore-microtubule interactions and delays the metaphase to anaphase transition until bipolar attachments are achieved (for review, see (Lew and Burke, 2003)).

An important regulator of both kinetochore attachment and the spindle checkpoint is the Ipl1/Aurora B protein kinase, a component of the conserved

chromosomal passenger complex (for review, see (Vagnarelli and Earnshaw, 2004)). Ipl1/Aurora B facilitates proper attachments by destabilizing kinetochore-microtubule interactions that do not generate tension, such as monopolar attachments (also called syntelic or mono-oriented) in which kinetochores bind microtubules emanating from the same pole (Biggins et al., 1999; Chan and Botstein, 1993; Dewar et al., 2004; Lampson et al., 2004; Pinsky et al., 2003; Tanaka et al., 2002). Despite the presence of improper attachments that should activate the spindle checkpoint, cells with impaired Ipl1/Aurora B function proceed through the cell cycle due to a defect in detecting a lack of tension (Biggins and Murray, 2001; Carvalho et al., 2003; Ditchfield et al., 2003; Hauf et al., 2003; Lens et al., 2003).

Ipl1 activity is opposed by Glc7 (Francisco et al., 1994), the sole, essential protein phosphatase I (PP1) catalytic subunit in budding yeast (Cannon et al., 1994). Glc7 regulates numerous cellular processes including mitosis, meiosis, glycogen and sugar metabolism, transcription, translation, and mRNA processing through its interactions with specific regulatory subunits that target the phosphatase to appropriate substrates (for review, see (Ceulemans and Bollen, 2004)). Although the Glc7 mitotic regulatory subunit has not yet been identified, many *glc7* alleles cause cells to arrest in mitosis (Andrews and Stark, 2000; Baker et al., 1997; Black et al., 1995; Hisamoto et al., 1994; MacKelvie et al., 1995), suggesting that Glc7 substrates must be dephosphorylated to allow cell cycle progression. Furthermore, impairing Glc7 function suppresses the *ipl1* temperature sensitive growth defect and restores the phosphorylation of the Ipl1 target, histone H3, indicating that Glc7 likely has a

specific role antagonizing Ipl1-mediated phosphorylation (Francisco et al., 1994; Hsu et al., 2000). Consistent with this, several *glc7* mutants have phenotypes that are the opposite of impaired Ipl1 function, including spindle checkpoint activation and reduced kinetochore binding to microtubules *in vitro* (Bloecher and Tatchell, 1999; Sassoon et al., 1999). However, the precise relationship between the kinase and phosphatase is not well understood and Glc7 regulation of Ipl1 function has not been examined.

Here we identify proteins that physically interact with Glc7 and likely regulate Glc7 localization to carry out its many functions. We further explore the relationship between Glc7 and Ipl1 and find that Glc7 is unlikely to directly modulate Ipl1 and instead opposes the essential functions of Ipl1 by dephosphorylating important substrates.

Results

ipl1-321 high copy suppressor screen

To achieve a better understanding of Ipl1's essential functions, we isolated dosage suppressors of the *ipl1-321* temperature sensitive growth defect (Biggins et al., 1999). From this analysis we found 10 genes that suppressed *ipl1-321* when present on a 2μ, high copy number plasmid (Table 5.1) (Figure 5.1A). Consistent with previous dosage suppressor screens using the temperature sensitive *ipl1-1* allele, we identified the dominant negative protein phosphatase I (PP1) allele *glc7Δ186-312*, as well as the *GLC8* and *SCD5* genes (Francisco et al., 1994; Tung et al., 1995). In addition to these known suppressors, we identified seven novel *ipl1-321* dosage suppressors: *SDS22, BUD14, YPL137c, FUN21, SOL1, SOL2,* and *PEX31*. At the restrictive temperature of 35 °C, all of the dosage suppressors restored *ipl1-321* growth to near wild type levels; while at 37 °C there were varying levels of partial suppression (Figure 5.1A).

Fun21, Sol1, and Pex31 physically interact with Glc7

It was proposed that a reduction of Glc7 function suppresses mutations in Ipl1 by restoring the balance of kinase/phosphatase activity (Francisco et al., 1994). Because the suppressors Glc8, Scd5, Sds22, Bud14, and Ypl137c physically interact with Glc7 (Chang et al., 2002; Gavin et al., 2002; Hazbun et al., 2003; Ho et al., 2002; Hong et al., 2000; Lenssen et al., 2005; Peggie et al., 2002; Ramaswamy et al., 1998; Tu et al., 1996; Uetz et al., 2000; Venturi et al., 2000; Walsh et al., 2002; Wu

and Tatchell, 2001), we tested by co-immunoprecipitation if the remaining dosage suppressors Pex31, Sol1, and Fun21 also interacted with Glc7. We generated strains co-expressing endogenous COOH-terminal fusions of Glc7-HA3 with Pex31-myc13, Sol1-myc13, and Fun21-myc13 and found that all three proteins immunoprecipitated Glc7 (Figure 5.1B). Therefore, like the known *ipl1* dosage suppressors, Pex31, Sol1, and Fun21 physically interact with Glc7.

Overexpression of FUN21 and YPL137c alters Glc7 localization

The observation that the overexpression of genes encoding a variety of Glc7 interacting proteins involved in disparate cellular processes (Table 5.1) suppress the *ipl1-321* temperature sensitivity suggests that these dosage suppressors may do so by inhibiting Glc7 function. This could be achieved by relocalizing Glc7 away from the targets relevant to Ipl1 function, such as kinetochores and the spindle. To test this hypothesis, a fully functional endogenous COOH-terminal fusion of Glc7 to triple green fluorescent protein (Glc7-GFP3) was localized in wild type cells and cells overexpressing galactose inducible Fun21 and Ypl137c (Figure 5.1C) which are reported to be in the cytoplasm (Table 5.1) (Huh et al., 2003). Nuclei were visualized by co-expressing the nuclear pore component Nic96 fused to cyan fluorescent protein (Nic96-CFP). Glc7-GFP3 was concentrated in the nucleus and also localized to the cytoplasm and bud neck, as reported previously (Bloecher and Tatchell, 2000). It also appeared to be excluded from the vacuole (Figure 5.1C). However, in cells overexpressing Fun21 and Ypl137c, the nuclear accumulation of

Glc7-GFP3 was not evident and the phosphatase was predominantly cytoplasmic. Therefore, overexpression of Fun21 and Ypl137c reduces the effective Glc7 concentration in the nucleus and suggests that *ipl1-321* dosage suppression may occur by spatially separating the phosphatase from its relevant targets opposing Ipl1 function.

Glc7 does not regulate Ipl1 activity or localization

Though the phosphorylation status of several proteins is modulated by the opposing activities of Glc7 and Ipl1 (Hsu et al., 2000; Kang et al., 2001; Sassoon et al., 1999), the precise relationship between the phosphatase and the kinase has not been studied. One possibility is that Glc7 directly inhibits Ipl1, so we determined if Glc7 physically interacts with Ipl1. Using strains co-expressing endogenous COOH-terminal fusions of Glc7-HA3 and Ipl1-myc13, we were unable to detect the physical association of Glc7 with Ipl1 (Figure 5.2A). In addition, Glc7 did not co-precipitate with the Ipl1 activator, Sli15 (data not shown). These data indicate that Ipl1 and Glc7 do not form a detectable complex *in vivo*.

We next examined whether Glc7 affects Ipl1 protein levels in strains carrying the temperature sensitive *glc7-10* allele. If Glc7 were a negative regulator of Ipl1 levels, we would expect the mutant cells to express more Ipl1 protein. The *glc7-10* mutant suppresses *ipl1-321* at high temperatures (see Figure 5.3A), and is defective in Glc7's known mitotic functions (Andrews and Stark, 2000; Sassoon et al., 1999). Wild type and *glc7-10* cells expressing Ipl1-myc13 were arrested in mitosis with

nocodazole to eliminate cell cycle variation, shifted to the restrictive temperature (37 °C) for two hours, and monitored for Ipl1 protein levels by α-myc immunoblotting (Figure 5.2B). Wild type and *glc7-10* cells expressed equal amounts of Ipl1, indicating that Glc7 does not regulate Ipl1 levels. We obtained similar results using cells asynchronously shifted to the restrictive temperature (data not shown).

We next tested whether Glc7 negatively regulates Ipl1 kinase activity. Wild type and *glc7-10* cells expressing Ipl1-myc13 were arrested in mitosis with nocodazole and shifted to the restrictive temperature (37 °C) for two hours. Ipl1-myc13 was immunoprecipitated from cell lysates and used in kinase assays *in vitro* with the substrate histone H3 (Figure 5.2C). There were equivalent amounts of Ipl1 kinase activity in both wild type and *glc7-10* mutant cells, suggesting that Glc7 does not regulate Ipl1 activity. Cells asynchronously shifted to the restrictive temperature also contained equal amounts of Ipl1 kinase activity (data not shown).

Since the metaphase kinetochore localization of Ipl1 is thought to reflect its role in chromosome segregation, we analyzed Glc7 effects on this localization. We visualized triple GFP epitope-tagged Ipl1 (Ipl1-GFP3) in wild type and *glc7-10* cells co-expressing CFP tagged tubulin to mark spindles (Tub1-CFP) (Figure 5.2D). In wild type cells, Ipl1-GFP3 localized to kinetochores and microtubules on short (1.5-3 microns) metaphase spindles, as previously described (Buvelot et al., 2003). Ipl1-GFP3 localization was similar in the *glc7-10* cells indicating that Glc7 does not regulate Ipl1's metaphase localization. Reducing nuclear Glc7 by Fun21 overexpression also did not alter Ipl1 localization (data not shown). Taken together,

these data show that negative regulation of Ipl1 by Glc7 is unlikely to explain the relationship between the kinase and phosphatase.

Glc7 regulates Dam1 phosphorylation

Another possibility is that Ipl1 and Glc7 regulate a common set of substrates, as proposed (Francisco et al., 1994). Ipl1 is thought to promote proper chromosome segregation by phosphorylating components of the Dam1/DASH/DDD complex (Cheeseman et al., 2002; Shang et al., 2003), an essential regulator of kinetochore-microtubule interactions and microtubule function (Cheeseman et al., 2002; Cheeseman et al., 2001a; Janke et al., 2002; Kang et al., 2001; Li et al., 2005; Li et al., 2002; Miranda et al., 2005; Westermann et al., 2005). We therefore determined whether the balance of kinase and phosphatase regulates Dam1 phosphorylation. To do this we monitored *ipl1* and *glc7* mutants, as well as *ipl1 glc7* double mutants for Dam1 gel mobility, as previously described (Li et al., 2002). In addition to the *glc7-10* allele, we also analyzed *glc7-12* (MacKelvie et al., 1995), another mitotic defective allele that does not suppress *ipl1-321* (Figure 5.3A). Wild type, *ipl1-321, glc7-10, ipl1-321 glc7-10, glc7-12,* and *ipl1-321 glc7-12* cells expressing an endogenous COOH-terminal fusion of Dam1-myc9 were asynchronously shifted to the restrictive temperature (35 °C) for 3 hours. Dam1 displayed a series of slower migrating forms in wild type cells that were abolished in *ipl1-321* mutant cells as previously reported (Li et al., 2002) (Figure 5.3B). Though the slower migrating forms of Dam1 were not noticeably enhanced in *glc7-10* mutant cells, the Dam1

phospho-forms were more intense in the *glc7-12* cells, suggesting that this allele may retain less residual phosphatase activity at the restrictive temperature. Importantly, the Dam1 phospho-forms were restored to wild type levels in both the *glc7-10 ipl1-321* and *glc7-12 ipl1-321* double mutant cells. This restoration of phosphorylation indicates that the *ipl1-321* allele retains some enzymatic activity at higher temperatures and is consistent with Dam1 phosphorylation being regulated by the balance of Ipl1 kinase and Glc7 phosphatase *in vivo*.

Discussion

The opposing activities of the Ipl1/Aurora protein kinase and Glc7/PP1 protein phosphatase are required for accurate chromosome segregation. We show here that *ipl1* dosage suppressors encode Glc7 interacting proteins that likely restore the kinase/phosphatase balance by reducing Glc7 access to relevant substrates. Decreased Glc7 activity affected the phosphorylation of the Ipl1 substrate Dam1, but did not alter Ipl1 levels, localization, or kinase activity, supporting the proposal that Glc7 opposes Ipl1 function by regulating the phosphorylation of common targets.

A genetic screen for ipl1 dosage suppressors reveals novel Glc7 interacting proteins

Protein phosphatase 1 catalytic subunits, such as Glc7, control numerous cellular processes through their interaction with specialized regulatory subunits that target the phosphatase to appropriate substrates (for review see (Ceulemans and Bollen, 2004)). We find that the *ipl1* temperature sensitive growth defect is suppressed by the increased dosage of genes encoding Glc7 interacting proteins (Table I). These genes include previously described *ipl1* dosage suppressors *GLC8* and *SCD5*, as well as *SDS22, BUD14,* and *YPL137c*, newly identified dosage suppressors that encode known Glc7 interacting proteins (Chang et al., 2002; Gavin et al., 2002; Hazbun et al., 2003; Ho et al., 2002; Hong et al., 2000; Lenssen et al., 2005; Peggie et al., 2002; Ramaswamy et al., 1998; Uetz et al., 2000; Venturi et al., 2000; Walsh et al., 2002; Wu and Tatchell, 2001). In addition, we identified *FUN21, SOL1, SOL2,* and *PEX31* as *ipl1* dosage suppressors, and show that these genes also

encode proteins that physically interact with Glc7. We therefore propose that *FUN21, SOL1, SOL2,* and *PEX31* are previously unidentified Glc7 regulatory subunits and/or substrates for uncharacterized Glc7 functions.

It is likely that the mechanism of dosage suppression involves the redistribution of Glc7 away from the nuclear targets relevant to Ipl1's essential functions, such as kinetochores and the spindle. Consistent with this idea, cells overexpressing *FUN21* and *YPL137c* have reduced Glc7 in the nucleus and most of the other dosage suppressors encode cytoplasmic or membrane bound proteins that would be predicted to mislocalize Glc7 away from Ipl1 targets (see Table 5.1). In contrast, increased levels of the Glc7 regulatory subunit important for directing the phosphatase to oppose Ipl1-mediated phosphorylation should deliver more Glc7 to Ipl1 targets and exacerbate the *ipl1* temperature sensitivity. It is therefore unlikely that any of the dosage suppressors encode this Glc7 mitotic regulator. Though previous work suggested a role for Sds22 in activating Glc7 towards mitotic substrates in the nucleus, we isolated *SDS22* as an *ipl1* dosage suppressor. Because s*ds22* mutants also suppress the *ipl1* temperature sensitivity and result in Glc7 mislocalization, the proposal that Sds22 acts as a Glc7 chaperone is more consistent with the current observations (Peggie et al., 2002). Additional candidates for the Glc7 mitotic regulator have not yet been identified and are currently the subject of active investigation.

Ipl1 and Glc7 regulate a common set of substrates

Three simple models have been proposed that could account for the functional interaction between Glc7 and Ipl1 that allow the cell to maintain the balance of phosphorylation for proper chromosome segregation; 1) Glc7 negatively regulates Ipl1. 2) Ipl1 negatively regulates Glc7. 3) Ipl1 and Glc7 modulate the phosphorylation status of a common set of substrates (Francisco et al., 1994). We describe here the first experiments that distinguish between these models in budding yeast. We found that Ipl1 levels, localization, and kinase activity are not affected by decreased Glc7 activity, suggesting that Glc7 does not oppose Ipl1 function by regulating Ipl1 itself. This is in contrast cultured vertebrate cells and *Xenopus* chromatin extracts where incubation with PP1 inhibitors resulted in elevated Aurora B kinase activity (Murnion et al., 2001; Sugiyama et al., 2002). In addition, we did not observe a change in the levels or localization of Ipl1 in mitotic cells with reduced Glc7 function, as has been described for Aurora B in meiotic *C. elegans* cells treated with PP1 *RNAi* (Rogers et al., 2002). It is possible that these results reveal true differences in Ipl1 and Aurora B regulation among organisms, though they may also represent a lack of inhibitor specificity or the limitations of our Ipl1 kinase and localization assays. We were also unable to detect a physical interaction between Glc7 and Ipl1 when expressed at endogenous levels. However, Aurora B interacted with each of the three PP1 isoforms (α, δ, and γ) when they were co-overexpressed in cultured cells (Sugiyama et al., 2002). It is not clear if the potential association of Glc7 with Ipl1 escaped our detection due to a weak, transient or cell cycle stage specific interaction, whether the overexpression studies promote an interaction that is

not present under normal conditions, or whether the interactions between Ipl1 and Glc7 are also organism specific. Consistent with our results indicating that Glc7 does not directly regulate Ipl1, the kinetochore associated PP1γ isoform localizes to a domain distinct from Aurora B in cultured cells (Trinkle-Mulcahy et al., 2003). We have not eliminated the possibility that Ipl1 negatively regulates Glc7 or its mitotic regulator, though Ipl1 does not phosphorylate Glc7 *in vitro* (data not shown) and Glc7 is not phosphorylated *in vivo* in budding yeast (Stuart et al., 1994).

It was previously shown that Ipl1/Aurora B and Glc7/PP1 regulate the phosphorylation of histone H3 in both budding yeast and *C. elegans* (Hsu et al., 2000), though this work did not differentiate between the models described above. However, these results suggest that the kinase and the phosphatase work in parallel to control the phosphorylation level of a common set of substrates. Consistent with this model, we found that impairing Glc7 function restores the phosphorylation of the Dam1 protein in *ipl1* mutant cells. Taken together, our results indicate that in the budding yeast, Ipl1 and Glc7 act on common targets to promote proper chromosome segregation.

Experimental Procedures

Microbial Techniques and Yeast strain construction. Media and microbial techniques were essentially as described (Rose et al., 1990; Sherman et al., 1974). Nocodazole was used at 10 μg/ml. Yeast strains are listed in Table 5.2 and were constructed by standard genetic techniques. All strains are isogenic with the W303 background and were generated for this study. The *glc7-10* (Sassoon et al., 1999), *glc7-12* (MacKelvie et al., 1995) (gifts from Michael Stark, University of Dundee, Dundee, U.K.), and *ipl1-321* (Biggins et al., 1999) alleles were crossed to make strains for this study. Strains containing *TUB1-CFP:URA3* were obtained by integrating plasmid pSB375 (a gift from Kerry Bloom, University of North Carolina, Chapel Hill, NC) digested with *StuI* at the *URA3* locus. *GLC7-GFP3* strains were made by integrating plasmid pSB881 digested with *EcoRI* at the *GLC7* locus. Insertion of the *pGAL* promoter, as well as HA3 and myc13 epitope tags were made using a PCR-based integration system (Longtine et al., 1998) and were confirmed by PCR. Specific primer sequences are available upon request. All fusion proteins are fully functional.

Plasmid Construction. The *GLC7-GFP3* integrating plasmid was made by PCR amplification of the C-terminal 400 base pairs of *GLC7* from pKC1048 (a gift from John Cannon, University of Missouri, Columbia, MO) using primers SB1047 and SB1048, that have *ClaI* and *BamHI* restriction sites engineered, respectively. The resulting PCR product was digested with *ClaI* and *BamHI* and ligated into the *ClaI*

and *BamHI* sites of PB1585 (a gift from David Pellman, Harvard Medical School, Boston, MA), to create pSB881.

ipl1-321 dosage suppressor screen. The *ipl1-321* strain (SBY1063) was transformed with a 2μ *URA3*-marked genomic yeast library, plated on selective media at the permissive temperature (23 °C) for three days, and then replica printed to the restrictive temperature (35.5 °C) for 1 day. Of the 48 temperature-resistant colonies identified, 29 showed temperature resistance that was plasmid dependent and were subjected to plasmid rescue and retransformation. The 26 remaining positives were grouped based on restriction mapping and representatives from each group were sequenced using primers SB359 and SB360. 12 genomic regions containing the following genes were identified; *IPL1* (4 times), *glc7Δ186-312* (1), *GLC8* (2), *SCD5* (2), *SDS22* (1), *BUD14* (2), *FUN21* (2), *PEX31* (1), *SOL1* (2), *SOL2* (1), *YPL137w* (7), *YOR342c* (1) (plasmids available upon request). *glc7Δ186-312, GLC8,* and *SCD5* were previously identified as *ipl1-1* dosage suppressors so their genomic regions were not further dissected. *FUN21* and *YPL137c* were confirmed to encode the dosage suppressors by generating a series of plasmid deletions retransforming into SBY1063, and testing for temperature resistance. To determine which genes encoded the remaining dosage suppressors, we obtained strains from the *GST-ORF* collection (a gift from Stan Fields, University of Washington, Seattle, WA) for each of the open reading frames in the above genomic regions. We isolated the *GST-ORF* plasmids, retransformed into SBY1063, and screened for temperature resistance. By

this method we identified *SDS22*, *BUD14*, *SOL1*, and *PEX31* as the dosage suppressors. Although *SOL2* is 78% identical to *SOL1* we have not eliminated the possibility that another gene in the genomic region is the dosage suppressor. We have not determined which gene in the *YOR342c* genomic region high copy suppresses *ipl1-321*.

Microscopy. Live microscopy was performed as described (Buvelot et al., 2003). At least 100 cells were analyzed for all reported experiments.

Protein and immunological techniques. Protein extracts were made and immunoblotted as described (Minshull et al., 1996). 9E10 antibodies that recognize the myc tag and 12CA5 antibodies that recognize the hemagglutinin (HA) tag were obtained from Covance and used at a 1:10,000 dilution. For immunoprecipitations, 50 ml cultures of mid-log cells were collected and lysates were prepared as described (Buvelot et al., 2003). 450 µl supernatant was incubated with 5 µl protein G dynabeads (Dynal Biotech, Inc.) and 5 µl of A-14 anti-myc antibody (Santa Cruz Biotechnology) for 2 hrs at 4 °C. The beads were washed 5x with 500 µl lysis buffer and the immunoprecipitates were separated by SDS-PAGE and immunoblotted as above. Kinase assays were performed as described (Buvelot et al., 2003), except 5 µg histone H3 (Roche) was used as substrate.

Table 5.1 *ipl1-321 dosage suppressors*

Gene	Description	Localization	Glc7 Interaction
glc7Δ186-312	Dominant negative allele of the type 1 phosphatase catalytic subunit *GLC7*	NA	NA
GLC8	*GLC7* regulatory subunit	cytoplasm, nucleus	2H, AP
SCD5	Multicopy suppressor of clathrin deficiency	cell cortex	2H, AP, Co-IP
SDS22	*GLC7* regulatory subunit	nucleus, cytoplasm	2H, AP, Co-IP
BUD14	Involved in bud site selection	bud site, mother-daughter bud neck	2H, Co-IP
YPL137c	uncharacterized ORF	cytoplasm	AP
FUN21	uncharacterized ORF	cytoplasm	Co-IP
SOL1	Regulator of tRNA function	nucleus, cytoplasm	Co-IP
SOL2	Regulator of tRNA function	cytoplasm	Unknown
PEX31	Peroxisomal integral membrane protein	peroxisomes	Co-IP

NA=Not Applicable
2H=2-Hybrid
AP=Affinity Precipitation
Co-IP= Co-immunoprecipitation

Table 5.2 *Yeast strains used in this study*

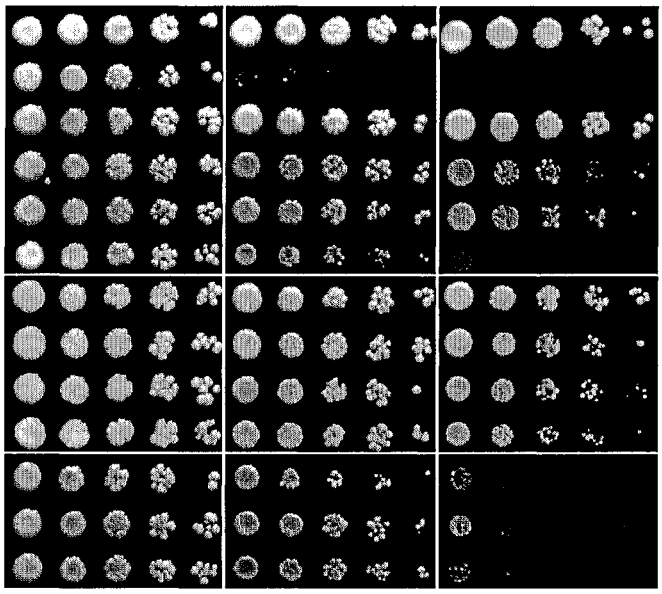
Strain	Genotype
SBY3	*MATa ura3-1 leu2,3-112 his3-11 trp1-1 ade2-1 can1-100 bar1Δ*
SBY214	*MATa ura3-1 leu2,3-112 his3-11:pCUP1-GFP12-lacI12:HIS3 trp1-1:256lacO:TRP1 lys2Δ ade2-1 can1-100 bar1Δ*
SBY322	*MATa ura3-1 leu2,3-112 his3-11:pCUP1-GFP12-lacI12:HIS3 trp1-1:256lacO:TRP1 lys2Δ ade2-1 can1-100 bar1Δ ipl1-321*
SBY625	*MATa ura3-1 leu2,3-112 his3-11 trp1-1 ade2-1 can1-100 bar1Δ GLC7-HA3:HIS3*
SBY1063	*MATa ura3-1 leu2,3-112:pGAL-ipl1(R343A):LEU2 his3-11:pCUP1-GFP12-lacI12:HIS3 trp1-1:256lacO:TRP1 lys2Δ ade2-1 can1-100 bar1Δ ipl1-321*
SBY1306	*MATa ura3-1 leu2,3-112 his3-11 trp1-1:glc7-10:TRP1 ade2-1 can1-100 bar1Δ glc7::LEU2*
SBY1308	*MATa ura3-1 leu2,3-112 his3-11 trp1-1:GLC7:TRP1 ade2-1 can1-100 bar1Δ glc7::LEU2*
SBY1994	*MATa ura3-1 leu2,3-112 his3-11 trp1-1:glc7-10:TRP1 ade2-1 can1-100 bar1Δ glc7::LEU2 ipl1-321*
SBY2055	*MATa ura3-1 leu2,3-112 his3-11 trp1-1 lys2Δ ade2-1 can1-100 bar1Δ DAM1-myc9:TRP1*
SBY2833	*MATa ura3-1:TUB1-CFP:URA3 leu2,3-112 his3-11 trp1-1 ade2-1 can1-100 bar1Δ IPL1-GFP3:HIS3*
SBY3675	*MATa ura3-1 leu2,3-112 his3-11 trp1-1:glc7-12:TRP1 ade2-1 can1-100 bar1Δ glc7::LEU2*
SBY4209	*MATa ura3-1 leu2,3-112 his3-11 trp1-1 ade2-1 can1-100 bar1Δ GLC7-HA3:HIS3 FUN21-myc13:KAN*
SBY4764	*MATa ura3-1 leu2,3-112 his3-11 trp1-1 ade2-1 can1-100 bar1Δ DAM1-myc9:TRP1 ipl1-321*
SBY4766	*MATa ura3-1 leu2,3-112 his3-11 trp1-1:glc7-10:TRP1 ade2-1 can1-100 bar1Δ glc7::LEU2 IPL1-myc13:KAN*
SBY4767	*MATa ura3-1 leu2,3-112 his3-11 trp1-1:GLC7:TRP1 ade2-1 can1-100 bar1Δ glc7::LEU2 IPL1-myc13:KAN*
SBY4801	*MATa ura3-1 leu2,3-112 his3-11 trp1-1:glc7-10:TRP1 ade2-1 can1-100 bar1Δ glc7::LEU2 ipl1-321 DAM1-myc9:TRP1*
SBY4822	*MATa ura3-1 leu2,3-112 his3-11 trp1-1 ade2-1 can1-100 bar1Δ GLC7-HA3:HIS3 IPL1-myc13:KAN*
SBY4826	*MATa ura3-1 leu2,3-112 his3-11 trp1-1:glc7-10:TRP1 ade2-1 can1-100 bar1Δ glc7::LEU2 DAM1-myc9:TRP1*
SBY4892	*MATa ura3-1 leu2,3-112 his3-11 trp1-1 ade2-1 can1-100 bar1Δ GLC7-GFP3:HIS3 NIC96-CFP:KAN*
SBY4893	*MATa ura3-1 leu2,3-112 his3-11 trp1-1 ade2-1 can1-100 bar1Δ GLC7-GFP3:HIS3 NIC96-CFP:KAN pGAL-HA3-FUN21:HIS3 PDS1-myc18:LEU2*
SBY4874	*MATa ura3-1 leu2,3-112 his3-11 trp1-1 ade2-1 can1-100 bar1Δ GLC7-HA3:HIS3 SOL1-myc13:KAN*
SBY4920	*MATa ura3-1 leu2,3-112 his3-11 trp1-1 ade2-1 can1-100 bar1Δ GLC7-HA3:HIS3 PEX31-myc13:KAN*
SBY4995	*MATa ura3-1 leu2,3-112 his3-11 trp1-1 ade2-1 can1-100 bar1Δ GLC7-GFP3:HIS3 NIC96-CFP:KAN pGAL-HA3-YPL137c:HIS3*
SBY4999	*MATa ura3-1:TUB1-CFP:URA3 leu2,3-112 his3-11 trp1-1:glc7-10:TRP1 ade2-1 can1-100 bar1Δ glc7::LEU2 IPL1-GFP3:HIS3*

Table 5.2 *continued*

Strain	Genotype
SBY5004	*MAT**a** ura3-1 leu2,3-112 his3-11 trp1-1:glc7-12:TRP1 ade2-1 can1-100 bar1Δ glc7::LEU2 ipl1-321*
SBY5032	*MAT**a** ura3-1 leu2,3-112 his3-11 trp1-1:glc7-12:TRP1 lys2Δ ade2-1 can1-100 bar1Δ glc7::LEU2 DAM1-myc9:TRP1 ipl1-321*
SBY5034	*MAT**a** ura3-1 leu2,3-112 his3-11 trp1-1:glc7-12:TRP1 lys2Δ ade2-1 can1-100 bar1Δ glc7::LEU2 DAM1-myc9:TRP1*

Figure 5.1 *ipl1-321* dosage suppressors encode Glc7 interacting proteins. (A) Glc7 regulators are high copy *ipl1-321* suppressors. 5-fold serial dilutions of wild type cells (SBY214), with an empty 2μ plasmid and *ipl1-321* cells (SBY1063) with an empty 2μ plasmid or 2μ plasmids containing the indicated dosage suppressors, were incubated for 2 days at 30, 35, and 37 °C. (B) Glc7 physically associates with Pex31, Sol1, and Fun21. Extracts from cells expressing Glc7-HA3 alone (SBY625) or in combination with Pex31-myc13 (SBY4920), Sol1-myc13 (SBY4874), and Fun21-myc13 (SBY4209), were immunoprecipitated with α-myc antibody. Extracts (Input) and immunoprecipitates (IP) were analyzed by α-HA and α-myc immunoblotting. The α-HA immunoblots of the Pex31 and Sol1 IPs were exposed 10 times longer than the Fun21 IP. (C) Overexpression of *FUN21* reduces nuclear Glc7. Wild type (SBY4892), galactose inducible *FUN21* overexpressing cells *(pGAL-FUN21)* (SBY4893), and galactose inducible *YPL137c* overexpressing cells (SBY4995) *(pGAL- YPL137c)* expressing Glc7-GFP3 and Nic96-CFP were grown in galactose for 4hrs at 30 °C. Bar, 5 μm.

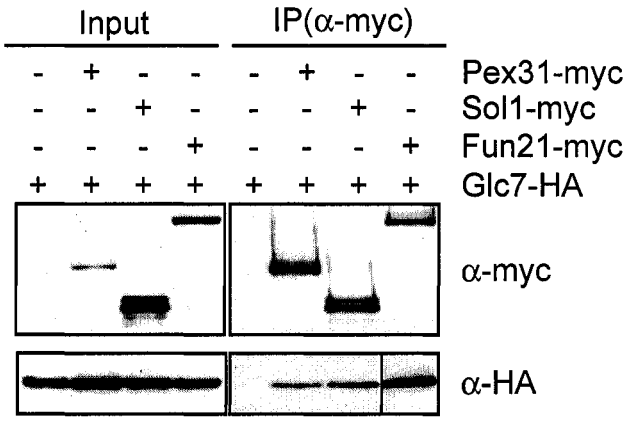
A



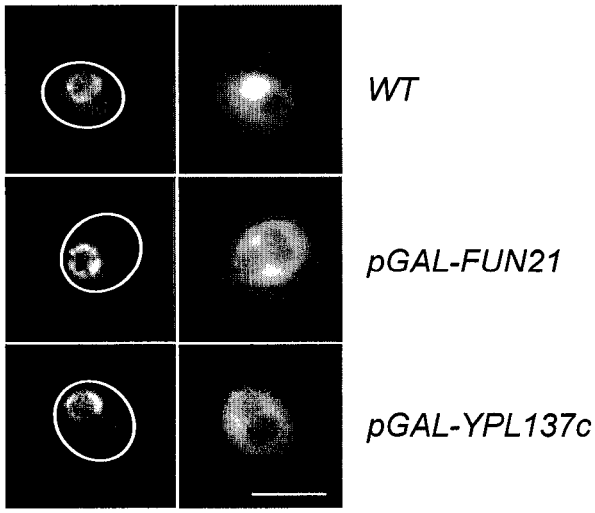
Genotype	2μ Plasmid
IPL1	*empty*
ipl1-321	*empty*
ipl1-321	*IPL1*
ipl1-321	*glc7Δ186-312*
ipl1-321	*GLC8*
ipl1-321	*SDS22*
ipl1-321	*SCD5*
ipl1-321	*BUD14*
ipl1-321	*YPL137c*
ipl1-321	*FUN21*
ipl1-321	*SOL1*
ipl1-321	*SOL2*
ipl1-321	*PEX31*

30°C 35°C 37°C

B



C



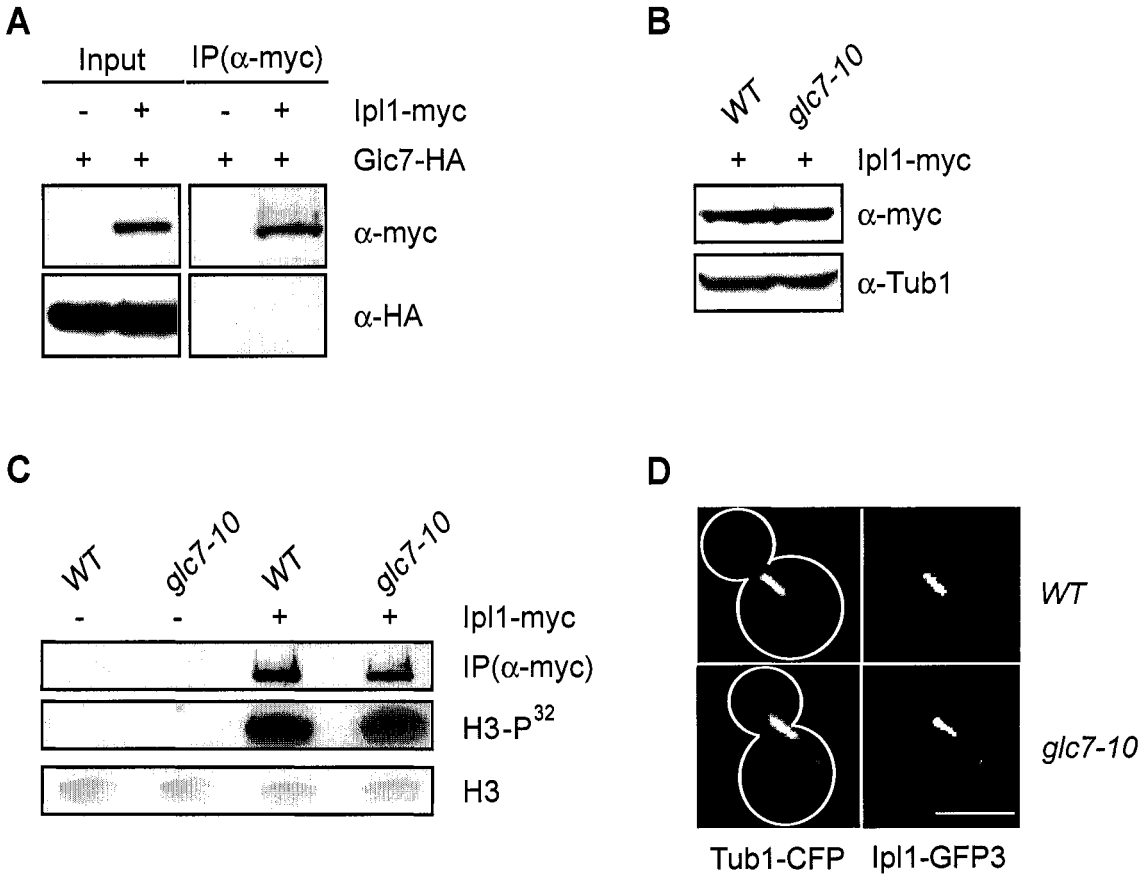


Figure 5.2 Glc7 does not regulate Ipl1 levels, activity, or localization. (A) Glc7 and Ipl1 do not form a detectable complex. Extracts from cells expressing Glc7-HA3 alone (SBY625) or in combination with Ipl1-myc13 (SBY4822), were immunoprecipitated with α-myc antibody. Extracts (Input) and immunoprecipitates (IP) were analyzed by α-HA and α-myc immunoblotting. (B) Glc7 does not alter Ipl1 protein levels. Wild type (SBY4767) and *glc7-10* mutant cells (SBY4766) expressing Ipl1-myc13 were arrested in mitosis with nocodazole, shifted to 37 °C for 2 hrs, and the extracts were analyzed by α-myc and α-Tub1 immunoblotting. (C) Glc7 does not regulate Ipl1 kinase activity. Wild type and *glc7-10* mutant cells expressing untagged Ipl1 (SBY1308 and SBY1306) or Ipl1-myc13 (SBY4767 and SBY4766) were grown as in (B). Extracts were immunoprecipitated (IP) with α-myc antibody and the IPs were analyzed by α-myc immunoblotting and used in kinase assays *in vitro* with the substrate histone H3. (D) Glc7 does not regulate Ipl1's metaphase localization. Wild type (SBY2833) and *glc7-10* cells (SBY4999) expressing Ipl1-GFP3 and Tub1-CFP were grown at 37 °C for 2 hrs. Bar, 5 μm.

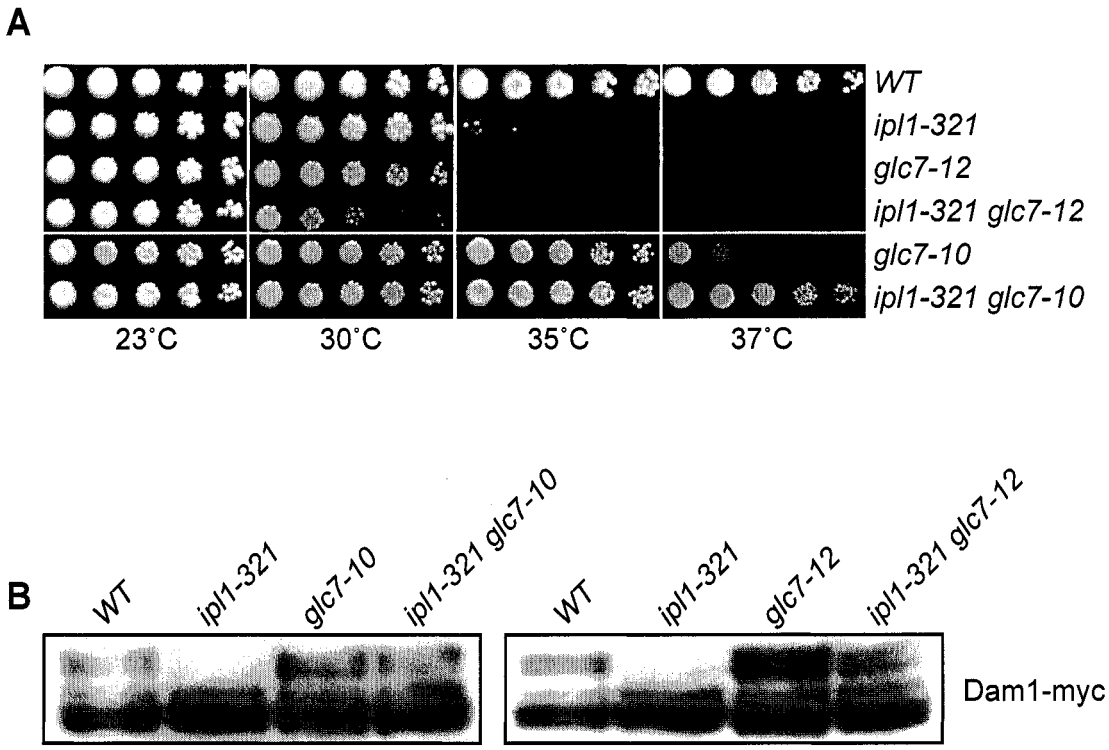


Figure 5.3 Ipl1 and Glc7 regulate Dam1 phosphorylation. (A) The *ipl1-321* temperature sensitivity is suppressed by *glc7-10* but not *glc7-12*. 5-fold serial dilutions of wild type (SBY214), *ipl1-321* (SBY322), *glc7-12* (SBY3675), *glc7-12 ipl1-321* (SBY5004), *glc7-10* (SBY1306), and *ipl1-321 glc7-10* (SBY1994) cells were incubated for 3 days at 23 °C and 2 days at 30, 35, and 37 °C. (B) The balance of Ipl1 kinase and Glc7 phosphatase controls Dam1 phosphorylation. Wild type (SBY2055), *ipl1-321* (SBY4764), *glc7-10* (SBY4826), *ipl1-321 glc7-10* (SBY4801), *glc7-12* (SBY5034), and *ipl1-321 glc7-12* (SBY5032) cells expressing Dam1-myc9 were grown at 35 °C for 3 hrs. Extracts were analyzed by α-myc immunoblotting for changes in Dam1 gel mobility and show that the upper Dam1 forms missing in *ipl1-321* cells are restored in *ipl1-321 glc7-10* and *ipl1-321 glc7-12* mutant cells.

CHAPTER 6:

The Ipl1/Aurora protein kinase and Glc7/Protein Phosphatase-1 regulate chromosome segregation through the phosphorylation of multiple targets

Summary

It has been proposed that the Ipl1/Aurora protein kinase and the Glc7/PP1 phosphatase facilitate proper kinetochore-microtubule attachments by regulating the phosphorylation status and kinetochore association of the Dam1 complex. Although Dam1 phosphorylation is affected, we find that impairing Ipl1 or Glc7 function does not alter the kinetochore localization of Dam1. Furthermore, genetic analysis of the *dam1* phosphorylation site mutants most closely representing the fully phosphorylated and unphosphorylated states suggests that Ipl1 and Glc7 likely have additional critical substrates. Consistent with this, the characterization of a *dam1* phospho-deficient mutant reveals phenotypes distinct from the *ipl1* mutant. We therefore propose that Ipl1 and Glc7 promote accurate chromosome segregation through the phosphoregulation of multiple important targets.

Introduction

The fidelity of chromosome segregation requires the precise regulation of kinetochore attachments to spindle microtubules. To ensure that the spindle forces on replicated chromosomes (sister chromatids) are directed away from one another, kinetochores must make bipolar attachments and interact with microtubules arising from opposite poles. The spindle checkpoint monitors these interactions and delays the metaphase to anaphase transition to prevent the untimely segregation of misattached chromosomes.

An important regulator of both kinetochore attachment and the spindle checkpoint is the Ipl1/Aurora B protein kinase, a component of the conserved chromosomal passenger complex (for review, see (Vagnarelli and Earnshaw, 2004)). Cells with impaired Ipl1/Aurora B function make kinetochore attachments to spindle microtubules emanating from the same pole (monopolar attachments) due to a defect in destabilizing improper kinetochore-microtubule interactions (Biggins et al., 1999; Chan and Botstein, 1993; Dewar et al., 2004; Lampson et al., 2004; Pinsky et al., 2003; Tanaka et al., 2002). Despite the presence of improper attachments, these cells fail to activate the spindle checkpoint and undergo catastrophic chromosome missegregation (Biggins and Murray, 2001; Carvalho et al., 2003; Ditchfield et al., 2003; Hauf et al., 2003; Lens et al., 2003).

An essential mediator of kinetochore-microtubule interactions and microtubule function in budding yeast is the Dam1/DASH/DDD complex (Cheeseman et al., 2002; Cheeseman et al., 2001a; Janke et al., 2002; Li et al., 2002).

Mutants in the Dam1 complex have various spindle abnormalities and chromosome segregation defects with the most severe alleles displaying a loss of spindle integrity, monopolar kinetochore attachments, and spindle checkpoint activation (Cheeseman et al., 2001a; Cheeseman et al., 2001b; He et al., 2001; Janke et al., 2002; Jones et al., 1999; Jones et al., 2001; Li et al., 2005; Li et al., 2002). Consistent with these phenotypes, the Dam1 complex localizes to both kinetochores and the spindle *in vivo* (Cheeseman et al., 2001a; Cheeseman et al., 2001b; Hofmann et al., 1998; Janke et al., 2002; Jones et al., 1999; Jones et al., 2001; Li et al., 2005; Li et al., 2002) and the reconstituted Dam1 complex forms a ring that can slide along microtubules and has microtubule stabilizing and bundling activities *in vitro* (Miranda et al., 2005; Westermann et al., 2005). In addition, the Dam1 complex is the only known kinetochore complex that requires microtubules for its kinetochore association (Janke et al., 2002; Li et al., 2002), indicating that Dam1 may directly link spindle microtubules to kinetochores.

Several studies suggest that Ipl1 regulates kinetochore-microtubule interactions through phosphorylation of the Dam1 complex (Cheeseman et al., 2002; Kang et al., 2001; Li et al., 2002). Though decreased Dam1 phosphorylation correlates with the segregation and checkpoint defects of *ipl1* mutant cells, a causal relationship was not established until Ipl1 phosphorylation sites on Dam1 were identified *in vitro* and phosphorylation site mutants were characterized *in vivo* (Cheeseman et al., 2002). Analysis of *dam1* phospho-deficient mutant cells revealed a temperature sensitive phenotype indistinguishable from the *ipl1* mutant phenotype

providing the strongest evidence that Dam1 is a critical downstream target of Ipl1 for its regulation of bipolar kinetochore attachment and the spindle checkpoint (Cheeseman et al., 2002).

It has been proposed that Ipl1-mediated phosphorylation reduces Dam1's affinity for the kinetochore (Shang et al., 2003). Consistent with this hypothesis, *dam1* phospho-mimetic mutant protein shows reduced interaction with the central kinetochore component Ndc80 *in vitro* and decreased association with the kinetochore *in vivo* (Shang et al., 2003). However, a contradictory report indicates that under-phosphorylated Dam1 from in *ipl1* mutant cells also displays limited kinetochore association that can be rescued by expressing the *dam1* phospho-mimetic mutant (Cheeseman et al., 2002).

This has led to speculation that the phosphoregulation of Dam1 is dynamic and requires both Ipl1 and the opposing Glc7 protein phosphatase (Cheeseman et al., 2002). Glc7 is the single, essential protein phosphatase I (PP1) catalytic subunit in budding yeast (Cannon et al., 1994), and is responsible for the regulation of numerous cellular processes including mitosis, meiosis, glycogen and sugar metabolism, transcription, translation, and mRNA processing (for review, see (Ceulemans and Bollen, 2004)). It is likely that Glc7 is the phosphatase that normally acts to antagonize essential Ipl1 functions because impairing Glc7 function suppresses the *ipl1* temperature sensitive growth defect (Francisco et al., 1994; Hsu et al., 2000). In addition, Glc7 regulates Dam1 phosphorylation *in vivo* (see Chapter 5), suggesting Dam1 may also be a critical target of the phosphatase. However, the

role of phosphorylation in Dam1 complex function remains unclear and the importance of Glc7 has not yet been determined.

To better understand Dam1 phosphoregulation, we examined the effects of Ipl1 and Glc7 activity on Dam1 localization, and further characterized several *dam1* phosphorylation site mutants. These studies reveal that although Ipl1 and Glc7 modulate Dam1's phosphorylation status, there are likely other essential Ipl1 and Glc7 substrates whose phosphorylation must be properly regulated to ensure accurate chromosome segregation.

Results

Dam1 localizes to kinetochores in ipl1 and glc7 mutant cells

To determine whether the kinetochore association of Dam1 is regulated by phosphorylation, we examined Dam1 localization by fluorescence microscopy in *ipl1* and *glc7* mutant cells. We utilized this independent localization technique because the previous conflicting reports analyzed kinetochore bound Dam1 by chromatin immunoprecipitation (ChIP). Wild type, *glc7-10*, and *ipl1-321* cells expressing triple GFP epitope-tagged Dam1 (Dam1-GFP3) and Tub1-CFP to mark spindles were shifted to the restrictive temperature (37 °C) for 2 hours (Figure 6.1). The *glc7-10* mutant suppresses *ipl1-321* at high temperatures and restores Dam1 phosphorylation to wild type levels (see Chapter 5). In wild type cells with short spindles, Dam1 localized to two spindle associated foci representing kinetochore clusters as described previously (Cheeseman et al., 2001a; Cheeseman et al., 2001b; Hofmann et al., 1998; Janke et al., 2002; Jones et al., 1999; Jones et al., 2001; Li et al., 2002). Surprisingly, Dam1 displayed kinetochore localization identical to wild type in all *glc7-10* and *ipl1-321* cells examined, indicating that phosphorylation does not significantly alter Dam1's association with the kinetochore. We have confirmed that these Dam1 clusters represent kinetochores because they are absent when kinetochore assembly is inhibited in *ndc10-1* mutant cells, though Dam1 still localized to spindles (data not shown). Furthermore, we could detect no difference in Dam1 localization when we compared synchronous cultures of wild type and *ipl1-321* cells released from G1 to the restrictive temperature (data not shown). In

contrast to the previous reports, we do not observe Ipl1 or Glc7 regulation of Dam1 localization.

Genetic analysis of dam1 phosphorylation site mutants suggests additional important Ipl1 and Glc7 targets

To further test the role of phosphorylation in Dam1 regulation we examined the genetic interactions of the *dam1* phosphorylation site mutants with *ipl1* and *glc7* mutants. We focused on two *dam1* mutants that were not previously analyzed for their genetic interactions and that most closely approximate the fully phosphorylated and unphosphorylated states. The *dam1* phospho-mimetic mutant has all four consensus Ipl1 sites changed to aspartic acid (*dam1 (S20D S257D S265D S292D)*), whereas the *dam1* phospho-deficient mutant has three out of the four Ipl1 consensus sites mutated to alanine (*dam1 (S257A S265A S292A) spc34 (T199A)*) in addition to the single site on Spc34, another essential component of the Dam1 complex (Cheeseman et al., 2002; Janke et al., 2002). The *dam1* mutant with all of the consensus Ipl1 sites changed to alanine is inviable and therefore has not been studied.

To facilitate genetic analysis, we backcrossed the *dam1* phospho-mimetic and -deficient mutants into the W303 strain background. We compared the backcrossed W303 strains with the original S288c strains and confirmed that the strain background difference did not substantially change growth and temperature sensitivity (Figure 6.2A). We next tested genetic interactions between these mutant

alleles and the *ipl1-321* and *glc7-10* alleles. These alleles must retain some enzymatic activity at restrictive temperatures since the double mutant is viable but strains completely lacking both *IPL1* and *GLC7* are inviable (data not shown and (Francisco et al., 1994)) (Figure 6.2B). If Dam1 were the essential target of Ipl1 and Glc7, the *dam1* phospho-mimetic mutant should suppress the *ipl1* mutant temperature sensitive growth defect and exacerbate the *glc7* mutant temperature sensitive growth defect. Conversely, the *dam1* phospho-deficient mutant should exacerbate the *ipl1* mutant growth phenotype and suppress the *glc7* mutant growth phenotype.

To test these predictions, we first examined the genetic interactions of the *dam1* phospho-mimetic mutant with *ipl1* and *glc7* mutants. In contrast to our expectations, *dam1 (S20D S257D S265D S292D)* did not suppress *ipl1-321* at 35 °C nor did it enhance the *glc7-10* temperature sensitivity (Figure 6.2B). Strikingly, the *ipl1-321* growth defect was still suppressed by *glc7-10* in the presence of *dam1 (S20D S257D S265D S292D)*, indicating that Ipl1 and Glc7 share other critical targets in addition to Dam1 or there are additional unidentified phosphorylation sites on Dam1.

We next analyzed the genetic interactions of the phospho-deficient *dam1* mutant with *ipl1* and *glc7* mutants. We attempted to generate an *ipl1-321 dam1 (S257A S265A S292A) spc34 (T199A)* mutant strain, but this combination was synthetically lethal, as expected (data not shown). We then tested if the *dam1* phospho-deficient mutant could suppress the *glc7-10* temperature sensitivity.

However, the *glc7-10 dam1 (S257A S265A S292A) spc34 (T199A)* mutant cells showed a synthetic growth defect at 35 °C instead of the expected suppression (Figure 6.2C). Surprisingly, the *glc7-10* mutant suppressed the *ipl1-321 dam1 (S257A S265A S292A) spc34 (T199A)* synthetic lethality at 23 °C although it did not display wild type growth at 30 °C (Figure 6.2D). Taken together, these genetic interactions are consistent with Ipl1 and Glc7 regulating other important substrates or additional Dam1 sites.

Impairing Ipl1 function exacerbates the dam1 phospho-deficient mutant chromosome segregation defect

These unexpected genetic interactions lead us to re-examine the phenotype of the *dam1* phosphorylation site mutant cells. Because aspartic acid mutations often do not perfectly mimic phosphorylation, we limited our analysis to the *dam1 (S257A S265A S292A) spc34 (T199A)* mutant. At elevated temperatures, these cells were reported to phenocopy *ipl1* mutant cells, displaying monopolar kinetochore attachments that fail to activate the spindle checkpoint. However, we found that the *dam1* phospho-deficient mutant activated the spindle checkpoint in an Ipl1-dependent manner (see Chapter 4), suggesting that these sites were not targets for Ipl1 checkpoint regulation.

The spindle checkpoint function of Ipl1 is likely coupled to its regulation of kinetochore attachment and chromosome segregation (see Chapter 4). We therefore compared the chromosome segregation defects of *dam1* phospho-deficient and *ipl1*

mutant cells using a fluorescently labeled chromosome IV (Chr IV). In this assay, sister chromatids display three potential outcomes at anaphase: 1) normal segregation of the sisters to opposite poles, 2) no segregation such that both sisters remain in the mother cell, or 3) abnormal segregation where both sisters move into the bud. Though both categories 2 and 3 represent missegregation, the overall pattern of segregation is also informative, as *ipl1* mutant cells display a segregation bias towards the bud (Pinsky et al., 2003; Tanaka et al., 2002). Wild type, *ipl1-321*, and *dam1 (S257A S265A S292A) spc34 (T199A) mad2Δ* mutant cells were arrested in G1 with mating pheromone to mark the mother cell with a mating projection, released to the restrictive temperature (37 ^{o}C), and assayed for the distribution of Chr IV (Figure 6.3A). Because the *dam1* mutant activates the spindle checkpoint, we eliminated the spindle checkpoint gene *MAD2* to allow cell cycle progression and comparison to the *ipl1* mutant cells. 120 minutes after release, just 3.5% of wild type cells showed chromosome missegregation and1.5% delivered both copies of Chr IV to the bud. In the *ipl1-321* cells, Chr IV was missegregated 71% of the time, with 44% of cells segregating both copies of Chr IV to the bud. In contrast, 48.5% of *dam1 (S257A S265A S292A) spc34 (T199A) mad2Δ* cells missegregated Chr IV and only 25% of these cells had both copies in the bud. Therefore, the *dam1* phospho-deficient mutant has a chromosome segregation defect that is distinct from *ipl1-321*.

We next determined whether impairing Ipl1 function exacerbates the *dam1* phospho-deficient mutant segregation defect. If Ipl1 regulates kinetochore

attachment via additional targets or phosphorylation sites on Dam1, then the double mutant should show the more severe *ipl1* mutant segregation defect. Because the *dam1* mutant is synthetically lethal with *ipl1-321*, we analyzed the consequences of Ipl1 loss of function in the phospho-deficient mutant background by using an analogue-sensitive *ipl1* allele (*ipl1-as5*) that has an enlarged ATP binding pocket to allow specific inhibition by bulky ATP analogue inhibitors (for review, see (Bishop et al., 2001)). Though kinase assays verified that the Ipl1-as5 protein was inhibited by the ATP analogue 1-NA-PP1 *in vitro*, the mutant had slightly reduced basal kinase activity in the absence of inhibitor (data not shown). To increase overall kinase activity *in vivo* and obtain conditional *ipl1 dam1* phospho-deficient mutant cells for analysis, we expressed *ipl1-as5* using the galactose-inducible promoter (*pGAL*). This promoter is highly transcriptionally active when cells are grown in galactose (inducing conditions) and is repressed when cells are grown in glucose (non-inducing conditions).

To determine the effect of impairing Ipl1 function on the chromosome segregation profile of the *dam1* phospho-deficient mutant, wild type, *dam1 (S257A S265A S292A) spc34 (T199A) mad2Δ*, *pGAL-ipl1-as5*, and *dam1 (S257A S265A S292A) spc34 (T199A) pGAL-ipl1-as5* cells containing fluorescently labeled Chr IV were arrested in G1 under inducing conditions and released into non-inducing media at the restrictive temperature (Figure 6.3B). Inhibitor was added upon budding to inactivate residual Ipl1-as5 protein. 120 minutes after release, just 8% of wild type cells showed chromosome missegregation and 2% moved both copies of Chr IV to

the bud. Consistent with the previous experiment, Chr IV was missegregated in 47% of the *dam1 (S257A S265A S292A) spc34 (T199A) mad2Δ* cells, and 63% of the *pGAL-ipl1-as5* cells, with 24% and 41% of cells segregating both Chr IV copies to the bud, respectively. Strikingly similar to the *pGAL-ipl1-as5* mutant, the *dam1 (S257A S265A S292A) spc34 (T199A) pGAL-ipl1-as5* cells missegregated Chr IV 66% of the time and had both copies of Chr IV in the bud in 38% of cells. These results complement the genetic analyses and indicate that Ipl1 regulates chromosome segregation through the phosphorylation of additional important substrates or sites on Dam1.

The dam1 phospho-deficient mutant has spindle defects similar to other Dam1 complex mutants

Our work indicates that the *dam1* phospho-deficient mutant does not have the *ipl1* mutant phenotype as previously suggested (Cheeseman et al., 2002). We therefore tested whether this mutant resembles other mutant components of the Dam1 complex and also has defects in spindle integrity (Cheeseman et al., 2001a; Cheeseman et al., 2001b; Janke et al., 2002; Jones et al., 1999; Jones et al., 2001; Li et al., 2002). To address this question, *dam1 (S257A S265A S292A) spc34 (T199A)* cells expressing GFP-Tub1 were arrested in G1, released to the restrictive temperature, and large budded cells were monitored for spindle morphology and DNA segregation 120 minutes after release (Figure 6.4). This is a time when the cells remain checkpoint arrested (see Chapter 4) and should therefore maintain a

short spindle. As a control, we utilized *cdc16* mutant cells that are conditionally defective in metaphase progression (Cohen-Fix et al., 1996). While 54% of *cdc16-1* cells arrest with a single mass of DNA and a short spindle positioned at the bud neck, only 13% of the *dam1 (S257A S265A S292A) spc34 (T199A)* mutant cells arrested with a short spindle. Instead, 70% of the dam1 phospho-deficient cells contained two unequally segregated masses of DNA and a broken down spindle. Therefore, *dam1 (S257A S265A S292A) spc34 (T199A)* mutant cells undergo premature spindle elongation and breakdown during a metaphase arrest, phenotypes that are common to numerous other mutants that impair the Dam1 complex.

Discussion

The phosphorylation of Dam1 is regulated by the opposing activities of the Ipl1/Aurora protein kinase and Glc7/PP1 phosphatase. We show here that Dam1's kinetochore localization is not changed in *ipl1* or *glc7* mutants, suggesting that Dam1 phosphorylation does not regulate its kinetochore association. Furthermore, the genetic and phenotypic characterization of *dam1* phosphorylation site mutants indicates that Ipl1 and Glc7 must regulate additional critical substrates to promote accurate chromosome segregation.

Ipl1 and Glc7 do not regulate Dam1's kinetochore localization

It was proposed that phosphorylation alters Dam1's kinetochore localization (Cheeseman et al., 2002; Shang et al., 2003), though the conclusions were contradictory. When we modulated Dam1 phosphorylation using *ipl1* and *glc7* mutants, we detected no difference in Dam1's kinetochore localization by fluorescence microscopy in live cells, consistent with previous Dam1 localization studies (Kang et al., 2001). It is therefore unlikely that either Ipl1 or Glc7 regulate the association of Dam1 with kinetochores.

Genetic analysis of dam1 phosphorylation site mutants indicates there may be additional critical targets of Ipl1 and Glc7

Previous genetic analysis of a series of *dam1* phosphorylation site mutants supported the hypothesis that Dam1 is a crucial downstream target of Ipl1, though

the mutants most closely approximating the fully phosphorylated and unphosphorylated states were not genetically characterized (Cheeseman et al., 2002; Shang et al., 2003). Our genetic analyses of these mutants indicate that Dam1 is likely one of several important Ipl1 and Glc7 targets. We found that the phospho-mimetic mutant *dam1 (S20D S257D S265D S292D)* mutant did not affect the growth defect of either *ipl1-321* or *glc7-10* mutant cells as predicted if it were the key target of these enzymes. Furthermore, the *ipl1-321 dam1 (S20D S257D S265D S292D)* double mutant could still be suppressed by impairing Glc7 function, suggesting that there are essential Ipl1 and Glc7 targets in addition to Dam1, or that there are additional Dam1 phosphorylation sites that have not been identified. The phospho-deficient *dam1 (S257A S265A S292A) spc34 (T199A)* mutant was synthetically lethal with *ipl1-321,* as expected, though the lethality could be suppressed by *glc7-10*, indicating that phosphorylation of other sites on Dam1 (such as S20), or sites on additional Ipl1 targets had been restored. In addition, the *dam1* phospho-deficient mutant exacerbated the *glc7-10* growth defect instead of suppressing it. Taken together, these observations suggest that Ipl1 and Glc7 must have additional targets and/or phosphorylation sites on Dam1 that must be properly regulated to ensure accurate chromosome segregation.

The dam1 phospho-deficient mutant resembles other mutants that impair Dam1 complex function

Thought it was reported that the *dam1* phospho-deficient mutant has a phenotype indistinguishable from the *ipl1* mutant (Cheeseman et al., 2002), our characterization revealed a constellation of phenotypes that showed only superficial similarity. Rather, the *dam1* phospho-deficient mutant exhibited the hallmark of mutations in Dam1 complex components, abnormal spindle elongation and breakdown in the presence of an activate spindle checkpoint (Cheeseman et al., 2001a; Cheeseman et al., 2001b; He et al., 2001; Janke et al., 2002; Jones et al., 1999; Jones et al., 2001; Li et al., 2002). These results suggest that this mutant does not separate Dam1's spindle functions from its role at the kinetochore, as previously suggested (Cheeseman et al., 2002). It is therefore unclear whether this mutant really represents the consequences of the lack of Dam1 phosphorylation by Ipl1. It is possible that the Dam1 phosphorylation site mutations create structural problems in addition to, or alternatively to phosphorylation defects that confound our characterization. For example, this phospho-deficient mutant eliminates phosphorylation on the mutated residues at all temperatures, yet displays a growth phenotype only at elevated temperatures. Furthermore, it is not known whether Ipl1 or Glc7 regulates the phosphorylation of these particular sites *in vivo*.

However, the *dam1* phospho-deficient mutant has an intermediate chromosome segregation defect that is consistent with Ipl1 regulating kinetochore attachment at least partially through Dam1 phosphorylation. In addition, the *ipl1 dam1* double mutant segregation defect is similar to the *ipl1* single mutant and is not

more severe, suggesting that Ipl1 and Dam1 function in the same pathway for chromosome segregation.

Models for Ipl1/Glc7 regulation of chromosome segregation

Our work suggests that the current models for the role of Ipl1/Glc7-mediated phosphoregulation of chromosome segregation are not yet fully understood. Because Dam1 is likely one critical target, it is possible that phosphorylation regulates some aspect of the kinetochore microtubule interface not revealed by simple localization. Alternatively, Dam1 phosphorylation might be crucial for non-essential functions that involve microtubule regulation.

Another possibility is that Ipl1 cooperates with additional kinases or phosphatases to regulate Dam1 phosphorylation. Protein kinase A (PKA) phosphorylates the Dam1 complex *in vitro* (Li et al., 2005) and the cyclin dependent kinase 1 (Cdk1) phosphorylates the Ask1 component of the Dam1 complex *in vivo* (Higuchi and Uhlmann, 2005; Li and Elledge, 2003). Therefore, continued analysis of the sites phosphorylated by Ipl1, Mps1, PKA, and Cdk1 *in vivo* and the identification of other Dam1 kinases and phosphatases may provide additional clues to the regulation of the Dam1 complex.

The model that we favor is that Dam1 phosphorylation is important only in the context of the phosphorylation of other Ipl1 substrates. Consistent with this idea, many other Ipl1 substrates have been identified *in vivo* (the Ipl1 regulator Sli15, histone H3 and the condensin subunit Ycg1) and *in vitro* (the centromeric H3 variant

Cse4, and the kinetochore proteins Ndc10, Ndc80, Mif2, and Dsn1) (Biggins et al., 1999; Buvelot et al., 2003; Cheeseman et al., 2002; Hsu et al., 2000; Kang et al., 2001; Lavoie et al., 2004; Westermann et al., 2003). To date, *DAM1* homologs have been identified only in fungi and Aurora B regulates at least some of its kinetochore functions in multicellular eukaryotes via the phosphorylation of the microtubule destabilizing kinesin, MCAK (Andrews et al., 2004; Lan et al., 2004). Our analysis suggests that there are multiple essential Ipl1 targets and the only way to suppress the *ipl1* phenotype is by altering the opposing phosphatase activity to maintain the kinase/phosphatase balance. We are therefore actively pursuing the identification of additional Ipl1 and Glc7 substrates to determine how these enzymes regulate chromosome segregation, the spindle checkpoint and other cellular processes.

Experimental Procedures

Microbial Techniques. Media and microbial techniques were essentially as described (Rose et al., 1990; Sherman et al., 1974). All experiments in which cells were released from a G1 arrest were carried out by α-factor arrest and release, using α-factor at 1 μg/ml. 1-naphthyl-pyrazolo[3,4-d]pyrimidine (1-NA-PP1) was used at 50 μM

Yeast strain construction. Yeast strains are listed in Table 6.1 and were constructed by standard genetic techniques. All strains were generated for this study and are isogenic with the W303 background, except *dam1 (S20D S257D S265D S292D)* (SBY3352=DDY2497) and *dam1 (S257A S265A S292A) spc34 (T199A)* (SBY3353=DDY2501) (Cheeseman et al., 2002) (a gift from David Drubin and Georjana Barnes, University of California, Berkeley, CA), which remain in the S288c background. The *dam1 (S257A S265A S292A) spc34 (T199A)* and *dam1 (S20D S257D S265D S292D)* strains in the W303 background were backcrossed at least 5 times. The *glc7-10* (Sassoon et al., 1999) (a gift from Michael Stark, University of Dundee, Dundee, U.K.) and *ipl1-321* (Biggins et al., 1999) alleles were crossed to make strains for this study. Strains containing *TUB1-CFP:URA3* were obtained by integrating plasmid pSB375 (a gift from Kerry Bloom, University of North Carolina, Chapel Hill, NC) digested with *StuI* at the *URA3* locus. Strains containing *GFP-TUB1:LEU2* were generated by integrating plasmid pSB340 digested with *AflII* at the *LEU2* locus. *DAM1-GFP3* strains were made by

integrating plasmid pSB867 digested with *SpeI* at the *DAM1* locus. Gene deletions were made using a PCR-based integration system (Longtine et al., 1998) and were confirmed by PCR. Specific primer sequences are available upon request. All fusion proteins are fully functional.

Plasmid Construction. The *DAM1-GFP3* integrating plasmid was made by PCR amplification of the C-terminal 700 base pairs of *DAM1* from pSF14 (a gift from Trisha Davis, University of Washington, Seattle, WA) using primers SB972 and SB973, the latter containing a *BamHI* restriction site. The resulting PCR product was digested with *EcoRI* and *BamHI* and ligated into the *EcoRI* and *BamHI* sites of PB1585 (a gift from David Pellman, Harvard Medical School, Boston, MA), to create pSB867.

Microscopy. Live microscopy was performed as described (Buvelot et al., 2003). Analysis of sister chromatids and GFP-Tub1 in fixed cells was performed as described (Biggins et al., 1999). DAPI (Molecular Probes) was used at 1μg/ml final concentration. At least 100 cells were analyzed for all reported experiments. The error bars indicate the 95% confidence interval.

Table 6.1 *Yeast strains used in this study*

Strain	Genotype
SBY214	*MAT**a** ura3-1 leu2,3-112 his3-11:pCUP1-GFP12-lacI12:HIS3 trp1-1:256lacO:TRP1 lys2Δ ade2-1 can1-100 bar1Δ*
SBY322	*MAT**a** ura3-1 leu2,3-112 his3-11:pCUP1-GFP12-lacI12:HIS3 trp1-1:256lacO:TRP1 lys2Δ ade2-1 can1-100 bar1Δ ipl1-321*
SBY1306	*MAT**a** ura3-1 leu2,3-112 his3-11 trp1-1:glc7-10:TRP1 ade2-1 can1-100 bar1Δ glc7::LEU2*
SBY1994	*MAT**a** ura3-1 leu2,3-112 his3-11 trp1-1:glc7-10:TRP1 ade2-1 can1-100 bar1Δ glc7::LEU2 ipl1-321*
SBY3352	*MAT**a** ura3-52 leu2,3-112 his3Δ200 ade2-1 dam1(S20D S257D S265D S292D):KAN*
SBY3353	*MAT**a** ura3-52:spc34(T199A):URA3 leu2,3-112 his3Δ200 ade2-1 dam1(S257A S265A S292A):KAN spc34::HIS3*
SBY3701	*MAT**a** ura3-52:spc34(T199A):URA3 leu2,3-112 his3-11 trp1-1 ade2-1 can1-100 bar1Δ dam1(S257A S265A S292A):KAN spc34::HIS3*
SBY3720	*MAT**a** ura3-1 leu2,3-112 his3-11 trp1-1 lys2Δ ade2-1 can1-100 bar1Δ dam1(S20D S257D S265D S292D):KAN*
SBY3849	*MAT**a** ura3-52:spc34(T199A):URA3 leu2,3-112 his3-11:pCUP1-GFP12-lacI12:HIS3 trp1-1:256lacO:TRP1 lys2Δ ade2-1 can1-100 bar1Δ dam1(S257A S265A S292A):KAN spc34::HIS3*
SBY3881	*MAT**a** ura3-1 leu2,3-112 his3-11 trp1-1 lys2Δ ade2-1 can1-100 bar1Δ dam1(S20D S257D S265D S292D):KAN ipl1-321*
SBY3958	*MAT**a** ura3-1 leu2,3-112 his3-11 trp1-1:glc7-10:TRP1 ade2-1 can1-100 bar1Δ glc7::LEU2 dam1(S20D S257D S265D S292D):KAN*
SBY4394	*MAT**a** ura3-1:TUB1-CFP:URA3 leu2,3-112 his3-11 trp1-1 ade2-1 can1-100 bar1Δ DAM1-GFP3:HIS3*
SBY4000	*MAT**a** ura3-1:GFP-TUB1:URA3 leu2,3-112 his3-11 lys2Δ ade2-1 can1-100 bar1Δ cdc16-1*
SBY4062	*MAT**a** ura3-52:spc34(T199A):URA3 leu2,3-112:GFP-TUB1:LEU2 his3-11 trp1-1 lys2Δ ade2-1 can1-100 bar1Δ dam1(S257A S265A S292A):KAN spc34::HIS3*
SBY4326	*MAT**a** ura3-52:spc34(T199A):URA3 leu2,3-112 his3-11:pCUP1-GFP12-lacI12:HIS3 trp1-1:256lacO:TRP1 lys2Δ ade2-1 can1-100 bar1Δ dam1(S257A S265A S292A):KAN spc34::HIS3 mad2::URA3*
SBY4425	*MAT**a** ura3-1:TUB1-CFP:URA3 leu2,3-112 his3-11 trp1-1 ade2-1 can1-100 bar1Δ DAM1-GFP3:HIS3 ipl1-321*
SBY4782	*MAT**a** ura3-52:spc34(T199A):URA3 leu2,3-112 his3-11 trp1-1:glc7-10:TRP1 ade2-1 can1-100 bar1Δ dam1(S257A S265A S292A):KAN spc34::HIS3 glc7::LEU2*
SBY4885	*MAT**α** ura3-1:TUB1-CFP:URA3 leu2,3-112 his3-11 trp1-1:glc7-10:TRP1 ade2-1 can1-100 bar1Δ glc7::LEU2 DAM1-GFP3:HIS3*

Table 6.1 *continued*

Strain	Genotype
SBY4887	*MAT**a** ura3-52:spc34(T199A):URA3 leu2,3-112 his3-11 trp1-1:glc7-10:TRP1 ade2-1 can1-100 bar1Δ dam1(S257A S265A S292A):KAN spc34::HIS3 glc7::LEU2 ipl1-321*
SBY4890	*MAT**a** ura3-1 leu2,3-112 his3-11 trp1-1:glc7-10:TRP1 lys2Δ ade2-1 can1-100 bar1Δ glc7::LEU2 dam1(S20D S257D S265D S292D):KAN ipl1-321*
SBY4997	*MAT**a** ura3-52:spc34(T199A):URA3 leu2,3-112 his3-11:pCUP1-GFP12-lacI12:HIS3 trp1-1:256lacO:TRP1 lys2Δ ade2-1 can1-100 bar1Δ dam1(S257A S265A S292A):KAN spc34::HIS3 pGAL-ipl1-as5:LEU2:HIS3:ipl1::KAN*
SBY4998	*MAT**a** ura3-1 leu2,3-112 his3-11:pCUP1-GFP12-lacI12:HIS3 trp1-1:256lacO:TRP1 lys2Δ ade2-1 can1-100 bar1Δ pGAL-ipl1-as5:LEU2:HIS3:ipl1::KAN*

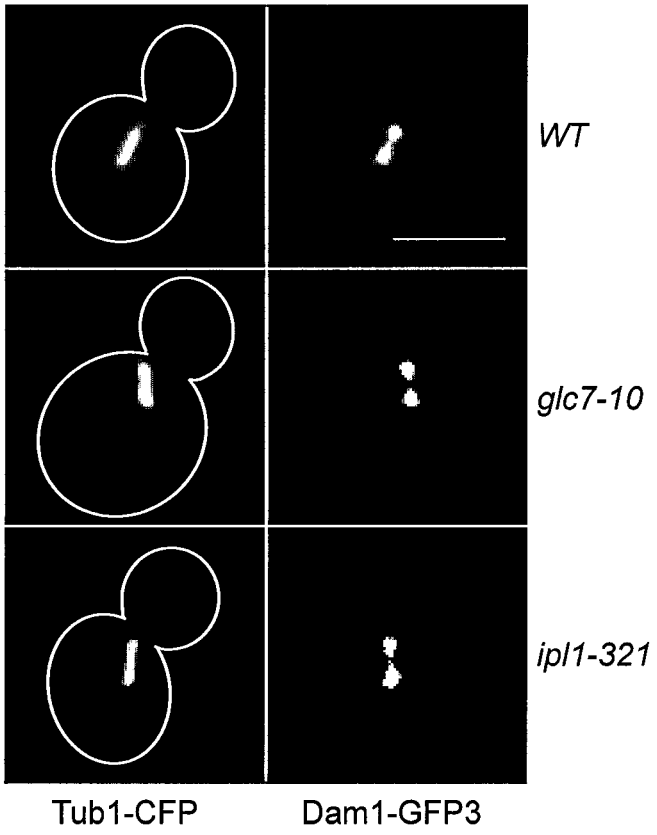
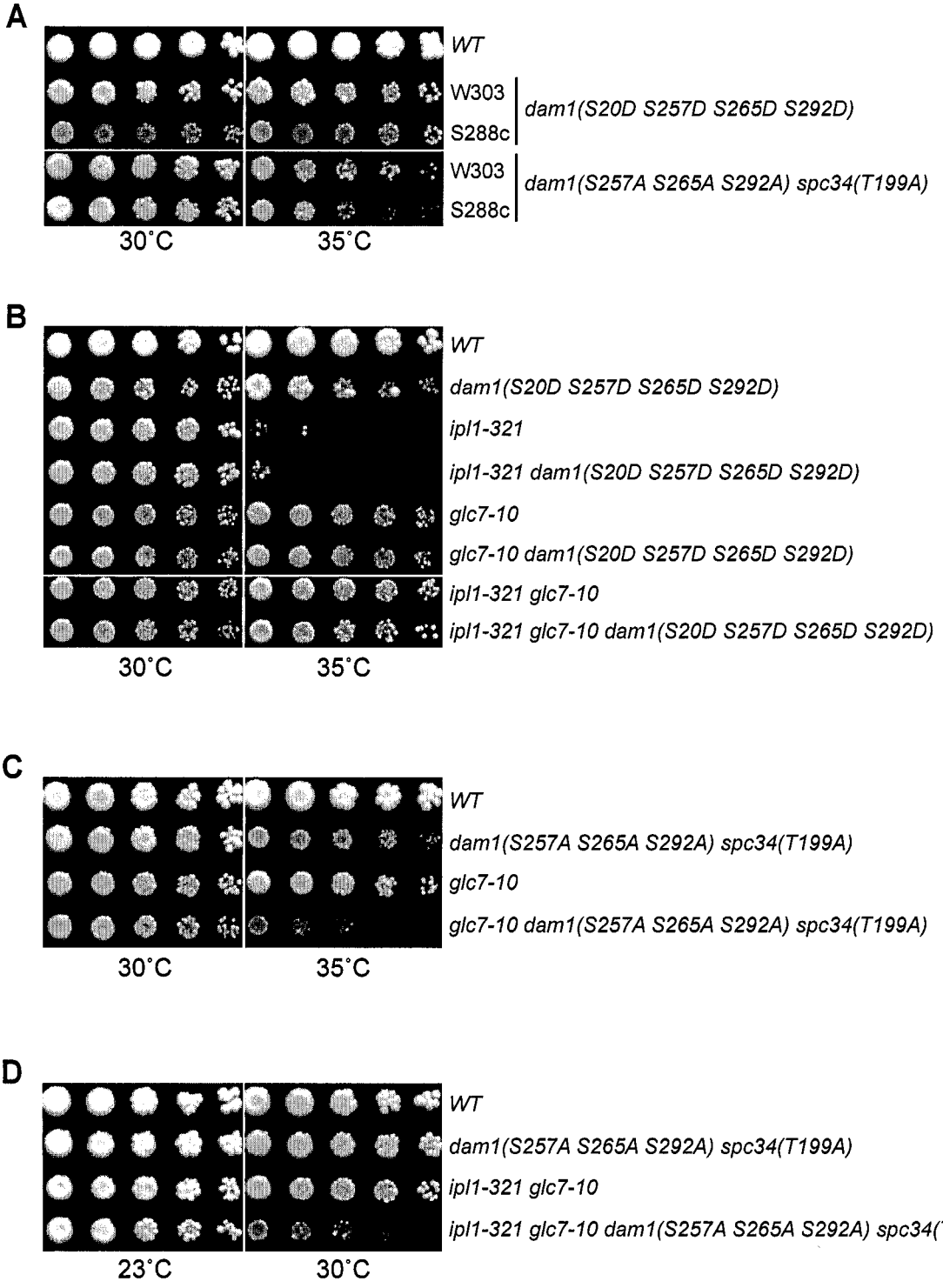


Figure 6.1 Phosphorylation does not regulate the kinetochore localization of Dam1. Wild type (SBY 4394), *glc7-10* (SBY4885), and *ipl1-321* cells (SBY4425) expressing Tub1-CFP and Dam1-GFP3 were incubated at 37 °C for 2 hrs. Cells with short spindles were analyzed for Dam1 localization. Bar, 5 μm.

Figure 6.2 genetic analyses of the *dam1* phosphorylation site mutants indicate that Ipl1 and Glc7 regulate chromosome segregation through additional important targets. (A) Differences in strain background do not significantly alter the growth and temperature sensitivity of the *dam1* phospho-mutants. 5-fold serial dilutions of wild type (SBY3), *dam1 (S20D S257D S265D S292D)* (W303=SBY3720, S288c=SBY3352), and *dam1 (S257A S265A S292A) spc34 (T199A)* (W303=SBY3701, S288c=SBY3353) were incubated for 2 days at 30 °C and 35 °C. (B) Impairing Glc7 function fully suppresses *ipl1-321* in the presence of the *dam1* phospho-mimetic mutant. 5-fold serial dilutions of wild type (SBY 214), *dam1 (S20D S257D S265D S292D)* (SBY3720), *ipl1-321* (SBY322), *ipl1-321 dam1 (S20D S257D S265D S292D)* (SBY3881), *glc7-10* (SBY1306), *glc7-10 dam1 (S20D S257D S265D S292D)* (SBY3958), *ipl1-321 glc7-10* (SBY1994), and *ipl1-321 glc7-10 dam1 (S20D S257D S265D S292D)* (SBY4890) cells were incubated for 2 days at 30 °C and 35 °C. (C) The *dam1* phospho-deficient mutant and *glc7-10* display a synthetic growth defect. 5-fold serial dilutions of wild type (SBY214), *dam1 (S257A S265A S292A) spc34 (T199A)* (SBY3849), *glc7-10* (SBY1306), and *glc7-10 dam1 (S257A S265A S292A) spc34 (T199A)* mutant cells (SBY4782) were incubated for 2 days at 30 °C and 35 °C. (D) The synthetic lethality of the *dam1* phospho-deficient mutant and *ipl1-321* is suppressed by impairing Glc7 function. 5-fold serial dilutions of wild type (SBY214), *dam1 (S257A S265A S292A) spc34 (T199A)* (SBY3849), *ipl1-321 glc7-10 (*SBY1994), and *ipl1-321 glc7-10 dam1 (S257A S265A S292A) spc34 (T199A)* mutant cells (SBY4887), were incubated for 3 days at 23 °C and 2 days at 30 °C.


A
WT
W303
S288c
dam1(S20D S257D S265D S292D)
W303
S288c
dam1(S257A S265A S292A) spc34(T199A)
30˚C
35˚C
B
WT
dam1(S20D S257D S265D S292D)
ipl1-321
ipl1-321 dam1(S20D S257D S265D S292D)
glc7-10
glc7-10 dam1(S20D S257D S265D S292D)
ipl1-321 glc7-10
ipl1-321 glc7-10 dam1(S20D S257D S265D S292D)
30˚C
35˚C
C
WT
dam1(S257A S265A S292A) spc34(T199A)
glc7-10
glc7-10 dam1(S257A S265A S292A) spc34(T199A)
30˚C
35˚C
D
WT
dam1(S257A S265A S292A) spc34(T199A)
ipl1-321 glc7-10
ipl1-321 glc7-10 dam1(S257A S265A S292A) spc34(T199A)
23˚C
30˚C

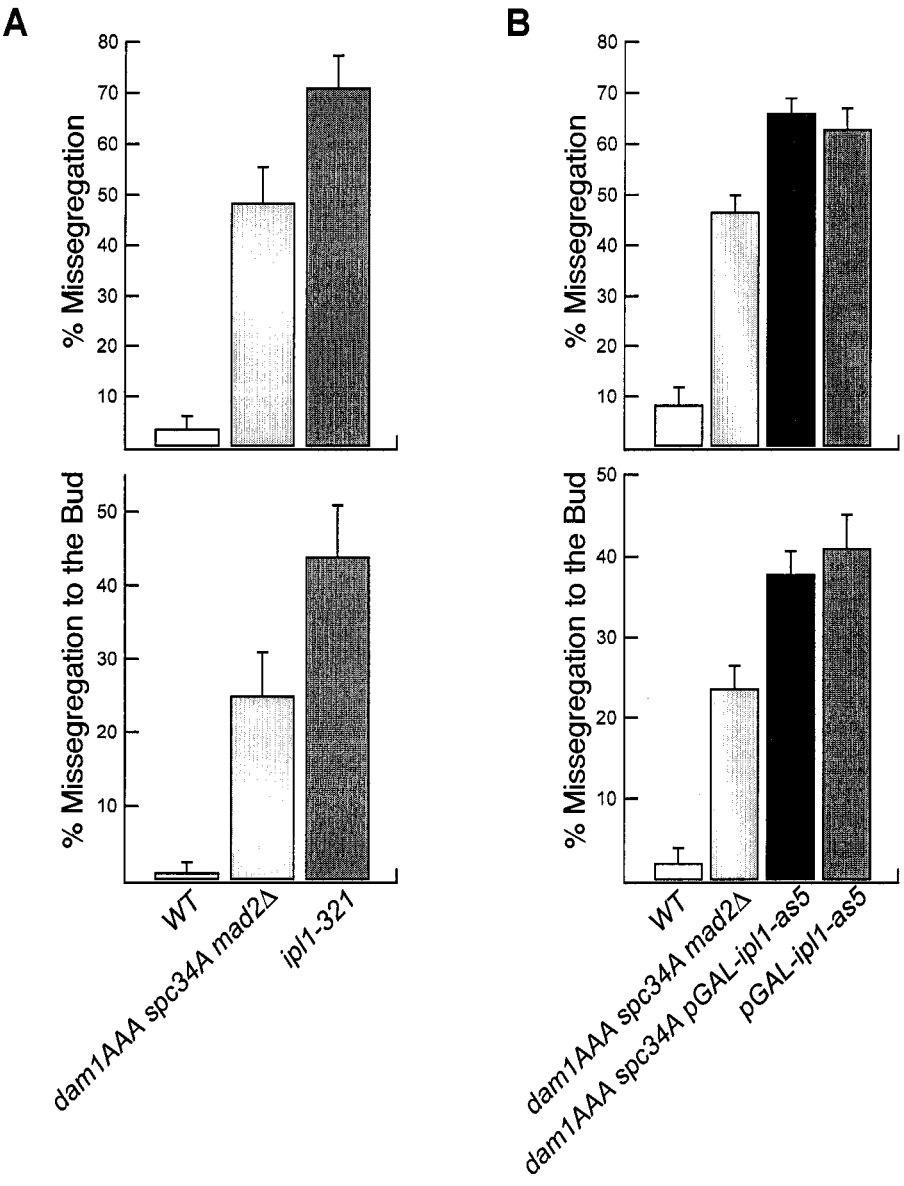


Figure 6.3 Chromosome segregation analysis of the *dam1* phospho-deficient mutant. (A) The *dam1* phospho-deficient mutant has a chromosome segregation defect distinct from *ipl1-321* cells. Wild type (SBY214), *dam1 (S257A S265A S292A) spc34 (T199A) mad2*Δ (SBY4326), and *ipl1-321* (SBY322) cells containing fluorescently marked chromosome IV (Chr IV) were arrested in G1 and released to 37 °C for 120 min. Large budded cells were monitored for the distribution of Chr IV. % Missegregation accounts for all cells in which either the mother cell or the bud contained both Chr IV sister chromatids. % Missegregation to the bud represents the cells where both sister chromatids went to the bud. (B) Ipl1 and Dam1 function in the same pathway to regulate chromosome segregation. Wild type (SBY214), *dam1 (S257A S265A S292A) spc34 (T199A) mad2*Δ (SBY4326), *dam1 (S257A S265A S292A) spc34 (T199A) pGAL-ipl1-as5* (SBY4997), and *pGAL-ipl1-as5* (SBY4998) cells containing fluorescently marked Chr IV were grown in inducing media, arrested in G1, and released to 37 °C in non-inducing media. 1-NA-PP1 was added upon budding and the cells were analyzed as in (A).

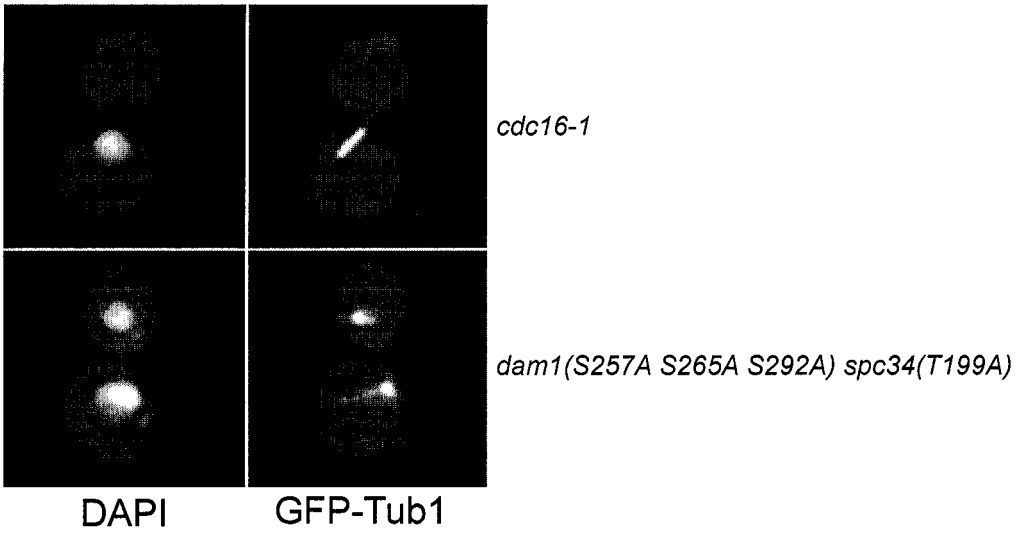


Figure 6.4 The *dam1* phospho-deficient mutant has spindle defects. *cdc16-1* (SBY4000) and *dam1 (S257A S265A S292A) spc34 (T199A)* mutant cells (SBY4062) expressing GFP-Tub1 were arrested in G1 and released to 37 °C for 120 min. DNA (DAPI) and spindles were analyzed in large budded cells. Bar, 5 μm.

CHAPTER 7:

Conclusions and Perspectives

Presented in this dissertation is a detailed analysis of the role of the Ipl1/Aurora B protein kinase in chromosome segregation and the spindle checkpoint. Below I highlight the important findings and speculate on future research.

The Mtw1 kinetochore complex

I describe here genetic and physical interaction data indicating that Mtw1, Dsn1, Nnf1, and Nsl1 comprise the budding yeast Mtw1/Mis12 kinetochore complex. Consistent with this work, the Mtw1 complex was concurrently identified by affinity purification by several other groups (De Wulf et al., 2003; Hazbun et al., 2003; Nekrasov et al., 2003; Westermann et al., 2003). In addition, Mtw1/Mis12 interacting proteins with similarity to the budding yeast components have been purified from fission yeast, human, and *C. elegans* cells, indicating that the complex may be conserved (Cheeseman et al., 2004; Kops et al., 2005; Nekrasov et al., 2003; Obuse et al., 2004).

The analysis of cells with impaired Mtw1 complex function revealed several striking phenotypes, including a severe spindle positioning defect and unattached chromosomes (Chapter 2). Because budding yeast undergo asymmetric cell divisions, the proper positioning of the short, metaphase spindle at the bud neck is critical for chromosome segregation fidelity. However, in *mtw1-1* cells, the short spindle migrates prematurely into the bud. Preliminary work indicates that this defect is suppressed by deleting the dynein microtubule motor and other regulators of dynein function (data not shown). These results provide an intriguing link

between kinetochore function and spindle positioning and suggest that Mtw1 inhibits dynein-mediated bud-directed forces or activates mother-directed forces that are opposed by dynein activity. Cells depleted for the cyclin, Clb5, show a similar positioning defect (Segal et al., 2000; Segal et al., 1998), and recent experiments indicate that Dsn1 is phosphorylated by the cyclin dependent kinase, Cdc28, *in vivo* (data not shown). Future work will examine the potential regulation of dynein by the Mtw1 complex and determine the role of Dsn1 phosphorylation in spindle positioning and kinetochore function.

Ipl1/Aurora regulation of kinetochore attachment and the spindle checkpoint

The demonstration that Ipl1 activity is responsible for the unattached chromosomes in *mtw1-1* mutant cells supported the hypothesis that Ipl1 promotes proper segregation by destabilizing kinetochore-microtubule interactions that are not under tension (Tanaka et al., 2002) (Chapter 2). Consistent with this proposal, analysis of representative mutants from each of the known kinetochore subcomplexes revealed that Ipl1 is required to generate unattached kinetochores in all mutants examined (Chapter 4). A substantial literature is devoted to assigning particular kinetochore proteins specific roles in microtubule attachment or kinetochore tension through the characterization of conditional mutants or *siRNA* depletion in a variety of model organisms. However, these experiments were carried out in the presence of active Ipl1/Aurora, suggesting that any observed attachment defects are the indirect result of Ipl1/Aurora-mediated kinetochore detachment. My

results suggest that most kinetochore proteins are not directly involved in microtubule attachment and impairing kinetochore component function generally results in tension defects that are monitored and corrected by Ipl1/Aurora. Therefore, the conclusions about the role of individual kinetochore complexes in microtubule attachment may need to be re-evaluated in the absence of Ipl1/Aurora activity.

I show here that the role of Ipl1 in destabilizing improper kinetochore attachments is strongly correlated with the Ipl1's function activating the spindle checkpoint, suggesting that Ipl1 regulates checkpoint activity by generating unattached kinetochores (Chapter 4). This is the strongest demonstration to date that the primary defect recognized by the spindle checkpoint is the unattached kinetochore. However, this argument relies on Ockham's razor (for review, see (Kaye and Martin, 2001)) and I have not ruled out the possibility that Ipl1 promotes spindle checkpoint activation independently of creating unattached kinetochores. There may still exist a special *IPL1* allele, or an allele of the *SLI15* or *BIR1* chromosomal passengers that separates these functions and allows tension defects to activate the checkpoint without kinetochore detachment.

Ipl1 regulation and tension sensing

Though this work presents a plausible mechanism to explain Ipl1-mediated correction of kinetochore tension defects, how Ipl1 recognizes and is regulated by tension is poorly understood. Ipl1 levels, activity, and localization in metaphase are

not affected by either the presence or absence of tension (data not shown and (Buvelot et al., 2003)), suggesting that Ipl1 inactivation might not be required to prevent the inappropriate destabilization of bipolar attachments. However, it is not clear if only a small pool of Ipl1 is tension-regulated and might escape detection due to the limited sensitivity of our assays. Alternatively, tension could regulate the Sli15 or Bir1 components of the passenger complex.

The role of Glc7/PP1 in antagonizing Ipl1 phosphorylation (Chapters 5 and 6) provides the possibility that it is the opposing phosphatase that responds to tension. In this scenario, tension defects would inhibit Glc7 and shift the kinase/phosphatase balance towards Ipl1. PP1 catalytic subunits are regulated by their interactions with specialized subunits that target the phosphatase to appropriate targets. However, the Glc7 mitotic regulatory subunit that localizes Glc7 to kinetochores has not yet been identified and the effects of tension on Glc7 function remain to be explored.

Another possibility is that tension controls the access of Ipl1 and Glc7 to their substrates but does not directly regulate either the kinase or the phosphatase. For example, bipolar tension-generating attachments might protect Ipl1 targets, whereas the absence of tension might expose relevant sites to Ipl1-mediated phosphorylation. Consistent with this, the Aurora B substrate MCAK, co-localizes with Aurora B only when kinetochores are not under tension (Andrews et al., 2004). Future experiments will differentiate between these models and uncover how Ipl1 detects and responds to tension defects.

Ipl1 substrates

The Dam1 kinetochore complex is reported to be the critical Ipl1 target in budding yeast (Cheeseman et al., 2002; Shang et al., 2003). Though Ipl1 regulates Dam1 phosphorylation *in vivo* (Chapter 5), the genetic and phenotypic analysis of the *dam1* phosphorylation site mutants clearly indicates that Ipl1 must have additional substrates or sites on the Dam1 complex that regulate kinetochore attachment and the spindle checkpoint (Chapters 4 and 6). Similarly, the characterization of MCAK phosphorylation site mutants suggests that Aurora B also has multiple kinetochore substrates (Andrews et al., 2004; Lan et al., 2004; Ohi et al., 2004). It is unlikely that the *ipl1/Aurora* mutant phenotype results simply from the reduced phosphorylation of Dam1 and MCAK in combination because Dam1 complex homologues have not been identified outside of fungi (Cheeseman et al., 2001b). Furthermore, the closest MCAK relative in budding yeast, the non-essential kinesin motor Kip3, does not play a significant role in kinetochore function and is not known to be phosphorylated by Ipl1 *in vivo* (data not shown and (DeZwaan et al., 1997)). Therefore, the future identification and careful analysis of additional Ipl1 targets will be invaluable for our understanding of chromosome segregation fidelity and will likely reveal the mechanisms underlying Ipl1-mediated attachment instability.

Aurora B and Cancer

The increased expression of Aurora B is associated with tumor-derived cell lines and primary cancer cells of several types, suggesting that Aurora B might act as an oncogene (Araki et al., 2004; Tatsuka et al., 1998). Consistent with this, Aurora B levels are tightly regulated and overexpression in cultured cells results in aneuploidy, anchorage-independent growth and tumor formation in nude mice (Nguyen et al., 2005; Ota et al., 2002). However, it is not clear whether the role of Aurora B overexpression in cellular transformation involves misregulation of kinetochore attachment or other Aurora B functions, such as cytokinesis.

The Aurora B gene maps to a chromosomal region (17p13) that is frequently deleted in cancer cells (Tatsuka et al., 1998). Furthermore, chromosome segregation errors and spindle checkpoint dysfunction are associated with many cancers (for reviews, see (Bharadwaj and Yu, 2004; Draviam et al., 2004)), suggesting that the loss of Aurora B function might also contribute to carcinogenesis. Though the Aurora B chromosomal region contains additional tumor-suppressor and tumor-related genes (Tatsuka et al., 1998), an intriguing possibility is that Aurora B inhibits tumor formation or progression through its role correcting improper kinetochore-microtubule interactions. However, a causal link between cancer development and Aurora B misregulation (both overexpression and loss of function) has not yet been firmly established. The basic Ipl1 findings presented here will likely inform future Aurora B studies and might ultimately influence the way we think about Aurora B in human disease.

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