Regulation of budding yeast kinetochore proteins by SUMO modification

by

Benjamen H.W. Montpetit

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ABSTRACT

Genome stability is a fundamental requirement for the stable propagation of eukaryotic cells, maintenance of phenotype, and cell viability. For example, aneuploidy (abnormal chromosome number), is a hallmark of most human cancers, and can be attributed to increased rates of chromosome instability in cancer cells. The multiprotein kinetochore complex contributes to faithful chromosome segregation by mediating the attachment of a specialized chromosomal region, the centromere, to the mitotic spindle. A dysfunctional kinetochore represents one possible source for chromosome instability and the generation of aneuploid cancer cells, due to a failure to properly mediate this attachment. To better understand the regulation of kinetochore proteins and their role in chromosome segregation, a series of genomic screens were performed with known kinetochore components in the budding yeast *Saccharomyces cerevisiae,* to identify genetic/physical interactions and novel functions that are critical for proper chromosome segregation. This work lead to the study of two distinct relationships: (1) between the kinetochore and the nuclear envelope, and (2) between kinetochore proteins and the ubiquitin-like protein SUMO.

In the first study, genes that are linked to chromosome stability were identified by performing genome-wide synthetic lethal screens using a series of novel temperature sensitive mutations in essential genes encoding a central (SPC24) and outer (SPC34) kinetochore protein. By performing these screens using different mutant alleles of each gene, we aimed to identify genetic interactions that revealed diverse pathways affecting chromosome stability. This study, which is the first example of genome-wide synthetic lethal screening with multiple alleles of a single gene, demonstrated that functionally

distinct mutants uncover different cellular processes required for chromosome maintenance. Two of our screens identified *APQ12*, a gene that encodes a nuclear envelope protein required for proper nucleocytoplasmic transport of mRNA, which was subsequently characterized with respect to chromosome stability. We found that *apq12* mutants are delayed in anaphase, re-replicate their DNA and re-bud prior to completion of cytokinesis, suggesting a defect in controlling mitotic progression. Overall, this analysis revealed a novel relationship between nucleocytoplasmic transport and chromosome stability.

In the second study, functional genomics lead to the identification of the kinetochore proteins Ndc10, Bir1, Ndc80, and Cep3 as being sumoylated substrates in budding yeast. This work demonstrated that Ndc10, Bir1, and Cep3, but not Ndc80, are differentially modified upon exposure to nocodazole, indicating distinct roles for SUMO modification in modulating kinetochore protein function and providing a potential link between sumoylation of kinetochore proteins and mitotic checkpoint function. Specific lysine to arginine mutations, were shown to eliminate sumoylation of Ndc10 and to cause chromosome instability, mis-localization of Ndc10 from the mitotic spindle, and abnormal anaphase spindles, suggesting that sumoylation of Ndc10 and other kinetochore proteins plays a critical role during the mitotic process. These results support the recent findings that post-translational modifications by the ubiquitin-like protein SUMO is an important regulator of many cellular processes including genome integrity.

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List of Abbreviations

αF	alpha factor
APC/C	Anaphase Promoting Complex / Cyclosome
bp	base pairs
CEN	centromere
CIN	Chromosome Instability
Cdk	Cyclin Dependent Kinase
CF	Chromosome Fragment
CFP	Cyan Fluorescent Protein
CTF	Chromosome Transmission Fidelity
CPP	Chromosome passenger proteins
DMSO	Dimethylsulphoxide
DNA	deoxyribonucleic acid
FACS	Fluorescence Activated Cell Sorting
G1	Gap 1 (Growth before DNA replication phase of cell cycle)
G2	Gap2 (Growth after DNA replication phase of cell cycle)
GFP	Green Fluorescent Protein
GDP	Guanine diphosphate
GTP	Guanine triphosphate
IP	Immunoprecipitation
LOH	Loss Of Heterozygosity
M	Mitosis
MIN	Microsatellite Instability

MTOC	Microtubule Organizing Center
NZ	Nocodazole
S	Synapsis (DNA replication phase of cell cycle)
SAC	Spindle assembly checkpoint
SCF	Skp1 / Cullin / F-box protein
SPB	Spindle Pole Body
SUMO	Small Ubiquitin-like modifier
UBC	Ubiquitin Carrying Enzyme
ULP	Ubiquitin-like protease
VFP	"Venus" yellow fluorescent protein
YFP	Yellow Fluorescent Protein
YPD	Yeast Extract Peptone Dextrose

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This work is dedicated to my loving wife Rachel

and our daughter Stella

Chapter 1:

Chromosome Segregation in *Saccharomyces cerevisiae*

Introduction

Maintenance of the genetic material is essential to an organism's health and survival. For this reason, cellular organisms have evolved an intricate program of events collectively referred to as the cell cycle, which in concert, ensure that each cell created by cell division has the correct complement of all cellular materials (e.g. chromosomes). In the case of the genetic material of eukaryotes, this includes making sure each chromosome is faithfully replicated, packaged, held together as sister chromatids, and then properly segregated to each daughter cell in the correct temporal order with high fidelity. Errors in any of these steps can result in aneuploidy, which has phenotypic consequences that include birth defects, such as Down Syndrome, and the development of cancers (Duesberg et al., 2006; Hassold and Hunt, 2001).

Genomic Instability and Disease

Genomic instability can be defined as an increase in the rate of change to the genetic material. This can occur at the level of single base pair changes (mutations) or can include the loss or gain of whole chromosomes or fragments thereof, both of which are associated with numerous diseases (Andrew and Peters, 2001). These two types of genomic instability are referred to as microsatellite instability (MIN) and chromosome instability (CIN), respectively. In colon cancer, MIN is the result of the inactivation of specific DNA repair genes that ultimately results in a mutator phenotype, where defects in repair leads to increased mutation rates throughout the genome (Lengauer et al., 1997). The mutator phenotype is also prevalent in numerous neurological diseases that are the result of repeat expansion events, and is associated with ~13% of all solid cancers; however, the majority of solid cancers (>80%) contain aneuploid cells that are likely the

result of CIN (Nowak et al., 2002). CIN can be defined as the loss or gain of whole chromosomes or parts thereof, and may function to induce a disease state by loss of heterozygosity (LOH) and the uncovering of recessive alleles, or by altering gene dosage balance between genes that have opposing function (e.g. tumor suppressors and oncogenes). In specific instances, these changes caused by genomic instability can confer an advantage to a cell and become fixed in the population through selection and clonal expansion. This process can repeat itself multiple times leading to the selection and expansion of an ever increasing abnormal population of cells, with the MIN or CIN phenotypes driving this process at an accelerated rate compared to that in normal cells, ultimately resulting in a disease such as cancer.

The genetic alterations responsible for MIN in colon cancers have been shown to result from mutations in the mismatch repair genes (e.g. *hMLH1* and *hMSH2)* (Fishel et al., 1993; Leach et al., 1993; Papadopoulos et al., 1994; Strand et al., 1993). In contrast, the cause of CIN in the vast majority of solid tumors is unknown, and due to the large number of genes that may give rise to a CIN phenotype, it is likely to be much more complicated than the case of MIN (Yuen et al., 2006). Current experimental evidence strongly supports the hypothesis that the CIN phenotype occurs early in the development of cancer, and represents an important step in the initiation and/or progression of the disease (Lengauer et al., 1998; Rajagopalan et al., 2004; Shih et al., 2001). For instance, the recent report that germline biallelic mutations in a spindle checkpoint gene, hBUB1B, is associated with inherited predispositions to cancer strongly supports a causal link between CIN and cancer development (Hanks et al., 2004); therefore, a major goal has been to determine the genetic basis of CIN in tumors.

One approach to identifying mutations responsible for CIN in cancer cells is to test for mutations in genes known to be important for chromosome segregation in human cells, or in human homologs of CIN genes discovered in model organisms, which serve as cross-species CIN candidate genes. For example, Vogelstein and colleagues initially showed that the hBUB1 (identified originally in yeast as a mitotic spindle checkpoint mutant) is mutated in a small percentage of colorectal tumors (Cahill et al., 1998). Additional mutation testing of candidate genes associated with genomic instability has provided evidence for somatic mutation in another 6 CIN genes (hCDC4, hRod, hZW10, hZwilch, hMRE11, and Ding) that together account for less than 20% of the CIN mutational spectrum of colon cancer (Rajagopalan et al., 2004; Wang et al., 2004). Thus, the genetic basis for CIN in colon cancer, and in all types of cancer, is largely unknown.

Candidate CIN genes encode proteins that function in all aspects of chromosome segregation, including proteins that function at kinetochores, telomeres, origins of replication, and in microtubule dynamics, sister chromatid cohesion, DNA replication, DNA repair, DNA condensation and cell cycle checkpoints. Of these, the kinetochore offers a logical choice for mutational testing since four out of seven CIN genes known to be mutated in CIN colon cancers encode kinetochore proteins. Furthermore, the ~100 predicted human genes that encode kinetochore components comprise a large mutational target that could be mutable to a CIN phenotype (Fukagawa, 2004). For example, kinetochore proteins constitute a significant portion of the collection of chromosome transmission fidelity (ctf) mutants identified in a classical genetic screen in yeast (9 out of the 24 CTF genes cloned and characterized to date) (Spencer et al., 1990). Thus, continued identification and characterization of conserved kinetochore components in

budding yeast and other model organisms provides an important source of information pertaining to CIN candidate genes that may be mutated in cancers.

The Cell Cycle

Genome stability is a product of the control imposed on a growing and dividing cell by the cell division cycle regulatory machinery to ensure that each event during cell division take place with high fidelity and in the correct temporal order (Murray and Hunt, 1993). This includes controlling the frequency and timing of each event to ensure that a particular event occurs only after the preceding event has been completed (e.g. DNA segregation does not occur until DNA replication is complete), and that certain events occur only once during the cell cycle (e.g. DNA replication). This involves passing checkpoints in the cell cycle, points at which cells will either irreversibly commit to the next phase of the cell cycle, or arrest until the previous events are completed or mistakes are repaired before proceeding further. In budding yeast, morphological changes occur to a yeast cell as it progresses through a cell cycle, which can be correlated with other events in the cell cycle including the process of DNA replication and segregation (Figure 1-1). These distinct morphologies were used to isolate mutations in key components that are essential for cell cycle progression in budding yeast, which have been shown to be highly conserved in all other eukaryotes (Hartwell, 1980; Lee and Nurse, 1987; Wood and Hartwell, 1982). This work resulted in 1/3 of the Nobel Prize in Physiology and Medicine each being awarded to Leland Hartwell and Paul Nurse in 2001 for the discoveries of key regulators of the cell cycle and their conservation.

The machinery controlling the cell cycle is highly conserved among all eukaryotes. This includes the Cyclin Dependent Kinases (Cdks), which serve as

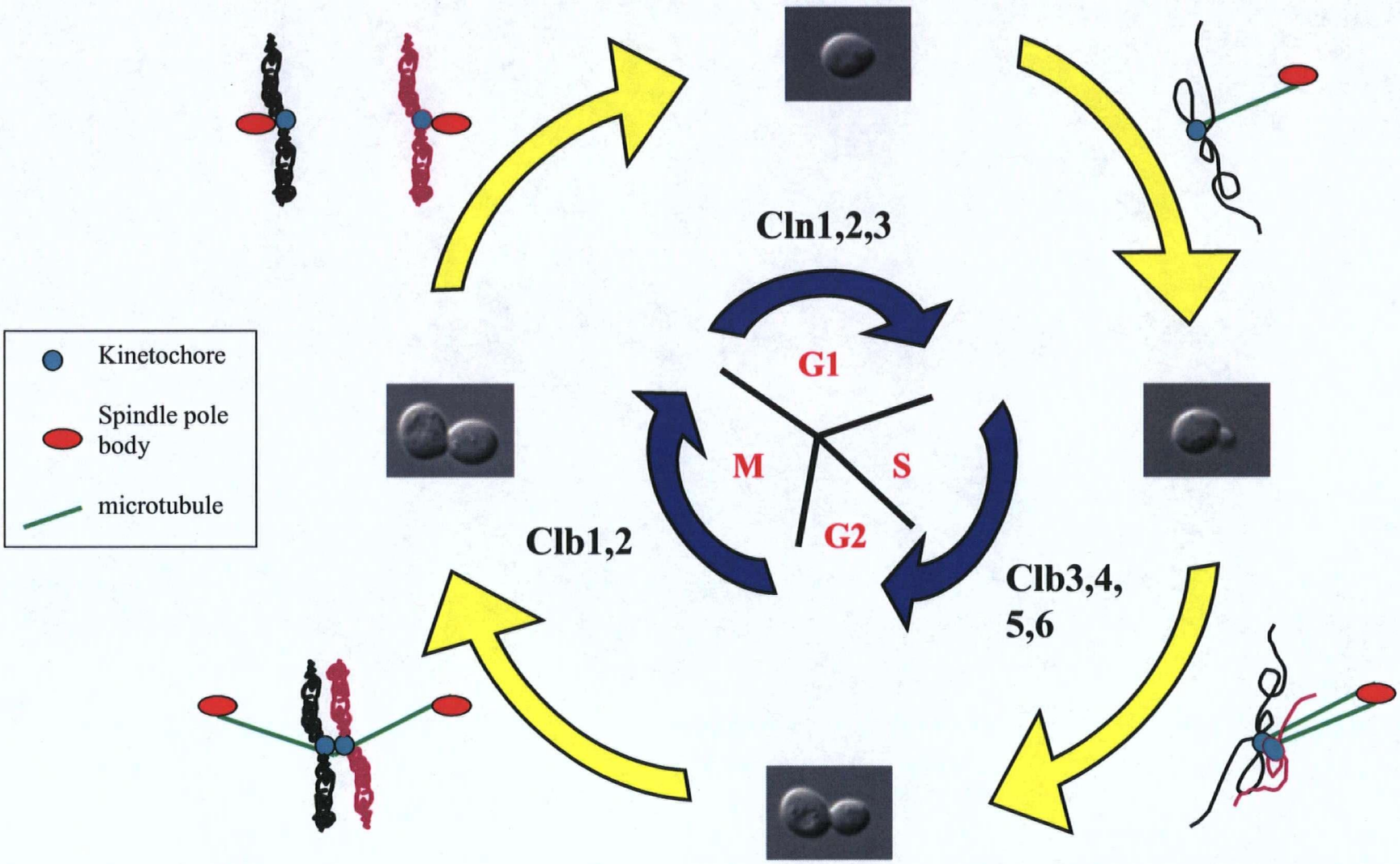


Figure 1-1 The budding yeast cell cycle. The cell cycle is divided into four stages [G1, S (DNA replication), G2 and M (mitosis)]. The size of the bud gives an approximate indication of cell cycle stage. During cell division, chromosomes undergo a replication and segregation cycle that is synchronized with the cell cycle. A specialized region of DNA, the centromere, onto which kinetochore proteins assemble (blue dot), is the site of attachment of chromosomes to microtubules of the mitotic spindle, emanating from the spindle pole body (green lines, red dot is the SPB). Each stage of the cell cycle is regulated by Cdk/Cyclin pairs as indicated (blue arrows).

regulators of the cell cycle in concert with a variety of cyclin subunits. The function of the cyclin subunits is to activate Cdk kinase activity towards substrates appropriate to that stage of the cell cycle. Thus, the combination of a particular cyclin/Cdk pair can drive the cell cycle by phosphorylating the appropriate substrates, and the periodic accumulation and degradation of cyclins allows Cdk activity to be regulated in phase with a given stage of the cell cycle (Figure 1-1). This periodic expression of cyclins was the basis for their discovery by Tim Hunt as proteins that accumulated and then disappeared in a cell cycle dependent manner (Evans et al., 1983; Hadwiger et al., 1989; Murray et al., 1989; Nash et al., 1988). This work was also recognized by 1/3 of the Nobel Prize in Physiology and Medicine being awarded to Tim Hunt in 2001 for the discovery of key regulators of the cell cycle.

Kinases, such as the Polo-like kinase(Plk)/Cdc5 and Aurora kinase/(Ipl1) are also emerging as key regulators of the cell cycle in addition to the Cdks (Donaldson et al., 2001; Katayama et al., 2003; Nigg, 2001). In budding yeast, Cdc28 and Pho85 are the Cdks involved in cell cycle progression with Cdc28 being the essential regulator (Mendenhall and Hodge, 1998). Cdc28, which is expressed at a high level throughout the cell cycle, is controlled by the G1 cyclins (Cln1, Cln2 and Cln3) (Cross, 1990; Hadwiger et al., 1989; Richardson et al., 1989), S phase cyclins (Clb5, Clb6, Clb3, and Clb4) (Dahmann et al., 1995; Richardson et al., 1989), and mitotic cyclins (Clb1 and Clb2) (Fitch et al., 1992; Ghiara et al., 1991). The targets of each stage specific Cdk/cyclin include transcription factors that when phosphorylated by Cdc28 control the expression of genes specific to and required for that particular stage of the cell cycle (Wittenberg and Reed, 2005). Proteolysis of stage specific gene products by the proteasome is also

required for proper cell cycle regulation (Vodermaier, 2004). For example, Sic1 is a Cdk kinase inhibitor that must be degraded during G1 in order for the G1/S phase transition to occur (Bai et al., 1996; Donovan et al., 1994; Schwob et al., 1994). Proteolysis of target proteins is an effective means of controlling the cell cycle since it provides an irreversible directionality to events (i.e. once a protein is destroyed its activity is irreversibly lost unless new transcription and translation occurs). Cell cycle regulated proteolysis is accomplished by targeting proteins for degradation by the 26S proteasome (Chun et al., 1996; Hershko, 1997). Targeting is accomplished by attaching ubiquitin to lysine residue(s) of a target protein, and once attached internal lysine residues in ubiquitin can then serve as sites for ubiquitin addition. Multiple rounds of this process lead to the creation of a poly-ubiquitin chain on the target protein that serves as a marker for degradation by the 26S proteasome. Ubiquitin mediated proteolysis is a highly conserved process in eukaryotes as is the underlying ubiquitin machinery. The ubiquitin machinery includes E1 ubiquitin activating enzymes that bind to ubiquitin and activate ubiquitin for subsequent binding to an E2 protein, a ubiquitin conjugating enzyme. An E3 complex, ubiquitin ligase, then brings substrate proteins and the E2-ubiquitin complex together to provide the specificity required to target select proteins for degradation..

E3 complexes that are important for cell cycle proteolysis include the Anaphase Promoting Complex / Cyclosome (APC/C) and the SCF complex (Skp1 / Cullin / F-box protein) (Page and Hieter, 1999; Peters, 1998; Vodermaier, 2004). Each complex contains a core set of subunits required for E3 activity, which then rely upon substrate specificity factors that bind the core APC/C or SCF complex to efficiently target proteins for degradation. APC/C specificity factors include Cdc20 and Cdh1 (Visintin et al., 1997),

and a meiosis specific factor, Ama1 (Cooper et al., 2000), while SCF specificity factors are recognizable as a set of F-box containing proteins that include Cdc4, Grr1, and Met30 (Skowyra et al., 1997). Each specificity factor contains protein-protein interaction domains such as WD-40 repeats, which contribute to protein-protein binding and are responsible for bringing substrates together with the ubiquitin machinery. For instance, Cdc20 is responsible for targeting proteins that are important for the metaphase to anaphase transition (e.g. Pds1) and passage through mitosis, whereas Cdh1 is responsible for proteolysis later in mitosis and during early G1 (Pfleger et al., 2001; Visintin et al., 1997). The post-translational modification of APC/C subunits is also critical for the proper regulation of E3 activity, and includes phosphorylation and sumoylation (Dieckhoff et al., 2004; Harper, 2002; Page and Hieter, 1999). Overall, regulation of the Cdk/cyclin and proteolysis machinery is key to controlling the cell division cycle, which make these complexes and their activities central targets for the action of cell cycle checkpoints.

Cell Cycle Checkpoints

A checkpoint is a mechanism to ensure that events in the cell cycle occur in the proper temporal order and that the next event in the cell cycle does not start before the previous event is complete with no detectable errors (Hartwell and Weinert, 1989). In the case of an error, the checkpoint will retard progress through the cell cycle to allow cells time to repair the defect(s). Checkpoints therefore are critical to the proper execution of the cell cycle since upcoming events in the cell cycle rely on the previous stage to have established conditions that are necessary for proper execution of the current event (e.g. segregation of one genomic complement to each cell during mitosis requires that the

genome was duplicated in S phase). Well-characterized checkpoints that are critical for maintaining euploidy include the DNA damage, spindle assembly and spindle position (mitotic exit) checkpoints. The DNA damage checkpoint monitors DNA replication and mediates DNA repair upon damage in G1, S, or G2/M (Longhese et al., 2003; Nyberg et al., 2002), the spindle assembly checkpoint ensures that the mitotic spindle is properly formed and all replicated sister chromatids are correctly attached to the spindle apparatus (Amon, 1999; Lew and Burke, 2003; Musacchio and Hardwick, 2002), while the spindle position checkpoint monitors proper spindle orientation, migration and completion of mitosis. In the context of these studies of kinetochore function and regulation, the critical checkpoint to consider is the spindle assembly checkpoint.

During mitosis, the mitotic spindle forms a polarized network of fibers that physically connects to sister chromatids and are used to pull replicated chromosomes apart. For proper segregation to occur every sister chromatid must be attached to this apparatus and sisters must be attached to opposite poles (bi-orientated) to allow equal segregation of the genetic material to each daughter cell. Monitoring of these attachments is accomplished by the spindle assembly checkpoint (SAC), which in the case of a defect, will arrest cells in G2/M by preventing the metaphase to anaphase transition until the defect is repaired. This checkpoint system is composed of a set of proteins that were first described in budding yeast but are largely conserved among eukaryotes (Amon, 1999; Smith, 2002). In budding yeast, five non-essential genes, *BUB1*, *BUB3* (budding uninhibited by benzimidazole), *MAD1*, *MAD2* and *MAD3* (mitotic arrest-deficient) encode essential components of the SAC machinery (Hoyt et al., 1991; Li and Murray, 1991). Deletion of these genes leads only to a modest increase in chromosome loss rates

in unperturbed conditions suggesting that in a "normal" cell cycle errors that require the action of the checkpoint are rare (Li and Murray, 1991; Warren et al., 2002). This is in contrast to mammalian cells, where the components of the SAC are essential for viability (Basu et al., 1999; Dobles et al., 2000; Kalitsis et al., 2000). This most likely reflects the fact that in budding yeast kinetochore microtubule attachments are maintained throughout the cell cycle and are present in S phase, so there are few chances for errors and ample time to facilitate proper bi-polar attachment (Winey and O'Toole, 2001). Where as in mammalian cells, kinetochore microtubule attachment are established only after nuclear envelope breakdown in mitosis, which provides only a small window of time for attachment and alignment of sister chromatids on the metaphase plate before progression into anaphase. Therefore, it is likely that in every mammalian cell mitosis, the SAC is required to retard the cell cycle until attachment and congression has occurred after nuclear envelope breakdown.

To function a checkpoint generally requires an ability to recognize the damage or defect and a method of transducing this signal to effector proteins, which ultimately arrest the cell cycle and mediate repair of the defect (Nyberg et al., 2002). The signal that is recognized by the SAC has been proposed to be either lack of tension exerted across sister chromatids or the lack of microtubule binding at the kinetochore(s) (Lew and Burke, 2003). The difficulty in distinguishing between these two possibilities is that both processes are intimately associated with one another. For example, unattached kinetochores are not under tension and it has been observed that tension can regulate microtubule attachment. In this regard, the Aurora kinase Ipl1 has been shown to destabilize kinetochore microtubule attachments that lack tension and signal this lack of

tension; therefore, it is possible that the transiently unattached kinetochore that is generated by Ipl1 activity may be the signal activating the checkpoint and not the lack of tension (Pinsky and Biggins, 2005; Tanaka et al., 2005). However, it is likely that both processes are important as spindle assembly checkpoint components have shown differences in sub-cellular localization dependent on the status of kinetochore microtubule attachment and tension (Lew and Burke, 2003), but exactly how a mechanical signal (lack of tension) is processed and turned into a biochemical signal is not known. Since the SAC is sensitive enough to detect even a single unattached kinetochore (Rieder et al., 1994), the signal that is detected by the checkpoint must lead to a potent and diffusible inhibitor of the cell cycle. This most likely occurs through a process of amplification through checkpoint transducers that include a number of kinases. In this regard, the recognition of an error by the SAC has been proposed to involve tension regulated phosphorylation (Li and Nicklas, 1997).

The checkpoint machinery requires the action of several kinases including Ipl1, Mps1, and Bub1(Lew and Burke, 2003). Bub1 is a kinase that is known to phosphorylate Bub3, while Mps1 can phosphorylate Mad1, and might function to recruit other checkpoint components to the kinetochore (Abrieu et al., 2001; Hardwick et al., 1996; Winey and Huneycutt, 2002). *MPS1* overexpression constitutively activates the checkpoint, and this activation requires the Mad and Bub components (Fraschini et al., 2001; Hardwick et al., 1996). Conversely, Mps1 proteolysis is required to silence the SAC, and is targeted by the APC/C for destruction in anaphase (Palframan et al., 2006). Loss of Mps1 activity in anaphase is necessary since once cohesion between sister chromatids has been resolved at the metaphase to anaphase transition, microtubule forces

are no longer opposed by cohesion and tension ceases at the kinetochore. This lack of tension would then be recognized by the SAC and cell cycle progression halted inappropriately if the checkpoint was not silenced.

Upon activation of the SAC, it is proposed that Mad2 dynamically interacts with unattached kinetochores rendering Mad2 competent to interact with the APC/C regulatory subunit Cdc20. Mad2 binding to Cdc20, leads to inhibition of APC/C activity, and stabilization of Cdc20-dependent APC/C substrates (Amon, 1999). Substrates stabilized by silencing the APC/C include Pds1 (Securin), which is a potent inhibitor of Esp1 (Separase), a protease required to resolve sister chromatid cohesion. Stabilization of Pds1, therefore blocks the metaphase to anaphase transition by preventing dissolution of sister chromatid cohesion (De Antoni et al., 2005; Lew and Burke, 2003; Luo et al., 2000). (Laloraya et al., 2000). The activated form of Mad2 may be an oligomerized complex, and the kinetochore might act as the catalytic site for polymerization of Mad2 subunits. Alternatively, the activated form of Mad2 may consist of a Mad-Bub complex (Shah and Cleveland, 2000). In either case, phosphorylation events, complex formation between the different checkpoint components, conformational changes induced by differential binding of components and effectors, and localization of these complexes all seem to be important in checkpoint activity (Musacchio and Hardwick, 2002).

In addition to the checkpoint components themselves, an intact kinetochore is also necessary for the checkpoint response as it provides a platform to allow detection of impaired kinetochore microtubule attachments and proper checkpoint signaling (Gardner et al., 2001; Goh and Kilmartin, 1993). Of the protein complexes at the kinetochore, the Cbf3 complex has a direct role in checkpoint function since one of its members, Skp1,

has been found to associate with Bub1, and this association is necessary for signaling kinetochore tension defects (Kitagawa et al., 2003). The Ndc80 complex also plays a role in checkpoint response by recruiting checkpoint components to the kinetochore and thereby regulating their activity (Janke et al., 2001; McCleland et al., 2003).

The Mitotic Spindle and Chromosome Segregation

The mitotic spindle is made up of individual microtubules (MTs) organized by two microtubule organizing centers (MTOCs), which are assembled during mitosis in a polarized manner to physically pull apart sister chromatids during anaphase (Gadde and Heald, 2004). In budding yeast, the mitotic spindle consists of microtubules that emanate from spindle pole bodies (SPBs) and bind to kinetochores (kinetochore microtubules), and MTs that form the central spindle, where microtubules from opposite spindle pole bodies interact in an anti-parallel array (interpolar microtubules). The spindle pole body is also the site of nucleation for astral microtubules that project out into the cytoplasm. Unlike higher eukaryotes, the yeast nuclear membrane does not break down during mitosis and the SPB remains embedded within the membrane during the whole cell cycle (Hoyt and Geiser, 1996; McIntosh et al., 2002). The SPB is made up of distinguishable layers, divided into three major plaques, which include the outer plaque (on the cytoplasmic face) and the inner plaque (on the nuclear face) that are linked by a central plaque embedded in the nuclear envelope (Francis and Davis, 2000; O'Toole et al., 1999).

Microtubules nucleated by the MTOC are composed of non-covalent hetero-dimer polymers of β-tubulin (Tub2 in budding yeast) and α-tubulin (Tub1 and Tub3 in budding yeast) (Desai and Mitchison, 1997). The α-tubulin and β-tubulin subunits are arranged in a head to tail conformation giving the microtubule polarity. Microtubule plus

ends are more dynamic and form the growing head of the filament structures, which associate to form a hollow cylindrical tube of a microtubule (Kline-Smith and Walczak, 2004). As dynamic structures, microtubules are governed by the rate of polymerization, the rate of de-polymerization, the frequency of catastrophe (switch from growth to collapse), and the frequency of rescue (switch from collapse to growth) (Desai and Mitchison, 1997; Gadde and Heald, 2004; Kline-Smith and Walczak, 2004). During polymerization, GTP bound β-tubulin is incorporated at the growing ends of microtubules, which is then converted to the GDP bound form of β-tubulin by hydrolysis. Once in the GDP form, β-tubulin does not exchange and thus forms the majority of the β-tubulin in the microtubule. A delay between incorporating the GTP bound tubulin and GTP hydrolysis provides a GTP cap on growing ends of microtubules that is thought to provide some stability to the growing plus end. When catastrophes occur, de-polymerizing β-tubulin rapidly exchanges GDP for GTP allowing tubulin subunits to be reused for polymerization if rescue occurs. Beyond the intrinsic properties of microtubules, microtubule-associated factors and motor proteins are critical for controlling the dynamic instability of microtubules (McIntosh and Hering, 1991; Mitchison and Kirschner, 1984), and allow these structures to be harnessed for work (e.g. chromosome segregation).

Dynamic instability is thought to allow rapid re-organization of microtubules in response to various stimuli and allow a process such as "search and capture" to occur (Kline-Smith and Walczak, 2004). "Search and capture" is a term used to describe the mechanism by which microtubules find and bind to kinetochores. This consists of microtubules alternately growing and shrinking, probing the nuclear space for

kinetochores until attachment occurs. As a result of sister chromatid cohesion, sister kinetochores are oriented away from each other which promotes attachments of sister kinetochores to microtubules emanating from opposite spindle poles (Tanaka et al., 2000). In many organisms, centromeres reside close to the centrosome even before mitosis, which likely mediates rapid capture of microtubules by kinetochores. In budding yeast, it appears that kinetochores may be attached to the SPB for most of the cell cycle (Winey and O'Toole, 2001). Once the sister kinetochore of a mono-oriented chromosome connects to a microtubule emanating from the opposite pole, tension results due to the opposition of microtubule pulling by cohesion. This tension is thought to further stabilize these attachments (Kline-Smith et al., 2005; Pinsky and Biggins, 2005). Failure to form bi-orientated attachment results in the activation of regulating factors such as the Ipl1/Aurora kinase, which destabilize kinetochore microtubule attachments allowing microtubule capture to reoccur (Biggins and Murray, 2001; Dewar et al., 2004; Pinsky et al., 2003; Tanaka, 2002). It is worth noting that microtubules in the absence of spindle poles and kinetochores have also been shown to self assemble into spindles (Heald et al., 1996) and mechanisms in addition to "search and capture" may be at work in mitotic spindle formation (Kapoor et al., 2006; Rieder and Alexander, 1990; Tanaka et al., 2005).

Although microtubules are highly dynamic, the exchange rate of tubulin subunits in kinetochore-associated microtubules is much slower than in unattached microtubules (McIntosh et al., 2002). Kinetochores thus have a stabilizing effect on kinetochore microtubules up to the onset of anaphase, after which depolymerization occurs. This suggests that there are factors at kinetochores that have the ability to influence

microtubule stability, which are regulated during mitosis to provide the motive force for chromosome segregation. One such known factor is the Dam1 outer kinetochore complex, which has been recently shown to form a collar around kinetochore microtubules functionally stabilizing kinetochore microtubules prior to anaphase. The Dam1 collar can then slide along microtubules during anaphase and can be used to capture the force generated by depolymerization (Asbury et al., 2006; Westermann et al., 2006). In support of this model, the Dam1 ring structure has been shown to interact with microtubules via the acidic C-tail of tubulin, rather than the microtubule lattice like most other microtubule-interacting factors. This allows passive one-dimensional diffusion of the Dam1 ring on the surface of the microtubule. Importantly, this diffusion becomes unidirectional when the microtubule depolymerizes and effectively pushes the ring at the speed of microtubule shrinkage. Cryo-electron microscopy studies of depolymerizing microtubules provide evidence of protofilaments peeling from the ends of microtubules. Overall, this provides an elegant model of force generation where depolymerizing protofilaments push the Dam1 ring and the associated kinetochore and sister chromatid in the minus end direction to facilitate chromosome segregation (Mandelkow et al., 1991; Nogales and Wang, 2006).

Movement of sister chromatids away from each other at anaphase proceeds in two stages: (1) anaphase A, where chromosomes move closer to the poles, and (2) anaphase B, where the interpolar distance is increased by pushing apart of the SPBs. At the end of anaphase, kinetochores are held very close to SPBs by short microtubules, and the remaining spindle consists of a few interpolar microtubules that interdigitate at the spindle mid-zone, where a number of proteins also reside, such as the yeast kinetochore

protein Ndc10 and the Aurora kinase Ipl1 (Buvelot et al., 2003). One of these proteins, Ase1, is degraded in an APC/C dependent manner, linking cell cycle progression control to the spindle cycle (Winey and O'Toole, 2001). Finally, spindle disassembly occurs, which is necessary for cytokinesis, through the depolymerization of the interpolar microtubules from their plus ends (Winey and O'Toole, 2001).

Two types of motors are thought to be involved in pole separation during anaphase B, the plus-end directed bimC/KinN kinesin-related motors, and the minus-end directed dynein. bimC/KinN motors are involved in spindle assembly and maintenance, and are thought to crosslink interpolar microtubules with subsequent sliding along two adjacent anti-parallel microtubules toward the plus end providing a pushing force to separate poles (Hoyt and Geiser, 1996). The minus-end directed motor dynein, and its associated activator complex dynactin, mediates movement of the nucleus into the bud, spindle elongation, and spindle positioning. To accomplish these tasks dynein associates with the cell cortex and with SPBs in a microtubule-dependent manner. When associated with microtubules, dynein is thought to slide along microtubules while being anchored at the cortex providing a pulling force on the SPBs that is also important for pole separation (Yeh et al., 1995). Motors have also been shown to be localized to kinetochores where they may contribute to microtubule attachment and provide some of the motive force required to move chromosomes along microtubules during anaphase.

The Kinetochore of Budding Yeast

The kinetochore (which consists of centromere DNA and associated proteins) is a macromolecular complex that is critical to the process of chromosome segregation; functioning to mediate the attachment of sister chromatids to the spindle microtubules

and direct chromosome movement during mitosis and meiosis (Nasmyth, 2002; Nasmyth et al., 2000). Remarkably, the kinetochore also monitors the state of MT attachment and is critical for sensing the completion of metaphase (bi-polar attachment of all chromosomes) before allowing anaphase to begin (Lew and Burke, 2003; Tanaka, 2002). To fulfill these critical tasks, the kinetochore acts as a central hub where kinetochore proteins, centromeric chromatin, cohesin, spindle checkpoint and MT-associated proteins gather to coordinate chromosome segregation.

The budding yeast centromere (CEN) is a "point" centromere and consists of three conserved elements (CDE I, CDE II, and CDE III) that span 125 base pairs (Loidl, 2003). CDEI and III are conserved sequences while CDEII is an A/T-rich region of conserved length and base composition (Hyman and Sorger, 1995). CDEI is not essential for centromere function, whereas the lack of CDEII and CDEIII or point mutations in CDEIII result in an inactive centromere (Hegemann et al., 1986; McGrew et al., 1986). How this small stretch of DNA facilitates assembly of a kinetochore is not known, but one model proposes that CDEI and III serve as sites of interaction with kinetochore proteins, while CDEII wraps around a specialized centromeric nucleosome (Cheeseman et al., 2002b). However, recent data supports a different model in which yeast centromere DNA is nucleosome-free (Espelin et al., 2003).

Proteins associated with budding yeast centromere DNA are assembled in a hierarchical manner in distinct biochemical sub-complexes (De Wulf et al., 2003), which can be categorized based on their proximity to DNA (Figure 1-2). These include inner kinetochore proteins that directly contact CEN DNA (or are in a complex with proteins that directly interact with CEN DNA), central kinetochore proteins that serve as

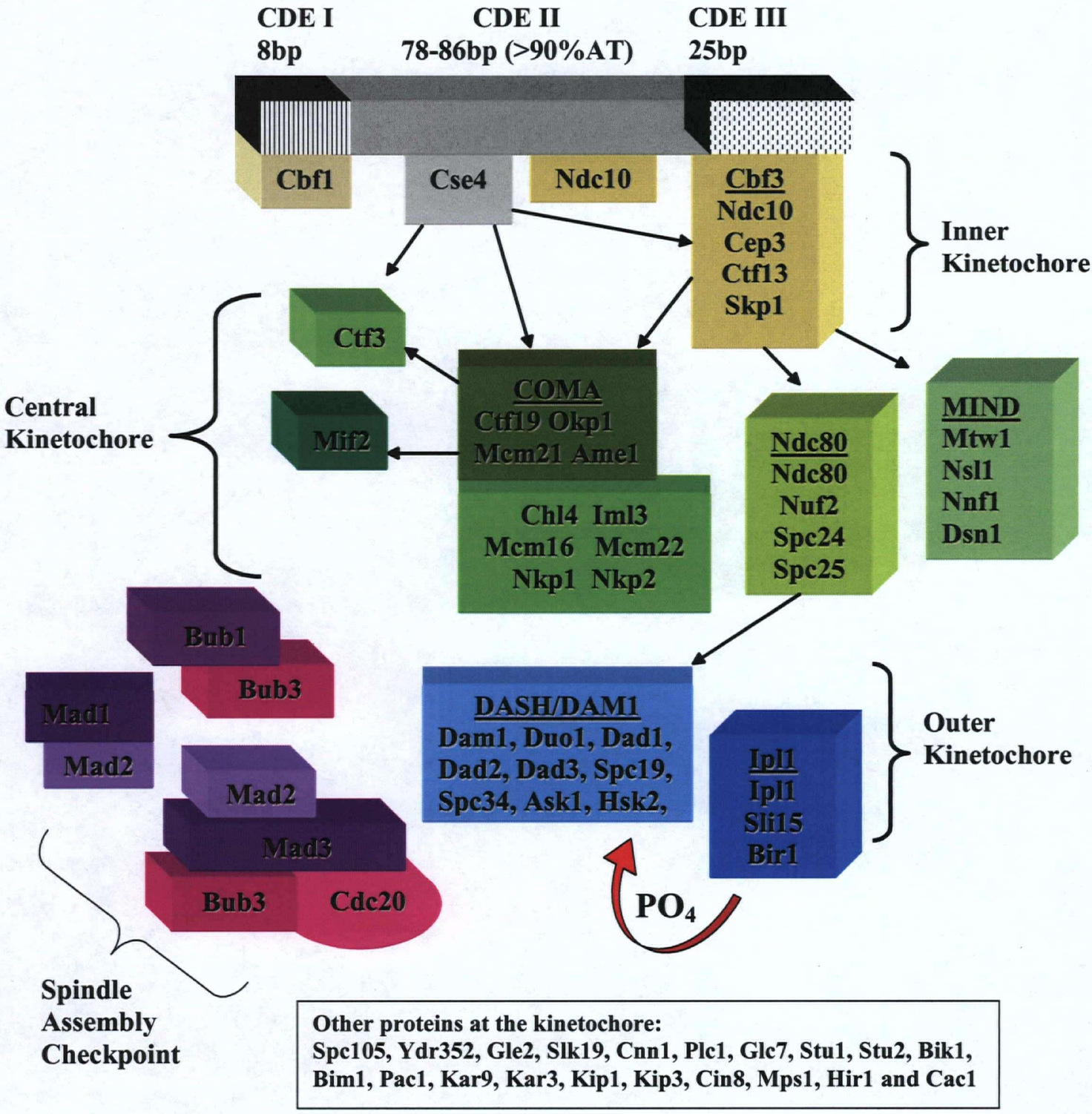


Figure 1-2. The budding yeast kinetochore. Organization of the budding yeast kinetochore with proteins grouped based upon biochemical purification. Black arrows indicate a dependence on localization and red arrow denotes a enzymatic activity. Figure adapted from (Chan et al., 2005).

scaffolding and link the inner kinetochore to the outer kinetochore proteins, and the outer kinetochore proteins that provide the interface required to interact with microtubules (Cheeseman et al., 2002b; Kitagawa and Hieter, 2001; McAinsh et al., 2003; Measday and Hieter, 2004). This general design of a kinetochore appears to be well conserved among other eukaryotes as defined by the continued discovery of conservation between individual kinetochore proteins and protein complexes of higher eukaryotes and yeast (Cheeseman et al., 2004; Hayashi et al., 2004; Kitajima et al., 2004; Kline et al., 2006; Obuse et al., 2004; Okada et al., 2006; Wei et al., 2005; Zhou et al., 2004). These data suggest that the basic building blocks and design of a kinetochore are conserved among eukaryotic organisms.

The budding yeast inner kinetochore consists of the CBF3 complex, the histone H3 variant Cse4, and Cbf1 (McAinsh et al., 2003). The CBF3 complex binds to the CDEIII region of the centromere and is composed of Ndc10, Ctf13, Cep3 and Skp1, whereas Cbf1 binds to the CDEI region of the centromere. Cse4 is the conserved histone H3 variant that replaces H3 in the histone octamer in and around the centromere region (Cheeseman et al., 2002b). In agreement with its position in the kinetochore, the CBF3 complex is required for the localization of all known central and outer kinetochore components (Cheeseman et al., 2002b; McAinsh et al., 2003).

The central kinetochore consists of at least three different sub-complexes based upon their biochemical purification properties, which include the NDC80 complex, the MIND complex and the COMA complex. Other proteins of the central kinetochore have been isolated, but have not yet been definitively placed into specific sub-complexes

(De Wulf et al., 2003; Measday et al., 2002; Pot et al., 2003). Many of the proteins of the central kinetochore are not essential for viability but the corresponding deletion mutants lose chromosomes at much higher rates than wild type strains. In the case of the NDC80 complex, all four subunits (Ndc80, Nuf2, Spc24, and Spc25) are essential and conserved in human cells (DeLuca et al., 2002; He et al., 2001; Janke et al., 2001; McCleland et al., 2003; Wigge and Kilmartin, 2001). Mutations in Spc24 or Spc25 result in the inactivation of the mitotic checkpoint due to the failure of the Mad and Bub spindle checkpoint proteins to localize to the kinetochore, suggesting that the Ndc80 complex is required for their interaction with the kinetochore (He et al., 2001; Janke et al., 2001).

The outer kinetochore consists of protein complexes that directly bind to microtubules. This includes the ten members of the DAM1 complex, which has been shown to bind the mitotic spindle, and Dam1 itself, which has been shown to bind microtubules *in vitro* (Cheeseman et al., 2002a; Kang et al., 2001; Li and Elledge, 2003). The localization of the DAM1 complex to the kinetochore is dependent on an intact mitotic spindle since the DAM1 complex forms a collar around microtubules that is important for capturing the force generated by depolymerizing microtubules for sister chromatid segregation (Miranda et al., 2005; Westermann et al., 2005; Westermann et al., 2006). Other proteins that have been localized to the kinetochore with microtubule binding capabilities include Stu2, Bik1, Bim1 and the kinesin related proteins Cin8, Kip1 and Kip3 (Cheeseman et al., 2002a; He et al., 2001).

Finally, there are a number of proteins that function at the kinetochore in a regulatory manner, and not in a structural capacity. These proteins tend to localize at the kinetochore transiently. This includes: (1) the Ipl1/Bir1/Sli15/ kinase complex, which

monitors tension across sister chromatids and (2) components of the mitotic spindle assembly checkpoint, which in budding yeast consists of Mps1, Mad1, Mad2, Mad3, Bub1 and Bub3 (Lew and Burke, 2003; Pinsky and Biggins, 2005). A subset of kinetochore proteins also re-localize to the mitotic spindle in anaphase and to the spindle mid-zone prior to cytokinesis, reflecting the localization of chromosome passenger proteins in higher eukaryotes (Adams et al., 2001a; Vagnarelli and Earnshaw, 2004). In yeast, the set of chromosome passenger like proteins includes the Aurora kinase Ipl1, the IAP repeat protein Bir1, and the inner kinetochore proteins Cep3 and Ndc10 (Biggins et al., 1999; Bouck and Bloom, 2005; Buvelot et al., 2003; Goh and Kilmartin, 1993; Huh et al., 2003; Montpetit et al., 2006; Widlund et al., 2006). The localization of these proteins to the mitotic spindle is thought to be important for regulating mitotic spindle dynamics and for cytokinesis (Adams et al., 2001a; Buvelot et al., 2003; Montpetit et al., 2006).

Scope of the Thesis

In the years preceding this work, application of technologies such as mass spectrometry on purified protein complexes led to the identification of more than 65 proteins that localize to the budding yeast kinetochore. The speed at which the new kinetochore subunits were identified far outpaced the detailed characterization that is needed to understand the function of each individual protein and how these functions are regulated. For this reason, I have focused on investigating a few essential budding yeast kinetochore proteins from the inner (Ndc10), central (Spc24), and outer kinetochore (Spc34). The general approach of this work focused on the genetic and physical interactions that these individual genes and gene products make, with the goal being to further our basic knowledge of these specific proteins. These basic studies of genes

required for chromosome transmission fidelity and their regulation are directly relevant to human biology and disease. In particular, the elucidation of the genetic basis of CIN in model organisms provides a mechanistic basis for understanding CIN in human cancers, and provides candidate genes for those CIN genes mutated in cancer.

Chapter 2:

Genome-wide synthetic lethal screens identify an interaction between the nuclear envelope protein, Apq12, and the kinetochore in *Saccharomyces cerevisiae*

This work is reprinted (with modifications) from the journal of *Genetics* (Montpetit B, Thorne K, Barrett I, Andrews K, Jadusingh R, Hieter P, Measday V. Genome-wide synthetic lethal screens identify an interaction between the nuclear envelope protein, Apq12, and the kinetochore in Saccharomyces cerevisiae. Genetics. 2005 Oct; 171(2):489-501) with permission from The Genetics Society of America. Data presented in this chapter are part of a collaboration with Dr.Vivien Measday (University of British Columbia), and as such, acknowledgements of other's work is given in each figure legend.

Introduction

Budding yeast undergo a "closed mitosis" in which the nuclear envelope (NE) does not breakdown during the course of mitosis, and the spindle pole bodies remain anchored in the nuclear membrane (Hurt et al., 1992). In contrast, mammalian cells undergo an "open mitosis" in which the NE breaks down and chromosomes and centrosomes become part of the cytoplasm. Although the NE does not break down in yeast there is evidence that suggests that proteins associated with the NE have an active role in controlling mitotic progression and chromosome segregation (Kerscher et al., 2001; Makhnevych et al., 2003; Ouspenski et al., 1999). This includes recently identified links between the nuclear pore complex (NPC) and the kinetochore providing a precedent for communication between the NE and the spindle checkpoint machinery (Loiodice et al., 2004; Rabut et al., 2004; Stukenberg and Macara, 2003). For example, the Mad1 and Mad2 spindle checkpoint proteins localize to the NPC in both yeast and mammalian cells (Campbell et al., 2001; Iouk et al., 2002). In budding yeast, upon activation of the spindle checkpoint, Mad2 and a portion of Mad1 re-localize to the kinetochore (Gillett et al., 2004; Iouk et al., 2002), while in human cells, checkpoint proteins localize to the NPC and upon NE breakdown re-localize to the kinetochore until MT attachment has occurred (Campbell et al., 2001). Studies in multiple eukaryotic systems have shown that the Ndc80 complex is required to localize spindle checkpoint proteins (Mads and Bubs) to the kinetochore and that cells carrying mutations in Ndc80 complex components are defective in checkpoint signaling (Gillett et al., 2004; Maiato et al., 2004). In addition to checkpoint proteins, other components have also been identified that interact with both the kinetochore and NE including Mps2, which localize to both the SPB and NE, are

required for SPB insertion into the NE (Munoz-Centeno et al., 1999; Winey et al., 1991). Interestingly, Mps2 interacts *in vivo* with Spc24, a component of the Ndc80 complex, supporting this kinetochore-NE connection (Le Masson et al., 2002).

NE associated proteins have also been identified that are required for chromosome stability, but do not appear to be components of the kinetochore. For example, the nucleoporin Nup170 was identified in a genetic screen for mutants that exhibit an increased rate of chromosome loss and was subsequently shown to be required for kinetochore integrity, despite the fact that Nup170 does not interact with centromere DNA (Kerscher et al., 2001; Spencer et al., 1990). Also in support of a kinetochore-NE connection, centromeres cluster near the SPB/NE in interphase and co-localize with SPBs during late anaphase providing the opportunity for a physical interaction between these two macromolecular protein complexes (Goshima and Yanagida, 2000; He et al., 2000; Jin et al., 2000; Pearson et al., 2001).

Our current understanding of chromosome segregation has benefited from many studies focused on protein purification and mass spectrometry analysis that have been instrumental in identifying structural components of the kinetochore based on the stoichiometric interaction of novel proteins with known kinetochore proteins (Biggins and Walczak, 2003; McAinsh et al., 2003). This includes the inner and central kinetochore complexes, which are assembled in a hierarchical manner onto *CEN* DNA and serve as a link to the Dam1 outer kinetochore complex that encircles MTs (Biggins and Walczak, 2003; McAinsh et al., 2003; Miranda et al., 2005; Westermann et al., 2005), and the Ndc80 complex, a highly conserved central kinetochore complex that is required for the Dam1 mediated kinetochore microtubule attachment (He et al., 2001; Janke et al.,

2001; Le Masson et al., 2002; Wigge and Kilmartin, 2001). However, the identification of proteins or pathways that affect chromosome segregation via transient or indirect interactions (e.g. nuclear pore proteins) has remained elusive. Genetic studies, however, have the ability to identify interactions that do not rely on direct protein-protein interaction yet still impact chromosome segregation. For this reason, synthetic genetic arrays (SGA) were used to identify nonessential mutants from the haploid yeast gene deletion set that have a role in chromosome stability (Tong et al., 2001). SGA is a method to automate the isolation of synthetically lethal (SL) or synthetically sick (SS) interactions that occur when the combination of two viable non-allelic mutations results in cell lethality or slower growth than either individual mutant. Two mutants that have a SL/SS interaction often function in the same or parallel biological pathways (Hartman et al., 2001). As a starting point for SGA analysis, three independent Ts alleles were created in members of both the Ndc80 and Dam1 kinetochore complexes. Genome-wide SL screens were performed using SGA methodology with all six Ts mutants as query strains.

This study represents the first series of comparative genome-wide SL screens performed on different mutant alleles of the same gene. Using this approach, a novel role for the nuclear envelope protein Apq12 was uncovered in maintaining chromosome stability and proper cell cycle progression through anaphase, providing a novel link between chromosome segregation and the nuclear envelope.

Materials and Methods

Creation of Ts mutants and integration into yeast strains

The *SPC24* and *SPC34* ORFs (642bp and 888bp, respectively) including ~250bp of upstream sequence were amplified by PCR and cloned into *pRS316* (Sikorski and Hieter, 1989) to create *pRS316-SPC24* (BVM93a) and *pRS316-SPC34* (BVM95c). BVM93a and BVM95c were both sequenced to ensure that they carried wild type *SPC24* and *SPC34* sequence, respectively. *SPC24* and *SPC34* were PCR amplified from BVM93a and BVM95c using mutagenic conditions [100ng template, Taq polymerase (BRL), 200μM of dNTPs with either limiting dATP (40μM) or dGTP (40μM), 2mM $MgCl_2$ and 25pmol primers]. Next, mutagenized *SPC24* and *SPC34* were cloned into *pRS315* using homologous recombination in strains YVM503 and YVM509 that contained deletions of *SPC24* and *SPC34* covered by the *URA3*-marked plasmids carrying wild type versions of *SPC24* (BVM93a) and *SPC34* (BVM95c) (Muhlrad et al., 1992). Wild type plasmids were removed from YVM503 and YVM509 by successive incubation on media containing 5-Fluoroorotic acid. Colonies now carrying mutagenized *pRS315-SPC24* or *pRS315-SPC34* as the sole source of either gene were incubated at 37° to identify Ts mutants, and plasmids rescued from these strains were retransformed to confirm the Ts phenotype. FACS analysis was performed on each Ts mutant after incubation at 37° for 2 to 6 hours. Mutants representing different FACS profiles at 37° - three *spc24* (*spc24-8*, *spc24-9*, *spc24-10*) and three *spc34* (*spc34-5*, *spc34-6*, *spc34-7*) mutants - were chosen for further analysis. Mutants were next integrated in the genome replacing the wild type *SPC24* or *SPC34* loci in both the SGA starting strain (Y2454) and our lab S288C background strain (YPH499) as described (Tong et al., 2001). Mutants

were sequenced and the corresponding amino acids changes are illustrated in Figure 2-1 and 2-2. All yeast strains used in this study are listed in Table 2-1.

SL screen using SGA methodology

The deletion mutant array was manipulated via robotics and the SL screens were performed as described (Tong et al., 2001). Each SL screen was performed twice and double mutants were detected visually for SS/SL interactions. For each query gene, all deletion mutants isolated in the first and second screen were condensed onto a mini-array and a third SL screen was performed. SS/SL interactions that were scored at least twice were first confirmed by random spore analysis (Tong et al., 2004) and then subsequently by tetrad analysis on YPD medium at 25°.

Two-dimensional (2D) hierarchical cluster analysis

2D hierarchical clustering was performed as described (Tong et al., 2001; Tong et al., 2004).

Chromosome fragment loss assay

Quantitative half-sector analysis was performed as described (Hyland et al., 1999; Koshland and Hieter, 1987). Homozygous diploid strains containing a single chromosome fragment were plated to isolate single colonies on solid media containing limiting adenine (Spencer et al., 1990). Colonies were grown at either 30° or 35° (see Table 2-3) for three days before incubation at 4° for red pigment development. Chromosome loss or 1:0 events were scored as colonies that were half red and half pink, nondisjunction or 2:0 events were scored as colonies that were half red and half white and over-replication or 2:1 events were scored as colonies that were half-white and half-pink.

TABLE 2-1. Yeast strain list

Strain	Genotype	Reference
DBY1385	*MATa his4 ura3-52 tub2-104*	D. Botstein
DBY2501	*MATa ura3-52 ade2-101 tub1-1*	D. Botstein
IPY1986	MATa *ura3-52, lys2-801, ade2-101, his3Δ200, leu2Δ1, trp1Δ63 OKP1-VFP::kanMX6 SPC29-CFP::hphMX4*	P. Hieter
Y2454	*MATα mfa1Δ::MFA1*pr-*HIS3 can1Δ ura3Δ0 leu2Δ0 his3Δ1 lys2Δ0*	(Tong et al., 2001)
YIB329	*MATα mfa1Δ::MFA1*pr-*HIS3 can1Δ ura3Δ0 leu2Δ0 his3Δ1 lys2Δ0 spc24-9::URA3*	This study
YIB331	*MATα mfa1Δ::MFA1*pr-*HIS3 can1Δ ura3Δ0 leu2Δ0 his3Δ1 lys2Δ0 spc24-10::URA3*	This study
YIB338	*MATα mfa1Δ::MFA1*pr-*HIS3 can1Δ ura3Δ0 leu2Δ0 his3Δ1 lys2Δ0 spc24-8::URA3*	This study
YIB343	*MATα mfa1Δ::MFA1*pr-*HIS3 can1Δ ura3Δ0 leu2Δ0 his3Δ1 lys2Δ0 spc34-5::URA3*	This study
YIB351	*MATα mfa1Δ::MFA1*pr-*HIS3 can1Δ ura3Δ0 leu2Δ0 his3Δ1 lys2Δ0 spc34-6::URA3*	This study
YIB355	*MATα mfa1Δ::MFA1*pr-*HIS3 can1Δ ura3Δ0 leu2Δ0 his3Δ1 lys2Δ0 spc34-7::URA3*	This study
YM20	MATa *ura3-52, lys2-801, ade2-101, his3Δ200, leu2Δ1, trp1Δ63 OKP1-VFP::kanMX6 SPC29-CFP::hphMX4 apq12ΔTRP1*	This study
YM40	MATa *ura3-52, lys2-801, ade2-101, his3Δ200, leu2Δ1, trp1Δ63 spc34-5::kanMX6*	This study
YM41	MATa *ura3-52, lys2-801, ade2-101, his3Δ200, leu2Δ1, trp1Δ63 spc34-7::kanMX6*	This study
YM61	MATa *ura3-52, lys2-801, ade2-101, his3Δ200, leu2Δ1, trp1Δ63 SPC34::kanMX6*	This study
YM192	*MATa/α ura3-52 ura3-52/, lys2-801/ lys2-801, ade2-101/ ade2-101, his3Δ200 his3Δ200/, leu2Δ1/ leu2Δ1, trp1Δ63/trp1Δ63 spc24-8::kanMX61/ spc24-8::kanMX6 CFIII CEN3.d URA3 SUP11*	This study
YM194	*MATa/α ura3-52 ura3-52/, lys2-801/ lys2-801, ade2-101/ ade2-101, his3Δ200 his3Δ200/, leu2Δ1/ leu2Δ1, trp1Δ63/trp1Δ63 spc24-10::kanMX61/ spc24-10::kanMX6 CFIII CEN3.d URA3 SUP11*	This study
YM196	*MATa/α ura3-52 ura3-52/, lys2-801/ lys2-801, ade2-101/ ade2-101, his3Δ200 his3Δ200/, leu2Δ1/ leu2Δ1, trp1Δ63/trp1Δ63 SPC24::kanMX61/ SPC24::kanMX6 CFIII CEN3.d URA3 SUP11*	This study
YM160	*MATa/α ura3-52 ura3-52/, lys2-801/ lys2-801, ade2-101/ ade2-101, his3Δ200 his3Δ200/, leu2Δ1/ leu2Δ1, trp1Δ63/trp1Δ63 apq12ΔTRP1/apq12ΔTRP1 CFVII RAD2.d URA3 SUP11*	This study
YPH272	*MATa/α ura3-52 ura3-52/, lys2-801/ lys2-801, ade2-101/ ade2-101, his3Δ200 his3Δ200/, leu2Δ1/ leu2Δ1, trp1Δ63/trp1Δ63 CFVII RAD2.d URA3 SUP11*	P. Hieter
YPH499	MATa *ura3-52, lys2-801, ade2-101, his3Δ200, leu2Δ1, trp1Δ63*	P. Hieter
YPH982	*MATa/α ura3-52 ura3-52/, lys2-801/ lys2-801, ade2-101/ ade2-101, his3Δ200 his3Δ200/, leu2Δ1/ leu2Δ1, trp1Δ63/trp1Δ63 CFIII CEN3.L URA3 SUP11*	P. Hieter
YVM503	MATa *ura3-52, lys2-801, ade2-101, his3Δ200, leu2Δ1, trp1Δ63, spc24ΔHIS3, pBVM93a*	This study
YVM509	MATa *ura3-52, lys2-801, ade2-101, his3Δ200, leu2Δ1, trp1Δ63, spc34ΔHIS3, pBVM95c*	This study

YVM1363	MATa *ura3-52, lys2-801, ade2-101, his3Δ200, leu2Δ1, trp1Δ63 spc24-10::kanMX6*	This study
YVM1370	MATa *ura3-52, lys2-801, ade2-101, his3Δ200, leu2Δ1, trp1Δ63 SPC24::kanMX6*	This study
YVM1380	MATa *ura3-52, lys2-801, ade2-101, his3Δ200, leu2Δ1, trp1Δ63 spc24-9::kanMX6*	This study
YVM1448	MATa *ura3-52, lys2-801, ade2-101, his3Δ200, leu2Δ1, trp1Δ63 spc24-8::kanMX6*	This study
YVM1579	MATa *ura3-52, lys2-801, ade2-101, his3Δ200, leu2Δ1, trp1Δ63 SPC24-GFP::TRP1::kanMX6*	This study
YVM1585	MATa *ura3-52, lys2-801, ade2-101, his3Δ200, leu2Δ1, trp1Δ63 spc24-8-GFP::TRP1::kanMX6*	This study
YVM1764	MATa *ura3-52, lys2-801, ade2-101, his3Δ200, leu2Δ1, trp1Δ63 apq12::TRP1*	This study
YVM1864	MATa *ura3-52, lys2-801, ade2-101, his3Δ200, leu2Δ1, trp1Δ63 spc34-6::kanMX6*	This study
YVM1892	*MATa/α ura3-52 ura3-52/, lys2-801/ lys2-801, ade2-101/ ade2-101, his3Δ200 his3Δ200/, leu2Δ1/ leu2Δ1, trp1Δ63/trp1Δ63 spc24-9::kanMX61/ spc24-9::kanMX6 CFIII CEN3.d URA3 SUP11*	This study
YVM1893	*MATa/α ura3-52 ura3-52/, lys2-801/ lys2-801, ade2-101/ ade2-101, his3Δ200 his3Δ200/, leu2Δ1/ leu2Δ1, trp1Δ63/trp1Δ63 apq12ΔTRP1/apq12ΔTRP1 CFIII CEN3.L URA3 SUP11*	This study
YVM1902	MATa *ura3-52, lys2-801, ade2-101, his3Δ200, leu2Δ1, trp1Δ63 apq12::TRP1 SPC24::kanMX6*	This study
YVM1904	MATa *ura3-52, lys2-801, ade2-101, his3Δ200, leu2Δ1, trp1Δ63 apq12::TRP1 spc24-10::kanMX6*	This study
YVM1906	MATa *ura3-52, lys2-801, ade2-101, his3Δ200, leu2Δ1, trp1Δ63 apq12::TRP1 spc24-8::kanMX6*	This study
YVM1918	MATa *ura3-52, lys2-801, ade2-101, his3Δ200, leu2Δ1, trp1Δ63 SPC24-GFP::TRP1::kanMX6 apq12::TRP1*	This study
YVM1919	MATa *ura3-52, lys2-801, ade2-101, his3Δ200, leu2Δ1, trp1Δ63 spc24-8-GFP::TRP1::kanMX6 apq12::TRP1*	This study

Preparation of yeast cell lysate and immunoblot analysis:

Cells were grown to log phase and then lysed by bead beating (Tyers et al., 1992). 50μg of protein was loaded per lane, and western blots were performed with α-GFP monoclonal antibodies (Roche) and with α-Cdc28 antibodies (gift from Raymond Deshaies).

Cell cycle synchronization

To assess the arrest phenotype of *spc24* and *spc34* Ts mutants (Figures 2-1 and 2-2), strains were grown to an OD_{600} of 0.2 at 25° in YPD media. α-factor (αF) was added (5μg/μL) and cultures were incubated for 2 hours then released into YPD at 37°. Samples were taken every hour for FACS and immunofluorescence microscopy. For *apq12Δ* αF and nocodazole (NZ) arrest-release experiments (Figure 2-6), strains were grown to an OD_{600} of 0.3 at 30°. αF or NZ was added to a final concentration of 5μM and 20μg/mL, respectively. Cultures were incubated for 2 hours, and then released into YPD. Samples were taken every 20 minutes for FACS and fluorescence microscopy.

FACS analysis

FACS analysis was performed as described (Haase and Lew, 1997).

Microscopy

Genes carrying C-terminal epitope tags were designed according to (Longtine et al., 1998). For immunofluorescence microscopy, cells were fixed in growth media by addition of 37% formaldehyde to 3.7% final concentration, washed and spheroplasted. Yol 1-34 rat anti-tubulin antibody (Serotec, Oxford, UK) was used with a fluorescein-conjugated goat anti-rat secondary antibody to visualize MTs in combination with DAPI to stain DNA. Cells were imaged using a Zeiss Axioplan 2 microscope equipped with a

CoolSNAP HQ camera (Photometrics) and Metamorph (Universal Imaging) software. Cells used for fluorescence microscopy were fixed by adding an equal volume of 4% paraformaldehyde and incubating for 10 minutes at 25°. Typically, a stack of 10 images was taken at 0.3μm spacing and then the images were displayed as maximum projections for analysis. SPB to SPB distances were determined using Metamorph software by measuring the straight-line distance between the brightest Spc29-CFP pixels.

Results

Isolation of Ts alleles in central and outer kinetochore proteins

In order to identify pathways that are important for proper chromosome segregation, a series of SL screens were performed using mutants in essential kinetochore components as query strains. One member of the Ndc80 complex, Spc24, which has previously been shown to have a role in MT-attachment and spindle checkpoint control, and one member of the outer kinetochore Dam1 complex, Spc34, which is required for MT-attachment, spindle stability and prevention of monopolar attachment were selected as queries (Janke et al., 2001; Janke et al., 2002; Le Masson et al., 2002; Wigge and Kilmartin, 2001). Genome-wide screening using only a single mutant of *spc24* and *spc34* might limit the results, depending on the nature of the mutation and the resulting defect in protein function. Thus, a series of Ts mutations in both genes were created and selected for mutants that displayed different arrest phenotypes upon shift to non-permissive temperature. Three alleles were isolated in both *SPC24* (*spc24-8*, *spc24-9*, *spc24-10*) and *SPC34* (*spc34-5*, *spc34-6*, *spc34-7*) and their effects on DNA content and cell morphology at 37°C was assessed. Strains were arrested in G1 phase using the mating

pheromone αF, then released to 37°C, and monitored over a period of four hours. Multiple *spc24* Ts mutants have been created yet only one arrest phenotype has been published (Janke et al., 2001; Le Masson et al., 2002; Wigge and Kilmartin, 2001). Unexpectedly, all three of the *spc24* alleles displayed different arrest profiles (Figures 2-1A and B). The *spc24-10* mutant, which carries four point mutations (Figure 2-1C), duplicates its chromosomes, and elongates its spindles but DNA remains in the mother cells, suggesting a lack of MT-attachment (Figures 2-1A and B). In addition, DNA re-replicates to 4N due to continuation of the cell cycle despite the defect in chromosome segregation (Figure 2-1A). Previous groups have described the *spc24-10* mutant arrest phenotype and have shown that the failure to arrest at the metaphase to anaphase transition is due to a defect in activation of the spindle checkpoint (Janke et al., 2001; Le Masson et al., 2002; Wigge and Kilmartin, 2001). The other two Ts mutants of *spc24* exhibited previously undescribed *spc24* arrest phenotypes. *spc24-8* carries one mutation in the second predicted coiled-coil domain of Spc24 (Figure 2-1C) and arrests with a short spindle and DNA at the bud neck, indicative of an active checkpoint arrest. The third allele, *spc24-9*, carries one mutation in the C-terminus of Spc24 (Figure 2-1C) and displays a pleiotropic arrest phenotype. Three hours after shift to non-permissive temperature, ~30% of *spc24-9* cells have discontinuous spindles, which extend into the mother and break (Figure 2-1B). DAPI staining revealed that three hours after the temperature shift, unequal amounts of DNA have segregated, suggesting that partial MT attachment has occurred. The spindle defect phenotypes of *spc24-9* mutants are reminiscent of the effects of some mutations in the Dam1 complex (see below).

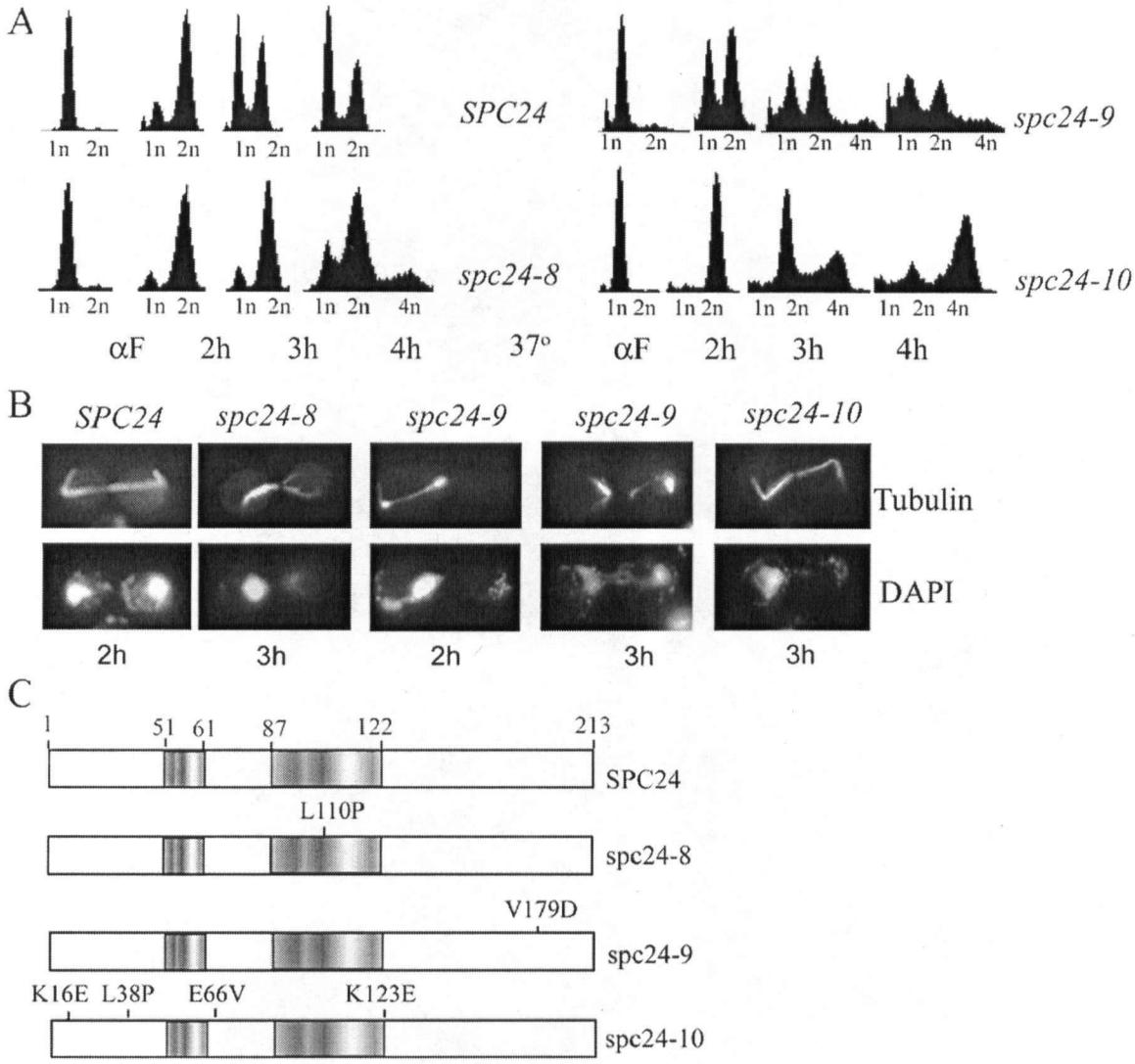


Figure 2-1. Characterization of *spc24* Ts mutants. (A) Wild type (*SPC24*), *spc24-8*, *spc24-9* and *spc24-10* strains were arrested with the mating pheromone αF at 25°, and then released to 37° and samples taken at 2hours (h), 3h and 4h. DNA content was analyzed by flow cytometry for 1N, 2N or 4N DNA content. (B) Immunofluorescence microscopy performed on cells incubated at 37° for 2h or 3h post αF release, as described in (A). Cells were stained for Tubulin and DNA (DAPI). (C) Schematic representation of amino acid (aa) substitutions in *spc24* mutants. Grey shading identifies aa regions of Spc24 predicted to be coiled-coil domains (aa51-aa61 and aa87-aa122) based on Multicoil analysis http://multicoil.lcs.mit.edu/cgi-bin/multicoil. Data in this figure was generated by Vivien Measday.

The three *spc34* mutants that were generated fall into two phenotypic classes. *spc34-6* and *spc34-7* mutants have a metaphase arrest phenotype at restrictive temperature with a short spindle and DNA at the bud neck as described for *spc24-8* (Figure 2-2A and B). In addition to having a similar arrest phenotype, *spc34-6* and *spc34-7* have a common amino acid mutation (S18P). However, they do not display similar genetic interactions (see below), possibly due to the K198E mutation in *spc34-6*, which is directly adjacent to an Ipl1 phosphorylation site (T199) (Cheeseman et al., 2002a). *spc34-5* mutants have a mixed cell population after 3 hours at non-permissive temperature that have either a discontinuous spindle in the mother that progresses into the daughter cell and breaks (Figure 2-2B, first three panels from the left), or a short spindle and a MT projection (Figure 2-2B, fourth panel from the left). The *spc34-5* mutant phenotypes are similar to those caused by the *spc34-3* allele described by Janke et al., 2002. Other members of the Dam1 complex have phenotypes similar to all three of the *spc34* mutants described here (Cheeseman et al., 2001a; Cheeseman et al., 2001b; Enquist-Newman et al., 2001; Hofmann et al., 1998; Janke et al., 2002; Jones et al., 1999).

Genome-wide SL screen with *spc24* and *spc34* alleles

The *spc24* and *spc34* mutations were introduced into the SGA query strain and reconfirmed by sequencing and checking the arrest phenotypes at restrictive temperature (data not shown). Genome-wide SL screens were performed twice using SGA methodology by mating each query strain to the yeast deletion set and selecting for double mutants (Tong et al., 2001). Since some of the *spc24* and *spc34* mutants are inviable at temperatures above 32°C, the SGA screens were performed at 25°C. Any nonessential mutants that were either inviable or slow growing in combination with *spc24*

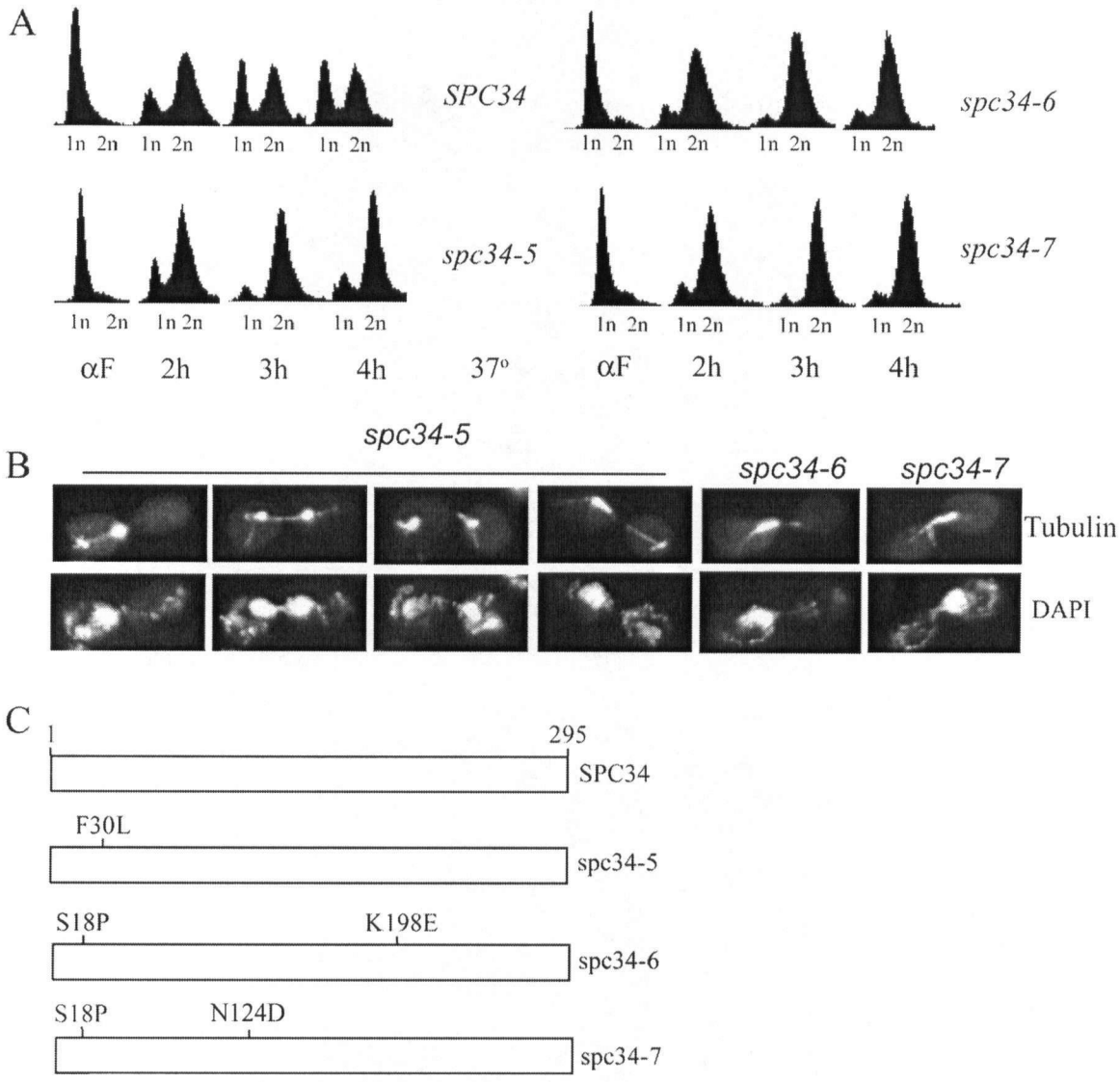


Figure 2-2. Characterization of *spc34* Ts mutants. (A) Wild type (*SPC34*), *spc34-5*, *spc34-6* and *spc34-7* strains were arrested with the mating pheromone αF at 25°, and then released to 37° and samples taken at 2h, 3h and 4h. DNA content was analyzed by flow cytometry for 1N, 2N or 4N DNA content. (B) Immunofluorescence microscopy performed on cells incubated at 37° at 3h post αF release, as described in (A). Cells were stained for Tubulin and DNA (DAPI). (C) Schematic representation of aa substitutions in *spc34* mutants. Data in this figure was generated by Vivien Measday.

or *spc34* mutants were placed on a miniarray and rescreened against the query strains. From these screens, positive genetic interactors were chosen when identified in at least two screens. Random spore analysis and then tetrad dissection were then performed to confirm double mutant phenotypes. Next, the data was organized by 2D hierarchical clustering, which orders both query genes and array genes based on the number of common interactions (Figure 2-3A). The profile of genetic interactions varied considerably depending on the allele screened.

Three central kinetochore mutants (*ctf3, ctf19* and *mcm21*) were identified in both *spc24-9* and *spc34-6* SL screens while two additional kinetochore mutants (*iml3* and *chl4*) were identified in the *spc24-9* screen alone. To determine if this is an allele specific interaction with *spc24-9* and *spc34-6* mutants, all three *spc24* mutants were tested directly for genetic interactions via tetrad analysis. Neither *spc24-8* nor *spc24-10* displayed an SL or SS interaction with the *chl4, ctf3, ctf19* or *mcm21* central kinetochore mutant or the *mad1* spindle checkpoint mutant at 25°, suggesting that the interactions with *spc24-9* are indeed allele specific at this temperature (Table 2-2). Thus, the defect in the *spc24-9* mutant is more sensitive to loss of central kinetochore proteins than the other *spc24* mutants.

The 2D clustering analysis revealed that the *spc24-9* and *spc34-6* mutants have the most genetic interactions in common (Figure 2-3B). Many of the genes have known roles in chromosome segregation, chromatin structure, MT dynamics and spindle checkpoint function. Three members of the GimC/Prefoldin complex were identified (*gim3*, *gim4* and *yke2*), which is a molecular chaperone that promotes MT and actin

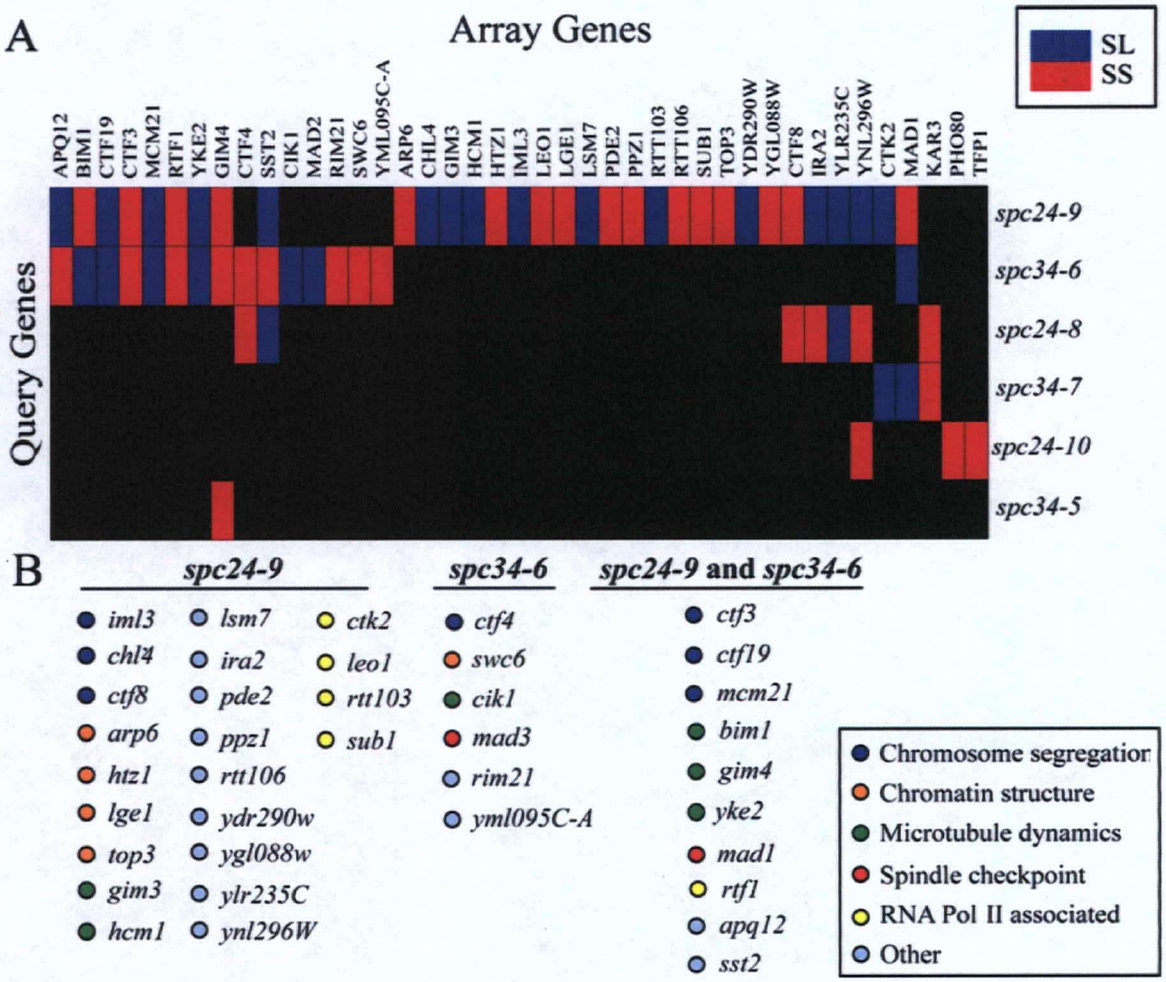


Figure 2-3. *spc24* and *spc34* genomic SL screen results. (A) 2D hierarchical cluster analysis of mutants identified in *spc24* and *spc34* SL screens. Rows are the *spc24* and *spc34* query genes and columns are the mutants, or array genes, identified in the SL screens. Blue represents SL genetic interactions whereas red represents SS genetic interactions. (B) Unique and common genes identified in the *spc24-9* and *spc34-6* screens. Genes are color coded based on their cellular roles. Data in this figure was generated by Vivien Measday.

TABLE 2-2. Genetic interactions between *spc24* and gene deletion mutants

ORF	SGD name	Function	Query Strain		
			spc24-9	***spc24-8***	***spc24-10***
YIL040W	*APQ12*	mRNA export	SL[a]	-	-
YDR254W	*CHL4*	Central kinetochore	SL	-	-
YLR381W	*CTF3*	Central kinetochore	SS	-	-
YPL018W	*CTF19*	Central kinetochore	SL	-	-
YGL086W	*MAD1*	Spindle checkpoint	SS	-	-
YDR318W	*MCM21*	Central kinetochore	SL	-	-

[a]All interactions were determined at 25° on YPD media, Synthetic lethal (SL), Synthetic sick (SS), No phenotype (-). Data in this table was generated by Ken Thorne.

filament folding (Geissler et al., 1998; reviewed in Hartl and Hayer-Hartl, 2002; Siegers et al., 1999; Vainberg et al., 1998). Genome-wide SL screens have been performed using members of the GimC/Prefoldin complex as query strains and have identified genetic interactors representing a wide variety of cellular processes (Tong et al., 2004). Two members of the SWR1 chromatin remodeling complex (*arp6, swc6*) were also identified, which function to incorporate the histone H2A variant, Htz1 (which was also found in this screen) into nucleosomes (Korber and Horz, 2004). Other genes identified include, two components of the Paf1 elongation complex, *rtf1* and *leo1*, plus three additional mutants that interact with RNA polymerase II (*ctk2*, *rtt103* and *sub1*) (Henry et al., 1996; Kim et al., 2004; Knaus et al., 1996; Mueller and Jaehning, 2002; Sterner et al., 1995). Interestingly, the *apq12* mutant was identified in both the *spc24-9* and *spc34-6* screens. *apq12* mutants are known to have defects in nuclear transport (Baker et al., 2004), and recent evidence suggests that nuclear transport is specifically regulated during mitosis (Makhnevych et al., 2003). Thus, this potential link between nuclear transport and kinetochore function was investigated further.

***APQ12* genetically interacts with the kinetochore**

APQ12 encodes a small 16.5kD protein with a predicted transmembrane domain that localizes to the NE (Baker et al., 2004; Huh et al., 2003). Recently, it was shown that *apq12* deletion mutants produce hyperadenylated mRNAs and accumulate poly(A)$^+$ RNA in the nucleus suggesting a defect in mRNA export (Baker et al., 2004). Unlike most mRNA transport mutants however, *apq12* mutants also have an aberrant cell morphology suggesting that Apq12 has additional cellular functions (Baker et al., 2004; Saito et al., 2004). *apq12* deletion mutants are specifically SS/SL in combination with

either *spc24-9* or *spc34-6* mutants but do not display a phenotype when combined with the other *spc24* or *spc34* mutants at 25° (Table 2-2 and data not shown). However, when tested for genetic interactions between *apq12Δ* and the other *spc24* alleles at temperatures higher than that used in the genome-wide screens (25°C), it was found that *spc24-10* was SS in combination with *apq12Δ* at 30°C and the *spc24-8 apq12Δ* double mutant was SS at 33°C (Figure 2-4A). Thus all three of the *spc24* mutants exhibit an unexpected genetic interaction with a mutant that has mRNA export defects. Conceivably, Apq12 could contribute to the nuclear export of *SPC24* mRNA and thereby affect Spc24 cellular protein levels. Combining an *SPC24* mRNA export defect with a *spc24* Ts mutation could result in low levels of the Spc24 protein, which is essential, and may explain the lethality of *apq12 spc24* Ts double mutants. To determine if Spc24 protein levels are perturbed in *apq12Δ* strains, the abundance and localization of GFP-tagged Spc24 in a log phase *apq12Δ* cell population was analyzed. Both Spc24-GFP and scp24-8-GFP protein levels and localization are not noticeably different between a wild type and *apq12Δ* strain (Figure 2-5A). spc24-8-GFP still remains Ts and the strain arrests with the same FACs profile as in Figure 1A suggesting that the GFP tag does not alter the behaviour of the *spc24-8* mutant (data not shown). Spc24-GFP localizes to punctate foci that are close to each other in small budded cells and separated in the mother and daughter in large budded cells (Figure 2-5B wild type panel). Spc24-GFP still localized to distinct foci in an *apq12* mutant suggesting that kinetochore clustering and the structure of the Ndc80 complex is not significantly altered in *apq12Δ* strains (Figure 2-5B, *apq12Δ* panel). Thus, the Spc24 protein stability and localization data suggests that the Spc24 protein is not severely affected in *apq12Δ* strains. Therefore, *apq12* mutants.

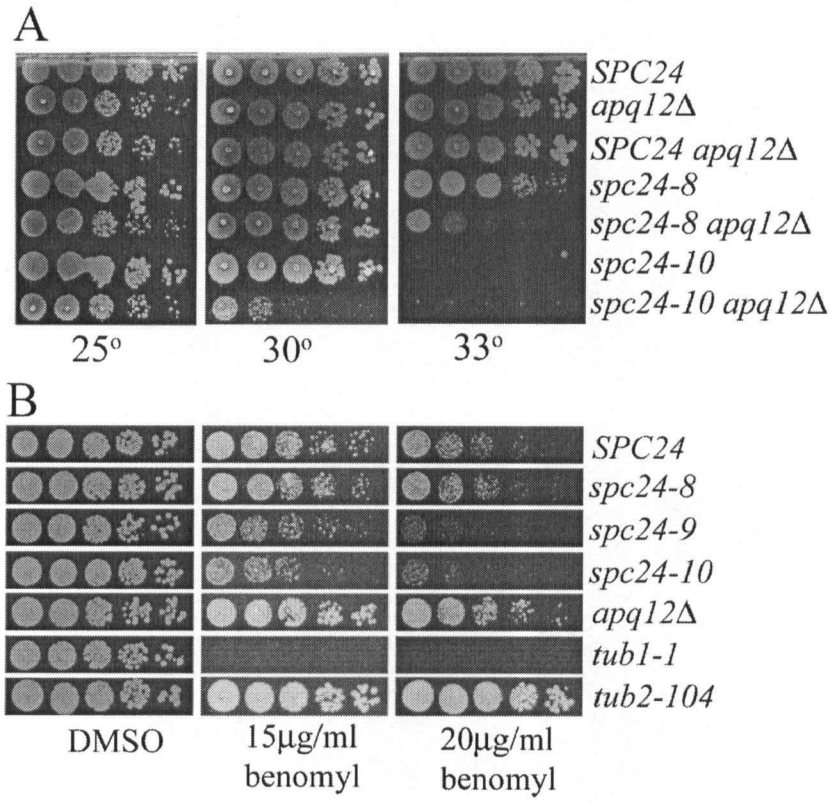


Figure 2-4. Growth defects and benomyl sensitivity of *spc24* and *apq12* mutants. (A) Wild type (*SPC24*), *apq12Δ* , *SPC24 apq12Δ*, *spc24-8*, *spc24-8 apq12Δ*, *spc24-10* and *spc24-10 apq12Δ* strains were grown to log phase, diluted to an OD_{600} of 0.1 and sequential five-fold dilutions were done. Cells were spotted onto YPD plates and incubated at 25°, 30° and 33° for two days. (B) Wild type (*SPC24*), *spc24-8*, *spc24-9*, *spc24-10*, *apq12Δ*, *tub1-1* and *tub2-104* strains were grown to log phase, diluted to an OD_{600} of 0.1, sequential five-fold dilutions were performed and spotted onto DMSO control, 15μg/ml and 20μg/ml benomyl plates and incubated at 30° for 2 days. Data in this figure was generated by Ken Thorne and Ben Montpetit.

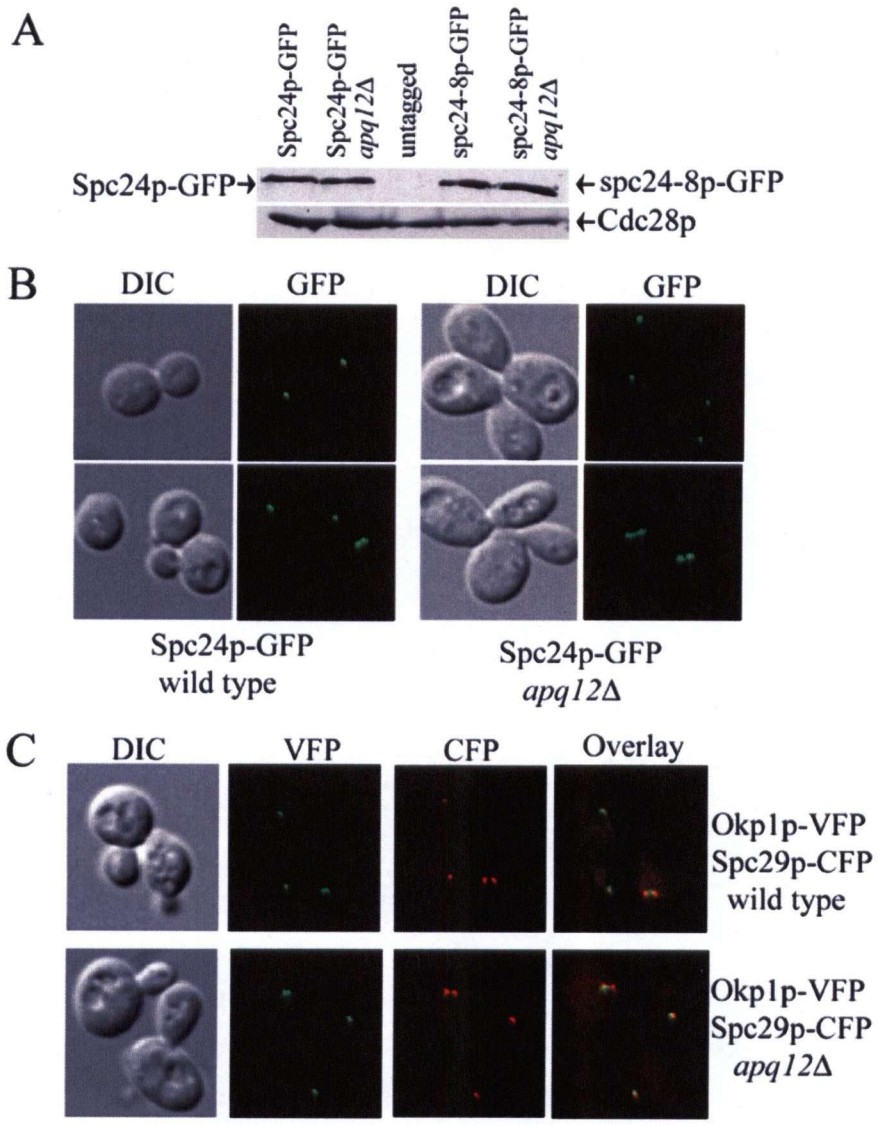


Figure 2-5. Spc24 protein levels and sub-cellular localization of Spc24 and Okp1 in *apq12* mutants. (A) Spc24-GFP and spc24-8-GFP protein levels in an *apq12Δ* strain. Spc24-GFP, Spc24-GFP *apq12Δ*, untagged, spc24-8-GFP and spc24-8-GFP *apq12Δ* strains were grown to log phase at 30° and lysates were prepared. Western blots were performed and immunostained with an anti-GFP antibody (upper panel). As a loading control, the anti-GFP blot was re-probed with an anti-Cdc28 antibody (lower panel). (B) Spc24-GFP sub-cellular localization in an *apq12Δ* strain. DIC and fluorescent images of Spc24-GFP localization in wild type and *apq12Δ* strains. GFP signal appears green. (C) Okp1-VFP Spc29-CFP subcellular localization in an *apq12Δ* strain. DIC and fluorescent images of Okp1-VFP Spc29-CFP localization in wild type and *apq12Δ* strains. VFP signal appears green and CFP signal appears red. Data in this figure was generated by Ben Montpetit.

are unlikely to have a specific defect in *SPC24* mRNA export

To determine if *apq12* mutants have a defect in overall kinetochore structure, the localization of the central kinetochore protein, Okp1-VFP, was analyzed in relation to the SPB protein Spc29-CFP in *apq12Δ* cells. Kinetochore proteins localize to the nuclear side of each SPB in cells with short spindles and co-localize with SPBs in cells with long spindles (Goshima and Yanagida, 2000; He et al., 2000; Pearson et al., 2001). Okp1-VFP Spc29-CFP localization was unperturbed in *apq12Δ* cells suggesting that kinetochore structure and dynamics are not greatly altered in *apq12* mutants (Figure 2-5C).

***apq12* mutants have aberrant chromosome segregation**

To determine if *apq12Δ* strains have a chromosome segregation defect, a colony color based half-sector assay was used to monitor the maintenance of a nonessential chromosome fragment (CF) (Koshland and Hieter, 1987). CF missegregation was also assayed in the three *spc24* Ts mutants to compare CF loss phenotypes between the different mutants. As expected, the *spc24* mutants showed an increased rate of chromosome loss (1:0 events) compared to wild type (Table 2-3). Interestingly, *spc24-9* had a much higher rate of chromosome loss (380 fold increase) compared to *spc24-8* and *spc24-10* (33 and 14 fold increase respectively) at 30°C. *apq12* mutants did not display a significant increase in chromosome loss compared to the wild type strain but did show a dramatic increase (58.8 fold over wild type) in 2:1 segregation events when plated at 35°C. For 2:1 segregation to occur in the first mitotic division (which gives rise to half-sectored colonies), the parental cell must over-replicate the CF to three or more copies and segregate two or more copies to one cell and one copy to the other cell. It is also possible that there might be a selective advantage for *apq12* deletion strains to acquire

TABLE 2-3. Chromosome loss events in *spc24* and *apq12* mutants

Strain Number	Genotype (a/α)	Temp (°C)	Total Colonies	Chromosome Loss (1:0 events)	Nondisjunction (2:0 events)	Over-Replication or Nondisjunction (2:1 events)
YM196	*SPC24::kanMX6/ SPC24::kanMX6* CFIII CEN3.d URA3	30°	24,976	2×10^{-4} (1.0)	2.8×10^{-4} (1.0)	0
YM192	*spc24-8::kanMX6/ spc24-8::kanMX6* CFIII CEN3.d URA3	30°	25,488	6.6×10^{-3} (33)	0	0
YVM1892	*spc24-9::kanMX6/ spc24-9::kanMX6* CFIII CEN3.d URA3	30°	7,606	7.6×10^{-2} (380)	1.3×10^{-4} (0.5)	0
YM194	*spc24-10::kanMX6/ spc24-10::kanMX6* CFIII CEN3.d URA3	30°	23,824	2.8×10^{-3} (14)	0	0
YPH982	wild type CFIII CEN3.L URA3	35°	17,440	4.6×10^{-4} (1.0)	4.1×10^{-3} (1.0)	1.7×10^{-4} (1.0)
YVM1893	*apq12Δ/apq12Δ* CFIII CEN3.L URA3	35°	47,612	6.5×10^{-4} (1.4)	2.0×10^{-3} (0.5)	1.0×10^{-2} (58.8)
YPH272	wild type CFVII RAD2.d URA	35°	29,224	1.7×10^{-4} (1.0)	1.4×10^{-5} (1.0)	1.7×10^{-4} (1.0)
YM160	*apq12Δ/apq12Δ* CFVII RAD2.d URA3	35°	35,759	8.1×10^{-4} (4.8)	0	7.4×10^{-3} (43.5)

Data in this table was generated by Irene Barrett.

an extra copy of the CF (CFIII CEN3.L) that contains part of chromosome III, even though the wild type copy of *APQ12* is located on chromosome IX and thus not on the CF. Therefore, the screen was also performed using a different CF (CFVII RAD2.d) and there was still a significant increase (43.5 fold over wild type) in 2:1 segregation events for *apq12Δ* strains (Table 2-3).

Mutants that have defects in chromosome segregation, including kinetochore mutants, are often sensitive to the microtubule depolymerizing drug benomyl. Although the precise mechanism of benomyl action is not known, evidence suggests that it may bind to the α and β-tubulin hetero-dimers thereby inhibiting MT formation (Richards et al., 2000). *apq12Δ* mutant strains and the three *spc24* Ts mutants, as well as tubulin mutant control strains were spotted on plates to test their sensitivity to benomyl. These tests surprisingly showed that *apq12Δ* strains are resistant to benomyl and grow nearly as well as a *tub2-104* resistant allele on 20ug/ml benomyl plates at 30°C (Figure 2-4B). In contrast, the *spc24-9* and *spc24-10* mutants are sensitive to 15ug/ml and 20ug/ml benomyl (Figure 2-4B). Resistance to benomyl suggests that *apq12* mutants may have stabilized MTs or high levels of tubulin. Anti-tubulin immunofluorescence was performed on fixed *apq12* log phase cells but no noticeable differences in MT formation were apparent (data not shown), although the resolution may not be sufficient to detect minor changes in MT levels or structure. Thus, *apq12Δ* strains may have stabilized MTs or be resistant to benomyl due to an indirect mechanism.

apq12 mutants have defects in exiting mitosis

The 2:1 CF segregation phenotype and benomyl resistance of *apq12Δ* mutants are indicative of problems during mitosis. Thus, the progression of a wild type and *apq12Δ*

strain through the cell cycle was monitored by synchronizing cells in G1 with the mating pheromone αF and releasing them into the cell cycle. Each strain carried a kinetochore and a SPB marker (Okp1-VFP and Spc29-CFP, respectively). Samples were taken every 20 minutes for DNA profiling by FACS analysis and for kinetochore-SPB localization analysis by fluorescence microscopy. After release from the αF arrest, wild type and *apq12Δ* strains showed similar timing of bud emergence and SPB duplication by analyzing fixed cells (data not shown). FACS analysis indicated that the timing of DNA replication was also similar (Figure 2-6A, compare 40 minute time points). However, *apq12Δ* cells showed a delay in the reapperance of 1N cells by ~20 minutes (Figure 2-6A, compare 100 minute time points), and that the population of 1N cells remained small compared to wild type throughout the time course (Figure 2-6A). Wild type cells showed a transient appearance of 4N DNA which disappeared by 160 minutes, whereas the *apq12Δ* cells had a 4N population of cells from 40 minutes onwards suggesting that a percentage of *apq12* mutants are re-replicating DNA prior to exiting mitosis (Figure 2-6A). *apq12Δ* cells also had an increased proportion of 2N cells when arrested with αF, suggesting that a percentage of *apq12Δ* G1 cells are carrying an extra copy of all chromosomes or that a percentage of *apq12Δ* cells do not respond to mating pheromone (Figure 2-6A, 0 minutes, *apq12Δ* panel). These observations are consistent with a delay for *apq12* mutants in progression through mitosis and a failure to complete mitosis prior to initiating replication. To define the stage of mitosis that is delayed in *apq12Δ* mutants, a similar experiment using the MT-depolymerizing drug NZ was performed, which arrests cells in metaphase. *apq12* mutants arrest primarily with a 2N population of cells after two hours of exposure to NZ and with a peak of 4N cells that persists throughout the

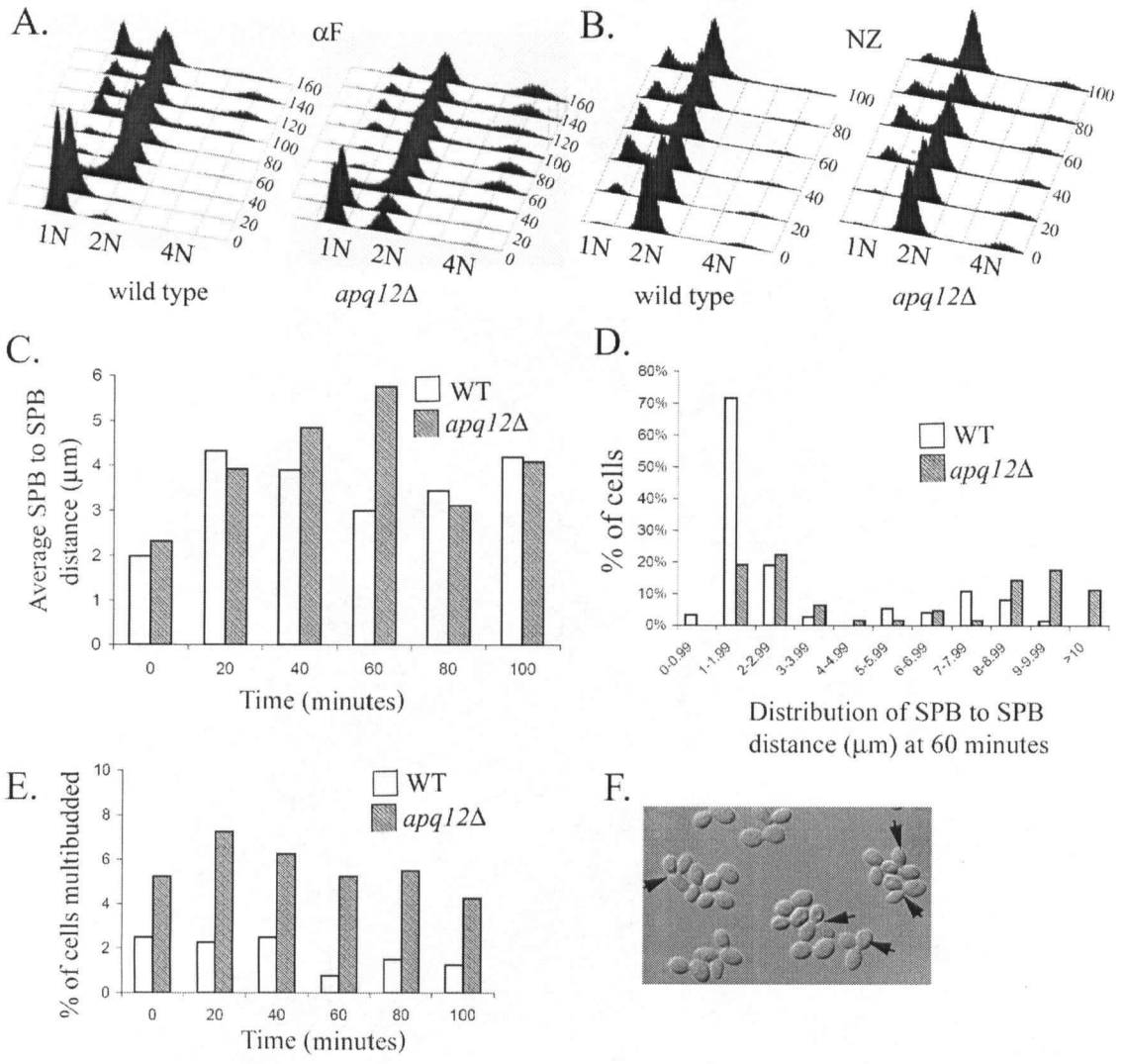


Figure 2-6. *apq12* mutants are delayed in anaphase and prematurely enter a new cell cycle. WT and *apq12Δ* cells were released from αF or NZ arrest and sampled every 20 minutes. (A) αF and (B) NZ treated cells were assessed for DNA content by FACS analysis. (C) Average SPB to SPB distances in cells sampled from each NZ arrest time point. SPBs were marked with Spc29-CFP and the distance was quantified from immunofluorescent images. (D) Distribution of SPB to SPB distances at 60 minutes after NZ release. (E) Percentage of multi-budded cells at each time point after NZ release. (F) DIC image of multi-budded cells at 60 minutes post NZ release in an *apq12Δ* mutant. For each time point (C-E) 100 cells were analyzed. Experiments were performed in duplicate with representative data from one experiment shown. Data in this figure was generated by Ben Montpetit.

apq12Δ time course (Figure 2-6B). Although a small peak of 4N cells is detectable in wild type cells responding to NZ, a greater proportion of *apq12Δ* cells contain a 4N quantity of DNA in the NZ imposed G2 arrest (Figure 2-6B). The DNA profile of *apq12Δ* cells released from NZ arrest showed a~20 minute delay in the reappearance of 1N cells compared to wild type cells as was seen in the G1 synchronization experiment (Figure 2-6B, compare 20 and 40 minute time points). The distance between two Spc29-CFP foci was also monitored, as an indicator of spindle length, and it was found that wild type and *apq12Δ* cells entered anaphase with similar kinetics as judged by spindle elongation. However, anaphase spindles persisted longer in the *apq12Δ* cell population suggesting that *apq12Δ* cells are delayed during mitosis (Figure 2-6C). More specifically, at 60 minutes post NZ release, 70% of wild type cells had a spindle length of 1 to 1.99μm suggesting that they had progressed from anaphase to G1 (Figure 2-6D). In contrast, only 19% of the *apq12Δ* cells had a 1 to 1.99μm spindle and 28% of the cells had a spindle between 8 and 10μm suggesting that the cells were still in anaphase (Figure 2-6D). In addition, 12% of *apq12* mutants had spindle lengths greater than 10μm whereas none of the wild type spindles reached this length. Finally, the *apq12* mutant population contained multi-budded cells throughout the time course, even immediately after release from NZ exposure, suggesting that some cells might be breaking through the mitotic checkpoint arrest (Figure 2-6E). The appearance of *apq12Δ* cells with multiple buds and 4N DNA content suggests that cells are attempting to re-enter the cell cycle prior to completing cytokinesis and that the spindle checkpoint is not sensing the defect causing *apq12Δ* cells to spend extended time in anaphase.

Discussion

A genome-wide SL screens using novel mutations in kinetochore proteins was employed to uncover novel pathways important for chromosome stability when kinetochore function is compromised. A component of the NE, Apq12, was identified that had not been previously linked to chromosome segregation. The data demonstrates that Apq12 has a role in the timely execution of anaphase and maintenance of chromosome stability and provides evidence that the NE is intimately linked with chromosome segregation.

The results of the SL screens highlight the importance of using multiple alleles of essential genes as queries in SGA analysis. Moreover, allele specific interactions provide information about functional domains of the query protein. For instance, the SL data suggests that Spc24 can be divided into distinct functional domains. *spc24-9*, which carries a mutation in the C-terminus of Spc24, was SS or SL with the *chl4*, *ctf3*, *ctf19*, *iml3* and *mcm21* central kinetochore mutants. *spc24-8* and *spc24-10*, which carry mutations in the N-terminal region of Spc24 that contains two coiled-coil domains, did not display genetic interactions with central kinetochore mutants at 25°C. *spc24-9* mutants also have a much higher rate of chromosome loss than *spc24-8* and *spc24-10* mutants (Table 2-3). Thus, it is likely that the C-terminal mutation in *spc24-9* affects a different Spc24 function or protein-protein interaction than the *spc24-8* and *spc24-10* mutants. This data is consistent with a recently published structural analysis of the Ndc80 complex which demonstrates that the C-terminus of Spc24 is a globular domain that likely interacts with the kinetochore (Wei et al., 2005).

In addition to identifying numerous central kinetochore mutants, two negative

regulators of the cAMP pathway were also identified, *ira2* and *pde2* in the *spc24-9* genome-wide SL screen (Figure 2-3). Interestingly, *PDE2* was recently identified as a high copy suppressor of Dam1 kinetochore complex mutants (Li et al., 2005). Five negative regulators of the cAMP pathway, including *ira2* and *pde2*, were also identified as benomyl sensitive mutants in genome-wide screens (Pan et al., 2004). Thus, upregulation of the cAMP pathway by mutation of its negative regulators appears to have a deleterious affect on kinetochore function.

The *apq12* mutant, which has defects in mRNA nucleocytoplasmic transport (Baker et al., 2004), was identified in both the *spc24-9* and *spc34-6* screens. Given the role of Apq12 in mRNA transport, one possibility is that Apq12 could direct the export of specific mRNAs expressing kinetochore proteins. In this hypothesis, mutation of *APQ12* could cause nuclear retention of these mRNAs and improper expression of their protein products. However, Spc24 protein levels and localization are not altered in *apq12* mutants suggesting that Spc24 protein expression is not affected . In addition, the Okp1 central kinetochore protein displayed a typical kinetochore localization pattern in *apq12Δ* cells suggesting that the kinetochore is intact. Finally, *apq12* mutants are resistant to benomyl, whereas cells carrying mutations in kinetochore components or spindle checkpoint proteins are often benomyl sensitive, suggesting that *apq12* mutants do not contain reduced levels of kinetochore and spindle checkpoint proteins.

Since *apq12* mutants appear to have normal levels of kinetochore proteins, Apq12 could have a direct role in chromosome segregation and cell cycle regulation by coordinating the localization of specific protein components to the NE. Recently identified links between the NPC and the kinetochore give precedent for communication

between the NE and the spindle checkpoint machinery (Loiodice et al., 2004; Rabut et al., 2004; Stukenberg and Macara, 2003). For example, the Mad1 and Mad2 spindle checkpoint proteins localize to the NPC in both yeast and mammalian cells (Campbell et al., 2001; Iouk et al., 2002). The localization patterns of Mad1 and Mad2 were analyzed in *apq12* mutants during both normal cell growth and in response to the spindle checkpoint induced by NZ; however, no changes in Mad1 or Mad2 localization in *apq12Δ* strains were detected compared to a wild type strain suggesting that Apq12 is not required to sequester spindle checkpoint proteins in the NE (data not shown). Thus, Apq12 has an alternative role at the NE, perhaps by sequestering or trafficking other chromosome segregation proteins via the NE and NPC.

apq12 mutants are delayed during mitosis and accumulate in anaphase, suggesting a defect in mitotic exit. Using two methods of cell synchrony, it was found that a small percentage of *apq12* mutants re-replicate their DNA and rebud prior to completing cytokinesis. During mitosis, the transition from metaphase to anaphase is marked by degradation of the anaphase inhibitor protein Pds1 (Cohen-Fix et al., 1996; Yamamoto et al., 1996a; Yamamoto et al., 1996b). Stabilization of Pds1 is a hallmark of cells that are actively responding to the spindle checkpoint pathway, thus *pds1* mutants are defective in the spindle checkpoint response. Recent genetic studies identified an SL interaction between *pds1* and *apq12* and between *mad2* and *apq12* (Sarin et al., 2004). Therefore, the mitotic defects of *apq12Δ* mutants render the spindle checkpoint pathway essential during normal cell growth.

Apq12 is one of a growing member of NE associated proteins that have a role in chromosome stability and mitotic progression. For example, Sac3 is a nuclear pore

associated protein that connects transcription elongation with mRNA export (Fischer et al., 2002). *sac3* deletion mutants accumulate in mitosis as large budded cells with extended MTs, are resistant to benomyl and have an increased rate of chromosome loss compared to wild type strains (Bauer and Kolling, 1996; Jones et al., 2000). In a previous genome-wide SL screen, a genetic interaction between *sac3* and *cep3-2* (an inner kinetochore protein) was also identified, further supporting a role for Sac3 in chromosome segregation (Measday et al., 2005b). A component of the SPB called Mps3 is another example of the connection between the NE and chromosome. Mps3 interacts with the Ctf7 cohesin protein and is required to maintain wild type levels of cohesion between chromosomes (Antoniacci et al., 2004).

In mammalian cells, where the NE disassembles, multiple proteins located at the NPC re-localize to the kinetochore upon NE breakdown (Stukenberg and Macara, 2003). Ran is a small GTPase that regulates the interaction of cargo proteins with nucleoporins. The Ran GTPase activating protein, RanGAP1 and its associated nucleoporin RanBP2 are targeted to kinetochores in a MT and Ndc80 complex dependent fashion (Joseph et al., 2004). In addition, RanGAP1 and RanBP2 are required for the kinetochore localization of both spindle checkpoint and kinetochore proteins and for maintaining kinetochore-MT interactions (Joseph et al., 2004). Although no nucleoporin has been shown to re-localize to the kinetochore in yeast, the Nnf1 kinetochore protein was originally identified from a purification of NE proteins and yeast cells depleted of Nnf1 accumulate poly(A)+ RNA (Shan et al., 1997) . The molecular mechanism by which NE proteins, such as Apq12 and kinetochore proteins interact may be a conserved cellular process that functions to promote proper chromosome segregation and mitotic progression.

Chapter 3:

Sumoylation of the budding yeast kinetochore protein Ndc10 is required for Ndc10 spindle localization and regulation of anaphase spindle elongation

The work presented in this chapter is reprinted (with modifications) from the *Journal of Cell Biology* (Montpetit B, Hazbun T, Fields S, Hieter P. Requirement for sumoylation of the budding yeast kinetochore protein Ndc10 for Ndc10 spindle localization and proper anaphase spindle elongation. J. Cell Biol. 2006 Sept; 174: 653-663.) with permission from The Rockefeller University Press, copyright 2006. I am solely responsible for all data presented in this chapter, data analysis, and manuscript writing with the exception of Table 3-3, which was generated by Tony Hazbun (Purdue University) and Stan Fields (University of Washington).

Introduction

Proper completion of mitosis depends on the proper co-ordination of chromosome (e.g. chromosome segregation) and cytoskeletal events (e.g. cytokinesis). The factors important for regulating chromosome related events are well known, and include the SAC, which monitors the attachment of sister chromatids to the spindle apparatus (Lew and Burke, 2003). However, less is known about the mechanism that coordinate the chromosome cycle with the mitotic spindle and cytokinesis. Candidates for regulating these events include chromosome passenger proteins (CPPs), which are proteins that localize to centromeres during early mitosis and then move to the spindle mid-zone during anaphase (Adams et al., 2001a; Vagnarelli and Earnshaw, 2004). This temporal re-localization provides an excellent mechanism to coordinate events at these different sub-cellular locations, in that CPPs tasks at the spindle mid-zone could not be completed until the CPPs are released from the centromere upon completion of the centromere related tasks. In yeast, the set of chromosome passenger like proteins includes the Aurora kinase Ipl1 and the IAP repeat protein Bir1, and the inner kinetochore protein Ndc10 (Biggins et al., 1999; Bouck and Bloom, 2005; Buvelot et al., 2003; Goh and Kilmartin, 1993; Huh et al., 2003; Widlund et al., 2006). The localization of these proteins to the mitotic spindle is thought to be important for regulating mitotic spindle dynamics and for cytokinesis (Adams et al., 2001b; Bouck and Bloom, 2005; Buvelot et al., 2003; Widlund et al., 2006). Indeed, temperature-sensitive alleles of *ndc10-1* or *bir1-33* cause cytokinesis defects at the restrictive temperature (Bouck and Bloom, 2005; Gillis et al., 2005). However, mutations in Bir1 that result in loss of Ndc10 from the mitotic spindle cause defects only in proper spindle elongation, suggesting that the function of the spindle

bound pool of Ndc10 is related to spindle function, not cytokinesis (Widlund et al., 2006).

Ndc10 and Cep3 are both members of the CBF3 inner kinetochore complex that is required for the localization of all other kinetochore proteins (He et al., 2001), with loss of function mutations disrupting microtubule kinetochore attachments and spindle assembly checkpoint function (Fraschini et al., 2001; Goh and Kilmartin, 1993). Recently, it was shown that the whole CBF3 complex is transported to the spindle mid-zone where it localizes with the plus-ends of microtubules. Furthermore, Ndc10 was shown to localize to MT plus-ends during spindle disassembly and along dynamic MTs in G1 (Bouck and Bloom, 2005). This pattern of localization highlights three possible roles the CBF3 complex, and Ndc10, could be playing when associated with MTs: 1.) CBF3 could contribute to spindle stability; 2.) mid-zone CBF3 could act as a signal to coordinate spindle disassembly with completion of chromosome segregation; and 3.) CBF3 associated with MTs plus-ends may represent a "pre-kinetochore" structure that participates in establishing MT attachment in the next cell cycle during the process of "search and capture".

To gain insight into the function of Ndc10 on the mitotic spindle, protein interacting partners of Ndc10 were identified with the goal of discovering proteins required for Ndc10's spindle localization and subsequent spindle functions. Using a genome-wide two-hybrid screen, multiple interactions between Ndc10 and the sumoylation machinery of budding yeast were found, and subsequent analysis demonstrated that Ndc10 is a target for sumoylation *in vivo,* as are other kinetochore proteins (Bir1, Cep3, and Ndc80).

Sumoylation plays a key role in many cellular processes including nuclear transport, signal transduction, transcriptional regulation, and maintenance of genome integrity (Hay, 2005; Johnson, 2004; Seeler and Dejean, 2003). Sumoylation is a process by which a **S**mall **U**biquitin-like **MO**difier protein, SUMO (Smt3 in yeast), is conjugated to a target protein at a lysine residue (Figure 3-1). SUMO belongs to a family of ubiquitin-like proteins (Ubls) sharing ~20% identity with ubiquitin, which was first discovered in mammals to be conjugated to RanGAP1 (Mahajan et al., 1997; Matunis et al., 1996). The conjugation of SUMO to target proteins makes use of an enzyme cascade that is similar to ubiquitination and includes an E1 activating enzyme that binds to SUMO and activates SUMO for subsequent binding to an E2 protein, or SUMO conjugating enzyme. An E3 complex, SUMO ligase, may then facilitate the modification of a target protein by mediating the interaction between the target and E2-SUMO complex. However, one of the unique features of the sumoylation process is the ability of the SUMO E2 (Ubc9) to recognize substrate proteins without the aid of an E3. Typically this occurs provided that the lysine to be modified is part of the SUMO conjugation motif ΨKxE where Ψ is any large hydrophobic residue and x is any residue (Rodriguez et al., 2001). It is worth noting that SUMO modification sites are known that do not conform to this consensus motif, nor are all matching consensus sites in a protein modified by SUMO. This suggests that although Ubc9 can recognize substrates, other factors are important for modification such as substrate presentation to Ubc9 or subcellular localization of the substrate.

Like most other ubiquitin-like proteins, SUMO must be post-translationally matured by proteolytic cleavage to reveal the di-glycine motif used to form the iso-

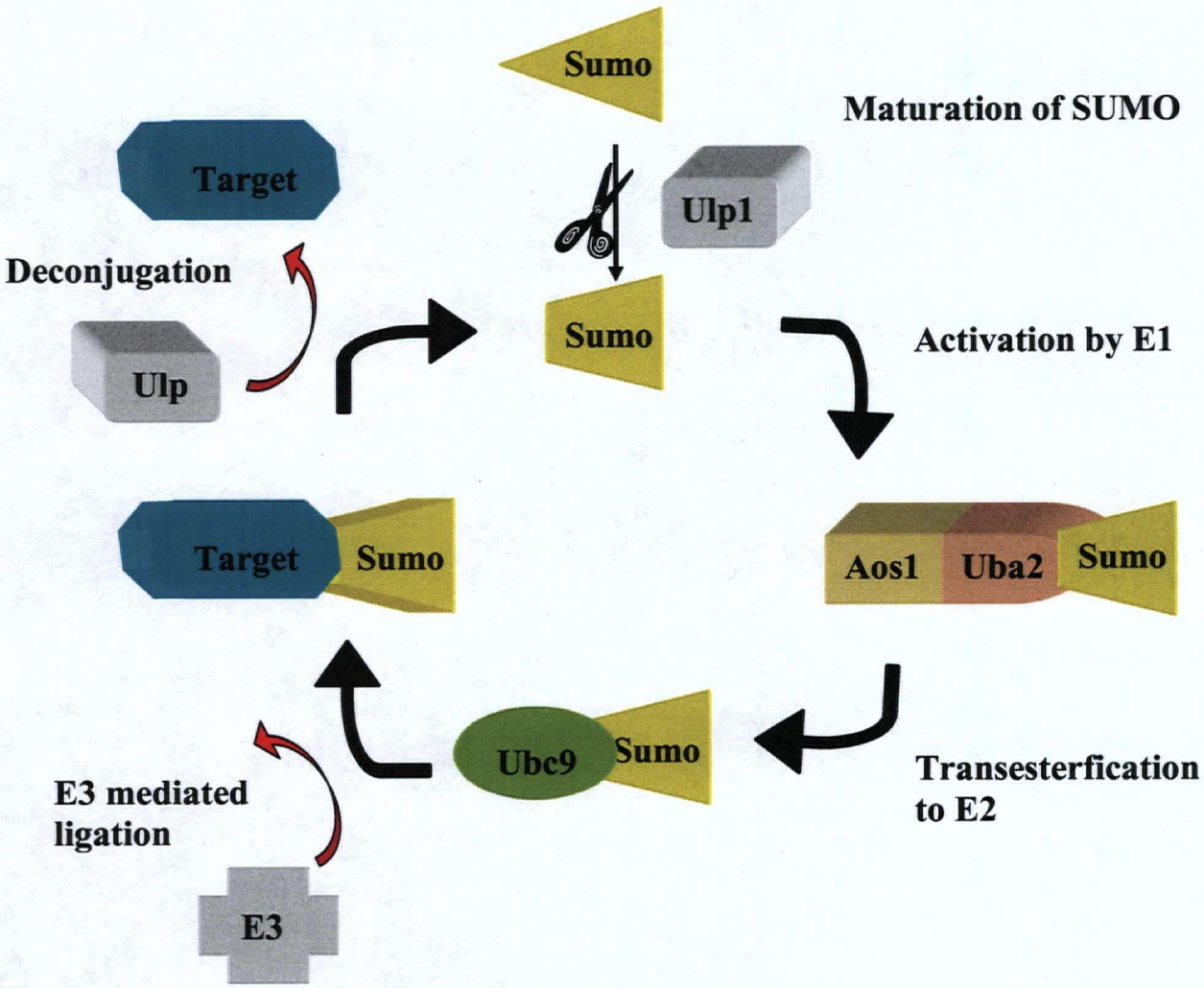


Figure 3-1. SUMO modification cycle. The process of sumoylation starts by proteolytic cleavage of Sumo to expose a di-glycine motif. Once matured, Sumo is activated and transferred to an E2 protein that then conjugates Sumo to the target protein via an iso-peptide bond with the help of an E3 ligase. Sumo modified substrates can then be deconjugated by the action of Ubiquitin-like proteases (Ulps) to regenerate Sumo and the unmodified form of the target.

peptide bond with its substrate. Maturation of SUMO is carried out by ubiquitin-like proteases (ULPs) that are also able to cleave SUMO from modified substrates making sumoylation a reversible process (Li and Hochstrasser, 1999; Li and Hochstrasser, 2000). The consequences of sumoylation are substrate specific, and can involve altering a substrate's interaction with other macromolecules (e.g. proteins and DNA), or blocking lysine residues on target proteins from being modified by other lysine-targeted modifications such as ubiquitin (Desterro et al., 1998; Hoege et al., 2002).

In mammalian cells, defects in sumoylation cause abnormal nuclear architecture, chromosome mis-segregation, and embryonic lethality (Nacerddine et al., 2005). In the budding yeast *Saccharomyces cerevisiae,* sumoylation is essential for viability and is required for proper chromosome segregation with sumoylation-deficient cells arresting in G2/M with short spindles and replicated DNA (Li and Hochstrasser, 1999; Li and Hochstrasser, 2000; Seufert et al., 1995). *SMT3*, which was originally identified as a high copy suppressor of a mutation in the kinetochore protein Mif2 (Meluh and Koshland, 1995), encodes the ubiquitin-like protein SUMO in yeast. These phenotypes highlight a role for SUMO modification in the cell cycle, and more specifically mitosis. For example, both DNA topoisomerase II and Pds5 have been shown to be sumoylated, with this modification being important for the role these two proteins play in sister chromatid cohesion (Bachant et al., 2002; Stead et al., 2003). Likewise, it was also found that DNA Topoisomerase II is a target for SUMO modification in Xenopus egg extracts, which is required for proper chromosome segregation. However, in budding yeast these proteins alone do not account for all the defects observed in SUMO deficient cells, thus the critical protein targets that lead to these phenotypes remain unknown. This is further

illustrated by the fact that in yeast strains defective for SUMO conjugation, APC/C activity is downregulated and targets critical for cell cycle progression (e.g. Securin and mitotic cyclins) are not degraded, but the actual targets being modified by SUMO that are required for APC/C activity are not known (Dieckhoff et al., 2004). Recent attempts in budding yeast to expand the number of SUMO substrates by proteomic means have identified greater than 400 potential sumoylation targets; thus, pinpointing the biologically relevant sumoylation events remains a challenge. (Denison et al., 2005; Hannich et al., 2005; Panse et al., 2004; Wohlschlegel et al., 2004; Wykoff and O'Shea, 2005; Zhou et al., 2004). Within this set of putative substrates are many proteins involved in chromosome segregation, including kinetochore proteins (Denison et al., 2005; Hannich et al., 2005; Hoege et al., 2002; Panse et al., 2004; Wohlschlegel et al., 2004; Wykoff and O'Shea, 2005; Zhou et al., 2004) However, the functional role(s) of sumoylation on these proteins are not understood.

Work presented in this chapter demonstrate that sumoylation of Ndc10, Ndc80, Cep3 and Bir1 is differentially regulated in response to checkpoint activation suggesting that sumoylation has distinct roles in modulating the function of these kinetochore proteins. Importantly, lysine residues required for Ndc10's sumoylation are necessary for Ndc10's proper localization to the mitotic spindle, suggesting that sumoylation plays a direct role in facilitating Ndc10's interaction with the spindle apparatus. Mis-localization of Ndc10 from the mitotic spindle is not associated with cytokinesis defects, suggesting that the spindle-bound form of Ndc10 does not have a role in cytokinesis as previously thought. Furthermore, loss of Ndc10's mitotic spindle association resulted in anaphase spindles of abnormal length, highlighting a role for Ndc10 in mitotic spindle dynamics.

Materials and Methods

Yeast Strains, plasmids, and microbial techniques

Yeast strains (S288C background) and plasmids used in this study are listed in Table 3-1 & 3-2. Mutant alleles were integrated into the yeast genome at the endogenous locus by co-transformation of a PCR product carrying the desired mutation(s) and a PCR product containing a nutritional marker (*URA3*). Integration of the desired mutation was confirmed by DNA sequencing. To arrest cell cultures, αF (1 mg/ml in methanol) or NZ (5 mg/ml in DMSO) was added and cultures were incubated for 2hrs at 30°C. To assay benomyl sensitivity, benomyl was added at the indicated concentration to YPD media, dimethyl sulfoxide was used in the control plate (0 μg/ml benomyl), and five fold serial dilutions were spotted on the plates and then grown at 30°C for 2 days. Flow cytometry analysis to monitor DNA content was performed as previously described (Haase and Lew, 1997).

Genome-wide Two-hybrid screen

NDC10 was cloned into pOBD2 and pBDC using standard techniques (Cagney et al., 2000; Millson et al., 2003). Fusion of the DNA binding domain to the C-terminus (pBDC), but not the N-terminus of Ndc10 (pOBD2), resulted in a functional protein as judged by the ability to rescue a ndc10Δ strain. Two independent genome-wide two-hybrid screens were performed using an activation domain array (Hazbun et al., 2003), as described previously (Uetz et al., 2000). The two-hybrid positives from these genome-wide screens were reconfirmed by repeating the two-hybrid assay. The identities of the activation domain fusions were confirmed by rescuing plasmids and sequencing.

TABLE 3-1. Yeast Strain List

Strain	Genotype	Source
DBY1385	*MATa his4 ura3-52 tub2-104*	D. Botstein
DBY2501	*MATa ura3-52 ade2-101 tub1-1*	D. Botstein
PWY 194[a]	MATa *ade2-1oc can1-100 his3-11,15 leu2-3,112 trp1-1ura3-1 ade3Δ bir1Δ::hphMX3 bir1-9A-13MYC ::kanMX6::URA3 lys2Δ::HIS3 NDC10-YFP::kanMX6*	(Widlund et al., 2006)
PWY 204[a]	MATa *ade2-1oc can1-100 his3-11,15 leu2-3,112 trp1-1ura3-1 ade3Δ bir1Δ::hphMX3 bir1ΔIAP-13MYC ::kanMX6::URA3 lys2Δ::HIS3*	(Widlund et al., 2006)
PWY93-3b[a]	MATa *ade2-1oc can1-100 his3-11,15 leu2-3,112 trp1-1ura3-1 ade3Δ BIR1-13MYC::kanMX6*	(Widlund et al., 2006)
YPH 1734	MATa *ura3-52, lys2-801, ade2-101, his3Δ200, leu2Δ1, trp1Δ63 NDC10-13MYC::kanMX6*	P. Hieter
YPH 1793	MATa *ura3-52, lys2-801, ade2-101, his3Δ200, leu2Δ1, trp1Δ63 SLI15-VFP::kanMX6 SPC29-CFP::hphMX3*	This study
YPH 1794	MATa *ura3-52, lys2-801, ade2-101, his3Δ200, leu2Δ1, trp1Δ63 CEP3-13MYC::His3MX6*	This study
YPH 1798	MATa *ura3-52, lys2-801, ade2-101, his3Δ200, leu2Δ1, trp1Δ63 ASE1-GFP::kanMX6*	This study
YPH 1799	MATa *ura3-52, lys2-801, ade2-101, his3Δ200, leu2Δ1, trp1Δ63 NDC10-13MYC::kanMX6 nfi1Δ::His3MX6*	This study
YPH 1800	MATa *ura3-52, lys2-801, ade2-101, his3Δ200, leu2Δ1, trp1Δ63 NDC80-13MYC::His3MX6*	This study
YPH 1801	MATa *ura3-52, lys2-801, ade2-101, his3Δ200, leu2Δ1, trp1Δ63 NDC10-13MYC::kanMX6 ulp2Δ::His3MX6*	This study
YPH 1802	MATa *ura3-52, lys2-801, ade2-101, his3Δ200, leu2Δ1, trp1Δ63 NDC10-3MYC::His3MX6*	This study
YPH 1803	MATa *ura3-52, lys2-801, ade2-101, his3Δ200, leu2Δ1, trp1Δ63 NDC10-13MYC::kanMX6 siz1Δ::His3MX6*	This study
YPH 1804	MATa *ura3-52, lys2-801, ade2-101, his3Δ200, leu2Δ1, trp1Δ63 NDC10-13MYC::kanMX6 pRS306-ULP2 (2μm,URA3)*	This study
YPH 1805	MATa *ura3-52, lys2-801, ade2-101, his3Δ200, leu2Δ1, trp1Δ63 NDC10-13MYC::kanMX6 pRS306-ULP1 (2μm,URA3)*	This study
YPH 1806	MATa *ura3-52, lys2-801, ade2-101, his3Δ200, leu2Δ1, trp1Δ63 ndc10 K556R-13MYC::kanMX6*	This study
YPH 1807	MATa *ura3-52, lys2-801, ade2-101, his3Δ200, leu2Δ1, trp1Δ63 ndc10 K779R-13MYC::kanMX6*	This study
YPH 1808	MATa *ura3-52, lys2-801, ade2-101, his3Δ200, leu2Δ1, trp1Δ63 ndc80 K231R-13MYC:: kanMX6*	This study
YPH 1809	MATa *ura3-52, lys2-801, ade2-101, his3Δ200, leu2Δ1, trp1Δ63 CDC10-GFP::His3MX6*	This study
YPH 1810	MATa *ura3-52, lys2-801, ade2-101, his3Δ200, leu2Δ1, trp1Δ63 CDC10-GFP::His3MX6 ndc10 K556,651,652, 779R::URA3*	This study
YPH 1811	MATa *ura3-52, lys2-801, ade2-101, his3Δ200, leu2Δ1, trp1Δ63 NDC10-13MYC::kanMX6 siz1Δ::TRP1 nfi1Δ::His3MX6*	This study
YPH 1812	MATa *ura3-52, lys2-801, ade2-101, his3Δ200, leu2Δ1, trp1Δ63 NDC10-13MYC::kanMX6 mms21-11::TRP1*	This study
YPH	MATa *ura3-52, lys2-801, ade2-101, his3Δ200, leu2Δ1, trp1Δ63 SLI15-*	This study

1814	*VFP::kanMX6 SPC29-CFP::hphMX3 ndc10 K556,651,652,779R::URA3*	
YPH 1816	MATa *ura3-52, lys2-801, ade2-101, his3Δ200, leu2Δ1, trp1Δ63 ASE1-GFP::kanMX6 ndc10 K556,651,652, 779R::URA3*	This study
YPH 1817	*MATa/α ura3-52/ura3-52, lys2-801/lys2-801, ade2-101/ade2-101, his3Δ200/his3Δ200, leu2Δ1/leu2Δ1, trp1Δ63/trp1Δ63 ndc10 K556,651,652,779R::URA3/ ndc10 K556,651,652,779R::URA3 CFIII CEN3.L TRP1 SUP11*	This study
YPH 1818	MATa *ura3-52, lys2-801, ade2-101, his3Δ200, leu2Δ1, trp1Δ63 ndc10 K651,652R- 13MYC::kanMX6*	This study
YPH 1819	MATa *ura3-52, lys2-801, ade2-101, his3Δ200, leu2Δ1, trp1Δ63 CEP3-GFP::His3MX6*	This study
YPH* 1820	MATa *ura3-52, lys2-801, ade2-101, his3Δ200, leu2Δ1, trp1Δ63 ndc10 K556,651,652,779R::URA3 CEP3-GFP::His3MX6*	This study
YPH 1821	MATa *ura3-52, lys2-801, ade2-101, his3Δ200, leu2Δ1, trp1Δ63 BIR1-GFP::His3MX6*	This study
YPH 1822	MATa *ura3-52, lys2-801, ade2-101, his3Δ200, leu2Δ1, trp1Δ63 BIR1-GFP::His3MX6 ndc10 K556,651,652, 779R::URA3*	This study
YPH 1823	MATa *ura3-52, lys2-801, ade2-101, his3Δ200, leu2Δ1, trp1Δ63 63 ndc10 K556,651,652,779R::URA3 BIR1-13MYC::His3MX6*	This study
YPH 1824	MATa *ura3-52, lys2-801, ade2-101, his3Δ200, leu2Δ1, trp1Δ63 CEP3-13MYC::His3MX6 ndc10 K556,651, 652,779R::URA3*	This study
YPH 1825	MATa *ura3-52, lys2-801, ade2-101, his3Δ200, leu2Δ1, trp1Δ63 BIR1-13MYC::His3MX6*	This study
YPH 1826	MATα *ura3-52, lys2-801, ade2-101, his3Δ200,leu2Δ1, trp1Δ63 BIR1-13MYC::His3MX6 ndc10-1*	This study
YPH 1827	MATa *ura3-52, lys2-801, ade2-101, his3Δ200, leu2Δ1, trp1Δ63 NDC80-13MYC::His3MX6 ndc10 K556,651, 652,779R::URA3*	This study
YPH 1828	MATa *ura3-52, lys2-801, ade2-101, his3Δ200, leu2Δ1, trp1Δ63 ndc10 K556,651,652,779R-3MYC::His3MX6*	This study
YPH 1829	MATa *ura3-52, lys2-801, ade2-101, his3Δ200, leu2Δ1, trp1Δ63 NDC10-13MYC::kanMX6 bir1Δ::His3MX6 pRS316-BIR1(*pPW02 CEN,URA3*)*	This study
YPH 1830	MATa *ura3-52, lys2-801, ade2-101, his3Δ200, leu2Δ1, trp1Δ63 NDC10-13MYC::kanMX6 bir1Δ::His3MX6 pRS316-bir1-9A (*pPW124 CEN,URA3*)*	This study
YPH 1831	MATa *ura3-52, lys2-801, ade2-101, his3Δ200, leu2Δ1, trp1Δ63 NDC10-13MYC::kanMX6 bir1Δ::His3MX6 pRS316-bir1-ΔIAP(*pPW08 CEN,URA3*)*	This study
YPH 1832	MATα *ura3-52, lys2-801, ade2-101, his3Δ200,leu2Δ1, trp1Δ63 BIR1-13MYC::His3MX6 cep3-1*	This study
YPH 1833	MATα *ura3-52, lys2-801, ade2-101, his3Δ200, leu2Δ1, trp1Δ63 BIR1-13MYC::His3MX6 ctf13-30*	This study
YPH 1834	MATa *ura3-52, lys2-801, ade2-101, his3Δ200, leu2Δ1, trp1Δ63 NDC10-GFP::His3MX6 CFP-TUB1::URA3*	This study
YPH 1835	MATa *ura3-52, lys2-801, ade2-101, his3Δ200, leu2Δ1, trp1Δ63 ndc10 K556,651,652,779R -GFP::His3MX6 CFP-TUB1::URA3*	This study
YBM 1015	MATa *ura3-52, lys2-801, ade2-101, his3Δ200, leu2Δ1, trp1Δ63 NDC10-3MYC::HISMX3 pGA1L-3XHA-CDC5::kanMX*	This study
YBM 186	MATa *ura3-52, lys2-801, ade2-101, his3Δ200, leu2Δ1, trp1Δ63 NDC10-13MYC::kanMX6 mad2Δ::HISMX3*	This study
YPH 499	MATa *ura3-52, lys2-801, ade2-101, his3Δ200, leu2Δ1, trp1Δ63*	P. Hieter

a = W303 background

TABLE 3-2. Plasmid list

Plasmid	Description	Source or Reference
BPH 995	pOBD2-NDC10	This study
BPH 996	pBDC-NDC10	This study
BPH 997	GST-NDC10 in pGex4t-2	This study
BPH 998	GST-ndc10 K556R in pGex4t-2	This study
BPH 999	GST-ndc10 K651,652A in pGex4t-2	This study
BPH 1000	GST-ndc10 K779R in pGex4t-2	This study
pPW02	*BIR1* in pRS316	(Widlund et al., 2006)
pPW124	*bir1-9xA* in pRS316	(Widlund et al., 2006)
pPW08	*bir1-ΔIAP* in pRS316	(Widlund et al., 2006)'

In vitro and *in vivo* sumoylation assays

GST-Ndc10 was expressed in *E. coli* strain BL21 from a pGex 4T-2 vector (Amersham Biosciences) and purified using glutathione beads. *In vitro* sumoylation reactions were carried out using bacterially expressed proteins (expression plasmids provided by Lawrence McIntosh, University of British Columbia, Vancouver, BC, Canada) as previously described (Macauley et al., 2005), and western blotting was performed with an Ndc10 antibody (Measday et al., 2005a). To detect sumoylation *in vivo*, yeast cells (100-200 OD_{600}) were lysed by bead beating (10 X 30sec) in 2.5 ml of lysis buffer (50 mM Tris-HCl [pH 8.0], 5 mM EDTA, 150 mM NaCl, 0.2% Triton X-100, Roche Complete protease inhibitor (1 tablet per 25mL), 10 mM N-ethylmaleimide [NEM], 2 mM phenylmethylsulfonyl fluoride, and 20 µg each of leupeptin, aprotonin and pepstatin per ml) on ice. Lysates were cleared by centrifugation at 30,000g for 20 min, and soluble protein concentrations were determined by protein assay (Bio-Rad). Equal amounts of protein were incubated with α-Myc-conjugated beads for 3hrs at 4°C, and then washed four times with cold lysis buffer for 2 minutes. Immunoprecipitated protein was then eluted with lysis buffer containing 2% SDS at 42°C for 15 minutes. 2.5µL of the eluted protein was used for western blotting with α-Myc Ab to confirm pull down of the tagged protein; 25µl was used for blotting with α –SUMO Ab to detect sumoylated protein. To detect the Ndc10 Bir1 interaction, 25µl of a Bir1 IP was used for western blotting with α-Ndc10 Ab. α -SUMO polyclonal antibodies were generated in rabbits (Covance Research Products) as previously described (Johnson and Blobel, 1999). Initial experiments also made use of an α -SUMO polyclonal antibody provided by Erica Johnson (Thomas Jefferson University, Philadelphia, PA).

Light, fluorescence and immunofluorescence microscopy

Strains used for microscopy were grown in either YPD (for synchronous culture experiments) or in FPM (minimal medium supplemented with 2X adenine and containing 6.5 g/L sodium citrate). Cells were imaged at room temperature using a Zeiss Axioplan 2 microscope with a PLANAPOCHROMAT 100X/1.4 DIC oil immersion objective with Zeiss filter set #38, #47, and Chroma filters 488000. 3D Images (0.25 μm steps) were acquired with a CoolSNAP HQ camera (Roper Scientific) and analyzed using Metamoprh software (Molecular Devices). Images are presented as maximum intensity two-dimensional projections. For fluorescence microscopy, wild type and mutant proteins were tagged at the endogenous locus with GFP or a GFP variant (Longtine et al., 1998). Tub1-CFP::URA3 containing strains were generated by integrating plasmid pSB375 (a gift from Kerry Bloom, University of North Carolina, Chapel Hill, NC) digested with StuI at the URA3 locus. Spindle length measurements were performed on asynchronous cultures of Tub1-CFP containing cells fixed in 70% ethanol and 200mM Tris-HCl [pH 8.0] judged to be in anaphase by the presence of part of the spindle in the daughter cell. To visualize haploid budding pattern, cells were incubated in PBS with 20 μg/ml Calcofluor white (Fluorescent Brightener 28; Sigma Aldrich) at 25°C for 5 min, washed in PBS, and visualized for bud scar staining.

Chromosome instability assays

Quantitative half-sector analysis was performed as described ((Hyland et al., 1999; Koshland and Hieter, 1987). To perform the diploid bimater assay, 10 single colonies were patched onto YPD plates and then replica plated to both MATa and MATα lawns and mating products were selected. The median number of mating products from

the 10 patches was compared to WT in order calculate the increase in frequency over mating of a WT diploid strain.

Results

Ndc10 interacts with multiple components of the sumoylation machinery

In two independent genome-wide two-hybrid screens using Ndc10 as bait, with the Gal4 DNA-binding domain fused to either the N- or C-terminus, ten proteins were identified as putative Ndc10 protein interactors (Table 3-3). Three of these interactions occurred with only one of the baits, suggesting that the presence of the Gal4 DNA-binding domain on the N- or C-terminus may be interfering with specific protein-protein interactions, as has been previously reported (Millson et al., 2003). Of these ten interacting proteins, Bir1 and Ubc9 were previously identified in two-hybrid screens with Ndc10 (Jiang and Koltin, 1996; Yoon and Carbon, 1999); Bir1 has also recently been shown to play a role in the localization of Ndc10 to the mitotic spindle (Bouck and Bloom, 2005; Widlund et al., 2006). To investigate the role the remaining proteins may have in mediating the spindle localization of Ndc10, proteins were tagged with CFP and microscopy was performed to assay for co-localization with Ndc10-VFP. Only Bir1-CFP co-localized with Ndc10 on the mitotic spindle as previously observed (Bouck and Bloom, 2005; Widlund et al., 2005), furthermore deletion of HEX3, NFI1, SAP1 or FIR1 resulted in no change in Ndc10-VFP localization (data not shown). Given that Ndc10 plays an essential role in chromosome segregation, deletion of HEX3, NFI1, SAP1, or FIR1 were also checked for CIN, but no increase in CIN was observed (data not shown). It is worth noting here, that both Fir1 and Nis1 localize to the bud neck, placing them in the correct locale to participate in the recently described roles for Ndc10 and the CBF3

TABLE 3-3. Ndc10 two-hybrid interactions

Prey	pOBD2-Ndc10[a,b]	pBDC-Ndc10[b,c]	Biological process[d]
BIR1	+++++	+++++	chromosome segregation, mitotic spindle elongation
CTF13	-	++	chromosome segregation, kinetochore assembly
FIR1	+++++	+++++	mRNA polyadenylylation
HEX3	+++	+++	protein sumoylation
NFI1	+++	+++	protein sumoylation
NIS1	+++	+++	regulation of mitosis
SAP1	++++	-	unknown
SLI15	+	+	chromosome segregation
SMT3	+	+	protein sumoylation
UBC9	-	++++	protein sumoylation

a = encodes Ndc10 with the GAL DNA binding domain fused to the N-terminus
b = two-hybrid positive colony growth from weak(+) to strong (+++++)
c = encodes Ndc10 with the GAL DNA binding domain fused to the C-terminus
d = obtained from the Saccharomyces Genome Database (http://www.yeastgenome.org/)

Data in this table was generated by Tony Hazbun (Purdue University).

complex in cytokinesis(Bouck and Bloom, 2005; Gillis et al., 2005). Within this set of two-hybrid interacting proteins, four components of the sumoylation machinery were also identified (Ubc9, Smt3, Hex3 and Nfi1). *SMT3*, which was originally identified as a high copy suppressor of a mutation in the kinetochore protein Mif2 (Meluh and Koshland, 1995), encodes the ubiquitin-like protein SUMO.

Ndc10 is sumoylated *in vitro* and *in vivo*

Given *SMT3*'s genetic interaction with the kinetochore, and the number of two-hybrid interactions between Ndc10 and the sumoylation machinery, it is likely that these interactions occur because Ndc10 is a target for sumoylation. To address this possibility, bacterially expressed GST-Ndc10 was tested as a substrate for sumoylation in an *in vitro* sumoylation reaction. Western blot analysis of the reaction products revealed two SUMO-modified forms of Ndc10 that were generated in an ATP-dependent manner, indicating that Ndc10 is sumoylated *in vitro* (Figure 3-2a). To determine whether Ndc10 is modified by SUMO *in vivo*, Ndc10 was immunoprecipitated (IP) from a yeast cell lysate and detected SUMO-modified proteins by western blot analysis using an antibody that recognizes the yeast SUMO protein. Two signals were apparent by western blotting that corresponded to the correct molecular weight to be SUMO-modified forms of Ndc10 (Figure 3-2b). Both of these signals changed electrophoretic mobility upon switching the tag on Ndc10 from thirteen to three copies of MYC, demonstrating that both of these signals represent *in vivo* SUMO-modified forms of Ndc10 and not a co-precipitated protein (Figure 3-2b). The presence of two modified forms of Ndc10 both *in vitro* and *in vivo* suggests that Ndc10 may be sumoylated on at least two lysine residues. Other post-translational modifications and/or poly-sumoylation on a single lysine residue, however,

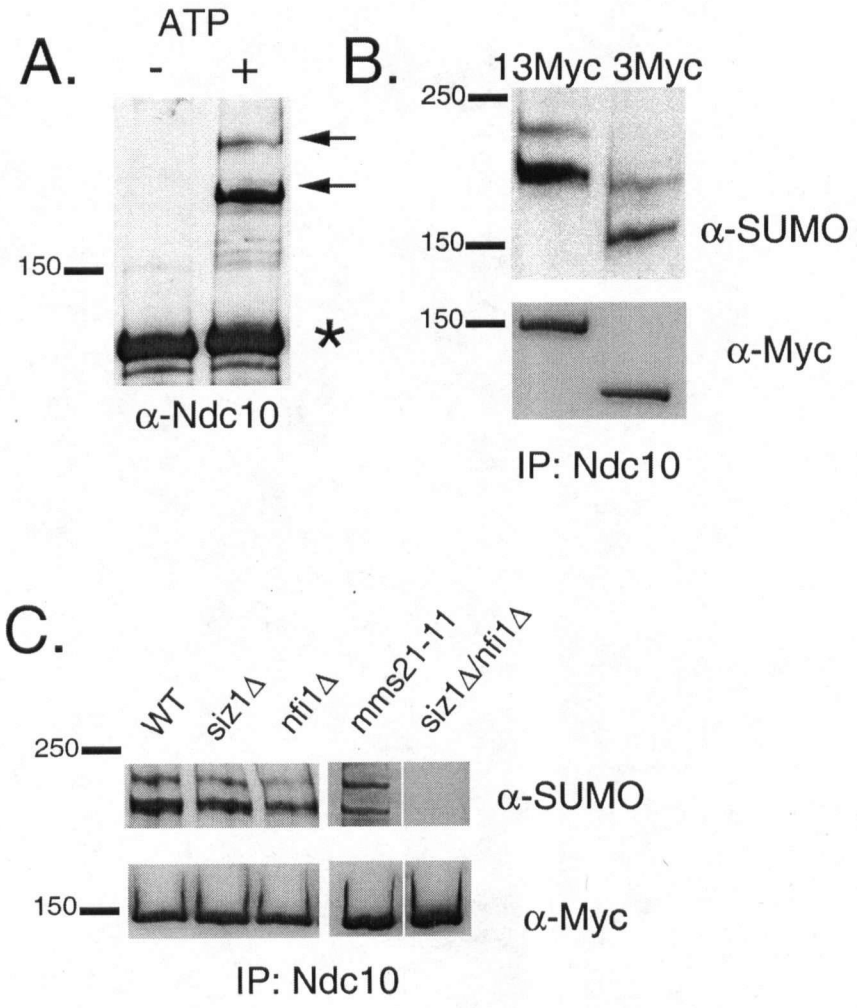


Figure 3-2. Sumoylation of the kinetochore protein Ndc10. (A) Ndc10 is sumoylated *in vitro*. ATP-dependent sumoylation reactions were performed with recombinant proteins purified from *E. coli* without (-) or with (+) ATP. The asterisk and arrows denote unmodified and SUMO modified forms of Ndc10, respectively. (B) Ndc10 is sumoylated *in vivo*. (C) The E3 proteins Siz1 and Nfi1 function in Ndc10 sumoylation.

cannot be ruled out as reasons for the appearance of multiple modified forms of Ndc10 from these data alone. The fraction of SUMO conjugated Ndc10 is estimated to be ~1% or less, which is consistent with the level of modification of most known SUMO substrates.

The addition of SUMO is often facilitated by a protein ligase (E3) (Hay, 2005; Johnson, 2004) and the observed two-hybrid interaction between the E3 protein Nfi1 and Ndc10 suggests that Nfi1 may act as an E3 for Ndc10 sumoylation. Mutation of known E3 proteins (*nfi1Δ* , *siz1Δ,* and *mms21-11*) individually had no effect on Ndc10 SUMO modification levels (Figure 3-2c); however, in the absence of both *NFI1* and *SIZ1,* Ndc10 sumoylation was reduced (Figure 3-2c), indicating a functional redundancy between Nfi1 and Siz1 in targeting Ndc10 for sumoylation. It is worth noting that Ndc10 has also been implicated as a substrate for ubiquitination (Kopski and Huffaker, 1997; Yoon and Carbon, 1995), raising the possibility of alternative regulation through both sumoylation and ubiquitination; however, in preliminary experiments a ubiquitinated form of Ndc10 has not been identified. During the course of this work, Ndc10 was also identified as a sumoylated protein in a proteomic analysis of sumoylated proteins in yeast (Wohlschlegel et al., 2004).

Dynamics of Ndc10 sumoylation

To begin investigating the possible function(s) of Ndc10 sumoylation, the modification state of Ndc10 at discrete arrest points within the mitotic cell cycle was assayed. The mating pheromone alpha factor (αF) and the microtubule-destabilizing drug nocodazole (NZ) were used to arrest cells in G1 and G2/M, respectively. The terminal

arrest of each population after treatment was verified by flow cytometry (data not shown). IP/westerns showed that sumoylation of Ndc10 was decreased to almost undetectable levels in those cells treated with NZ, while it was maintained in αF-treated cells (Figure 3-3a). Loss of sumoylation is not a general consequence of NZ treatment since other sumoylated proteins were not affected (see Ndc80, Figure 3-5a) or exhibit increased levels of sumoylation upon NZ treatment (Johnson and Blobel, 1999). Moreover, the loss of sumoylation is not a result of the G2/M arrest since Ndc10 does not become desumoylated in cells that are arrested in G2/M with temperature-sensitive alleles in the anaphase promoting complex/cyclosome (Figure 3-3a). To further analyze Ndc10 sumoylation during the cell cycle, the modification state of Ndc10 was monitored in a synchronized population of cells as they progressed through mitosis following release from αF arrest (Figure 3-3b). Timing of the progression of cells through the cell cycle was monitored by flow cytometry (Figure 3-3c). The modification state of Ndc10 remained relatively constant throughout the synchronized cell cycle, and in replicate experiments, the fluctuation observed between individual timepoints was not consistent, suggesting that Ndc10 sumoylation does not change dramatically over the cell cycle. These results suggest that the loss of Ndc10 sumoylation in NZ arrested cultures may be a direct consequence of NZ addition relating to checkpoint activation and/or loss of microtubules.

Specific cysteine proteases are responsible for cleaving SUMO from modified substrates and for maturation of the SUMO protein itself (Johnson, 2004). Of the two SUMO proteases in yeast, Ulp2 localizes to the nucleus, placing it in the proper location to mediate the removal of SUMO from Ndc10 during NZ exposure. In *ulp2Δ* cells with

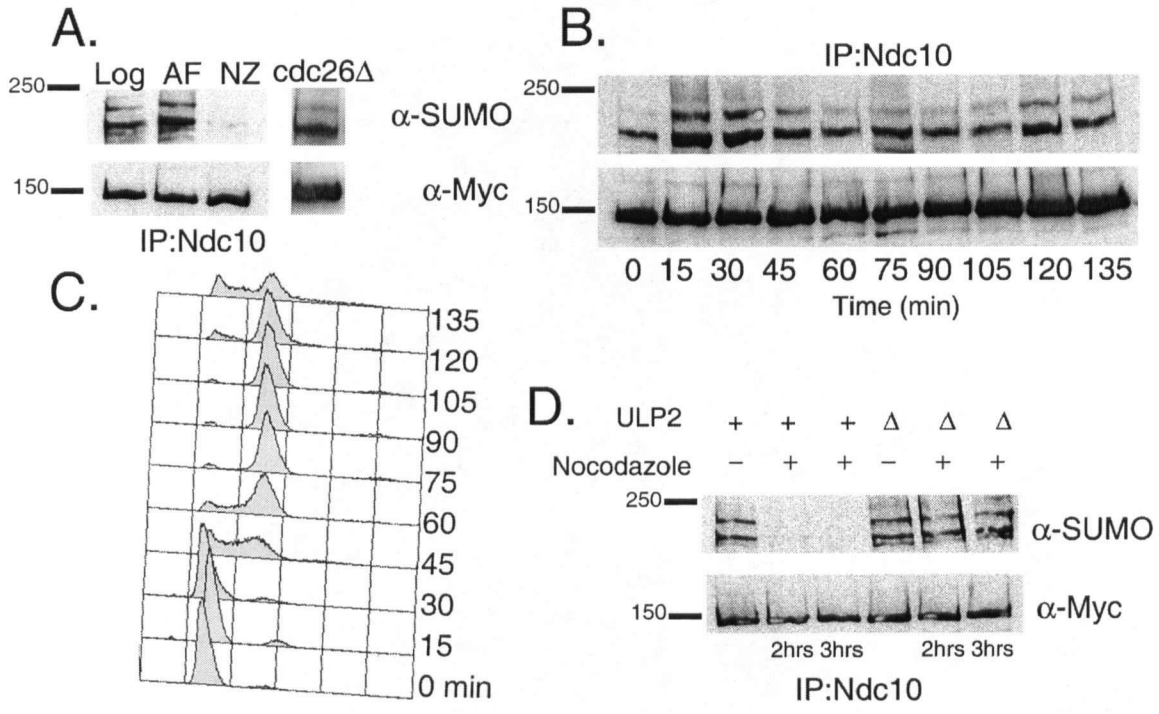


Figure 3-3. Dynamics of Ndc10 sumoylation. (A) Ndc10 sumoylation is reduced in NZ treated cells. IP/western blots were performed on protein extracts from logarithmic, G1 (αF), G2/M (NZ) arrested yeast cell cultures or cdc26Δ culture arrested in G2/M by shift to the non-permissive temperature of 37°C for 3hrs. (B) Ndc10 sumoylation is constant over an unperturbed cell cycle. An αF synchronized population was released into rich media at 30°C and samples for analysis by IP/western were taken every 15 minutes. (C) DNA content was analyzed at each 15 minute interval by flow cytometry to determine cell cycle progression. (D) Loss of sumoylation in NZ requires Ulp2. Cells wild-type (+) or mutant (Δ) for the SUMO protease Ulp2 were assayed for sumoylation after treatment of cells with DMSO (-) or DMSO + NZ (+).

NZ, it was found that the sumoylated forms of Ndc10 were still present (Figure 3-3d); therefore, Ulp2 is required for the loss of Ndc10 sumoylation in response to NZ.

Loss of Ndc10 Sumoylation is linked to checkpoint activity

To distinguish between the possibility that Ndc10 sumoylation is lost during NZ treatment due to checkpoint activation or the loss of microtubules, Ndc10 sumoylation was assayed in two conditions where microtubules are absent, but the spindle checkpoint remains inactivated. The first condition tested for a loss of Ndc10 sumoylation in G1 arrested cells (non-mitotic), and the second tested Ndc10 sumoylation in anaphase arrested cells (checkpoint is silenced through APC/C mediated proteolysis (Palframan et al., 2006)), which are arrested by controlling the expression of CDC5 through the GAL promoter. In both cases, after cells were arrested and treated with NZ there was no observable change in the level of Ndc10 SUMO modification (Figure 3-4a). This demonstrates that in response to nocodazole, Ndc10's SUMO modifications are not lost due to an absence of microtubules, but is likely removed in a checkpoint dependent fashion. To verify this, Ndc10 sumoylation was assayed in a yeast strain that has had the checkpoint abolished through deletion of MAD2. As expected, in the mad2Δ strain, the addition of nocodazole did not result in a loss of sumoylation supporting the conclusion that loss of Ndc10 sumoylation is checkpoint dependent (Figure 3-4b).

Kinetochore proteins Cep3 and Ndc80 are sumoylated

To understand if sumoylation is a common modification on kinetochore proteins, a panel of fourteen proteins comprised of inner (Cep3, Mif2), central (Chl4, Cnn1, Ctf3, Ctf19, Iml3, Ndc80, Nuf2, Spc24) and outer kinetochore components (Dam1, Spc34) were tested for modification by SUMO. The spindle checkpoint protein Bub1 and the

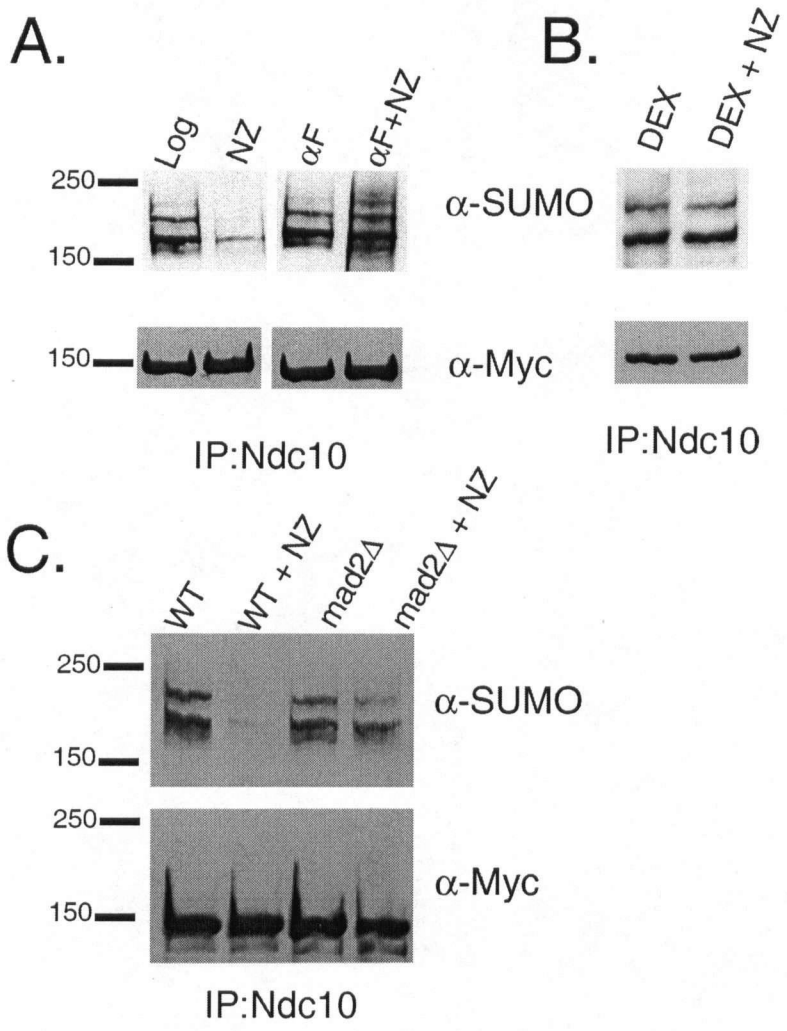


Figure 3-4. Loss of SUMO from Ndc10 in nocodazole treated cells is checkpoint dependent. (A) IP/western blots were performed on protein extracts from logarithmic or G1 arrested cultures with or without the addition of nocodazole (NZ). (B) Cells carrying GAL-3xHA-CDC5 were arrested in anaphase by growth in dextrose containing media to shutoff Cdc5 expression and tested for Ndc10 sumoylation in the absence or presence of NZ. (C) Cells wild-type (WT) or mutant (mad2Δ) for the checkpoint gene MAD2 were assayed for sumoylation after treatment with or without NZ.

kinetochore-associated microtubule-binding protein Bik1were also tested. Of all proteins tested, Cep3 and Ndc80 were found to be sumoylated by IP/western, with the detection of a doublet signal for Cep3, and a ladder of sumoylated forms of Ndc80 (Figure 3-5a). Like Ndc10, the fraction of SUMO conjugated Cep3 is 1% or less, while Ndc80 is modified at slightly higher levels (~1-5%). Ndc80 and Cep3 were also found to be sumoylated throughout the cell cycle, and in replicate experiments the fluctuations observed between individual timepoints was not consistent, suggesting that Ndc80 and Cep3 sumoylation, as observed for Ndc10, is not altered dramatically over the cell cycle (Figure 3-5b).

Ndc80 was recently identified as a sumoylated protein in a proteomic analysis of sumoylated proteins in yeast by mass spectrometry, along with the kinetochore proteins Bir1, Sli15, and Mcm21(Bachant et al., 2002; Denison et al., 2005; Hannich et al., 2005; Hoege et al., 2002; Panse et al., 2004; Stead et al., 2003; Wohlschlegel et al., 2004; Wykoff and O'Shea, 2005; Zhou et al., 2004). Analysis revealed that Cep3, a member of the CBF3 complex with Ndc10 that also localizes to the mitotic spindle (Bouck and Bloom, 2005), showed decreased sumoylation in response to NZ, an effect similar to that seen for Ndc10 (Figure 3-5a). For Ndc80, which does not localize to the mitotic spindle, sumoylation does not change in NZ-treated cells, suggesting that desumoylation during the NZ induced spindle checkpoint arrest (Figure 3-5a) may be associated only with those proteins interacting with the mitotic spindle. Furthermore, the differing response in NZ is indicative of a distinct role for Ndc80 sumoylation relative to that of Ndc10 and Cep3.

Identification of lysine residues required for Ndc10, Ndc80, and Cep3 sumoylation

Sumoylation often occurs on lysine residues found in the consensus motif ΨKxE

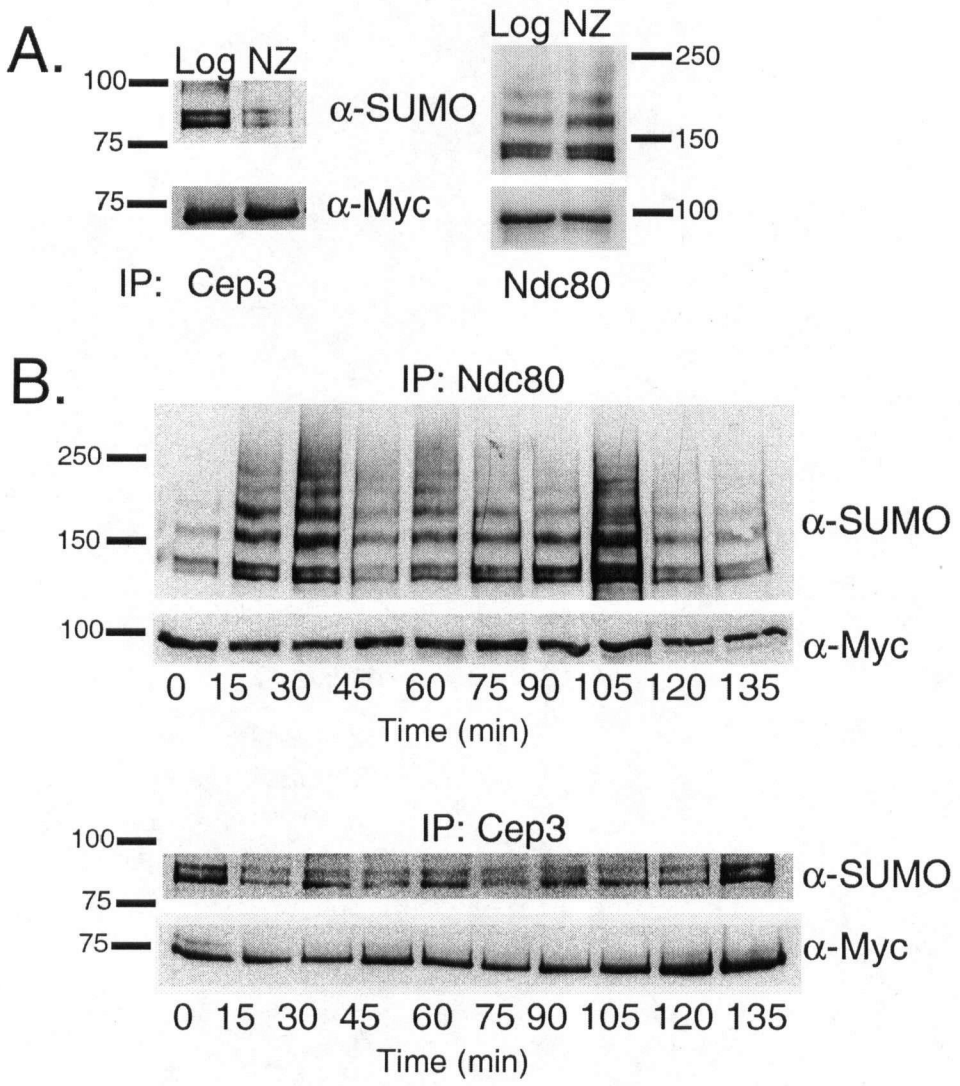


Figure 3-5. Sumoylation of the kinetochore proteins Cep3 and Ndc80. (A) Cep3 and Ndc80 are sumoylated *in vivo*. (B) Ndc80 and Cep3 sumoylation is constant over an unperturbed cell cycle. A αF synchronized population was released into rich media at 30°C and samples for analysis by IP/western were taken every 15 minutes. DNA content was analyzed at each 15 minute interval by flow cytometry to determine cell cycle progression (data not shown; see Figure 3-3c for reference).

where Ψ is any large hydrophobic residue and x is any residue (Johnson, 2004). To identify the possible site(s) of sumoylation in Ndc10, Ndc80, and Cep3, select lysine residues in amino acid sequences that resemble the consensus sequence for sumoylation were mutated. In total, 11, 14, and 6 potential sumoylation sites were mutated in Ndc10, Ndc80, and Cep3, respectively (Table. 3-4). Strains expressing each K→R mutant were assayed for growth at 37°C, chromosome instability (CIN), and changes in sumoylation by IP/western. None of the 31 K→R individual mutations in Ndc10, Cep3 or Ndc80 caused a Ts phenotype or CIN. However, lysine residues were identified that when mutated caused an overall decrease in the SUMO modification state of Ndc10 (K651, 652R and K779R) or abolished one specific SUMO-modified form of Ndc10 (K556R) (Figure 3-6a). Given Ndc10's localization to the mitotic spindle, these three Ndc10 K→R mutants were also assayed for proper sub-cellular localization. In all three cases, the localization of Ndc10 was indistinguishable from that of WT (data not shown).

Of the 14 K→R mutations in Ndc80, one lysine mutation (K231R) abolished the majority of Ndc80 sumoylation (Figure 3-6b), while none of the 6 K→R mutations in Cep3 affected its sumoylation state (data not shown). The complete loss of sumoylation in Ndc80 through a single mutation suggests that K231 may be required for SUMO modification of other lysine residues, which leads to the ladder of modified species, or that this site is poly-sumoylated. To distinguish between these possibilities, Ndc80 sumoylation was checked in a strain that carries a form of SUMO with mutations at positions K11, 15, and 19 that eliminate the formation of poly-sumoylated chains (Bylebyl et al., 2003). In Ndc80 IP/Westerns using this strain, the ladder of modified Ndc80 proteins recognized by the SUMO antibody remained present, indicating that the

TABLE 3-4. Potential sumoylation sites targeted by site directed mutagenesis

Ndc10	Ndc80	Cep3
K102R: E**K**RE	K155R: L**K**QP	K23R: V**K**CD
K240R: L**K**LG	K231R: I**K**LD	K265R: F**K**NF
K243R: G**K**RD	K292R: L**K**LE	K383R: Y**K**VD
K260,261R: E**KK**D	K305R: L**K**LG	K449R: A**K**SE
K410R: F**K**SP	K354R: L**K**SD	K503R: S**K**LD
K421R: A**K**KD	K377R: G**K**LE	K551R: L**K**ND
K556R: Q**K**QE	K382R: M**K**SE	
K606R: E**K**LE	K388R: L**K**EE	
K651,652R: I**KK**E	K448R: R**K**LE	
K695R: F**K**KD	K554R: L**K**HD	
K779R: L**K**RP	K566R: E**K**LE	
	K598R: E**K**ME	
	K627R: L**K**LE	
	K632R: L**K**VD	

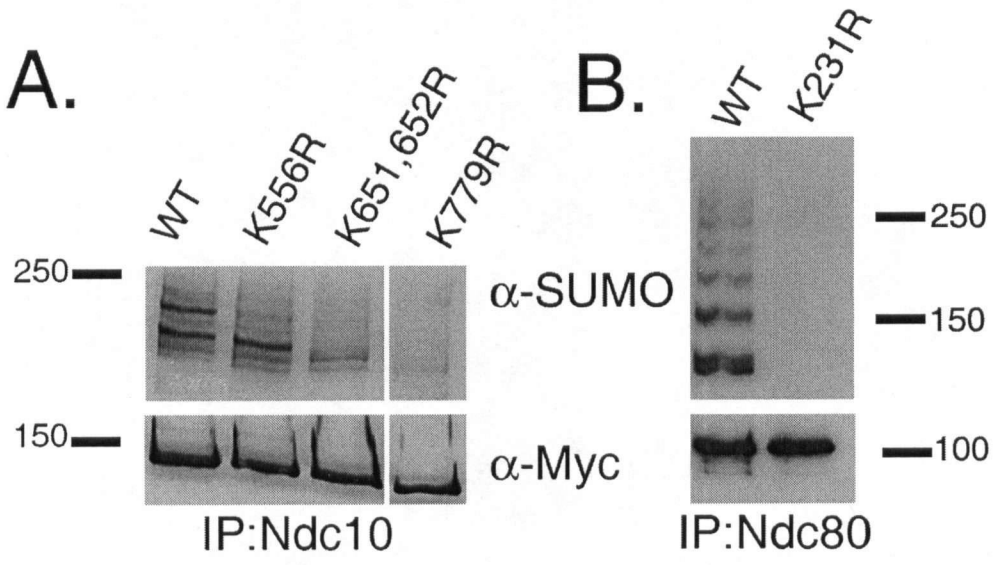


Figure 3-6. Identification of lysine residues in Ndc10 and Ndc80 that affect sumoylation. (A) Residues K556, K651-652, and K779 affect sumoylation of Ndc10 *in vivo*. (B) Residue K231 is required for sumoylation of Ndc80 *in vivo*. Mutations were introduced at the endogenous locus for each mutant strain and protein extracts were generated for assaying sumoylation by IP/western.

Ndc80 K231 site is not poly-sumoylated (data not shown).

Ndc10 mitotic spindle localization is lost in the *ndc10 4xK→R* mutant

Both *in vitro* and *in vivo* Ndc10 sumoylation data showed two modified forms of Ndc10, indicating that Ndc10 may be modified by SUMO on more than one site (Figure 3-2). Moreover, lysine mutations in single SUMO consensus sites within Ndc10 did not completely block sumoylation, suggesting that there is more than one sumoylation site in Ndc10 (Figure 3-6a). If each of these lysine residues represents a potential sumoylation site, then defects caused by loss of one site, may be masked due to sufficient levels of modification at one of the other two sites. To circumvent this problem, all three potential sumoylation site mutations were combined to create Ndc10-4xK→R, which carries four lysine to arginine mutations at residues 556, 651, 652 and 779. By IP/western, Ndc10-4xK→R lacked any detectable levels of sumoylation, indicating that these three sites are required for the majority of Ndc10 sumoylation (Figure 3-7a). Combining the mutations at residues 556, 651, 652 and 779 also caused a dramatic reduction in the amount of Ndc10 localized to the anaphase mitotic spindle both along the length of the spindle and at the spindle mid-zone (Figure 3-7b), which was not due to a change in overall protein levels of Ndc10-4xK→R (Figure 3-7c). Using a *cdc5-10* allele to arrest *ndc10-4xK→R* strains in anaphase with elongated spindles, the localization defects associated with these mutations were quantified and it was found that 57% of cells had undetectable levels of Ndc10-4xK→R on the spindle, while 43% had faint staining that was observable, but well below WT levels (n=200). This result is in contrast to WT cells, which showed robust spindle staining in 88% of cells, and faint staining in only 12% of the population (n=200). Ndc10 may be on the spindle as part of the CBF3 complex since Cep3 was

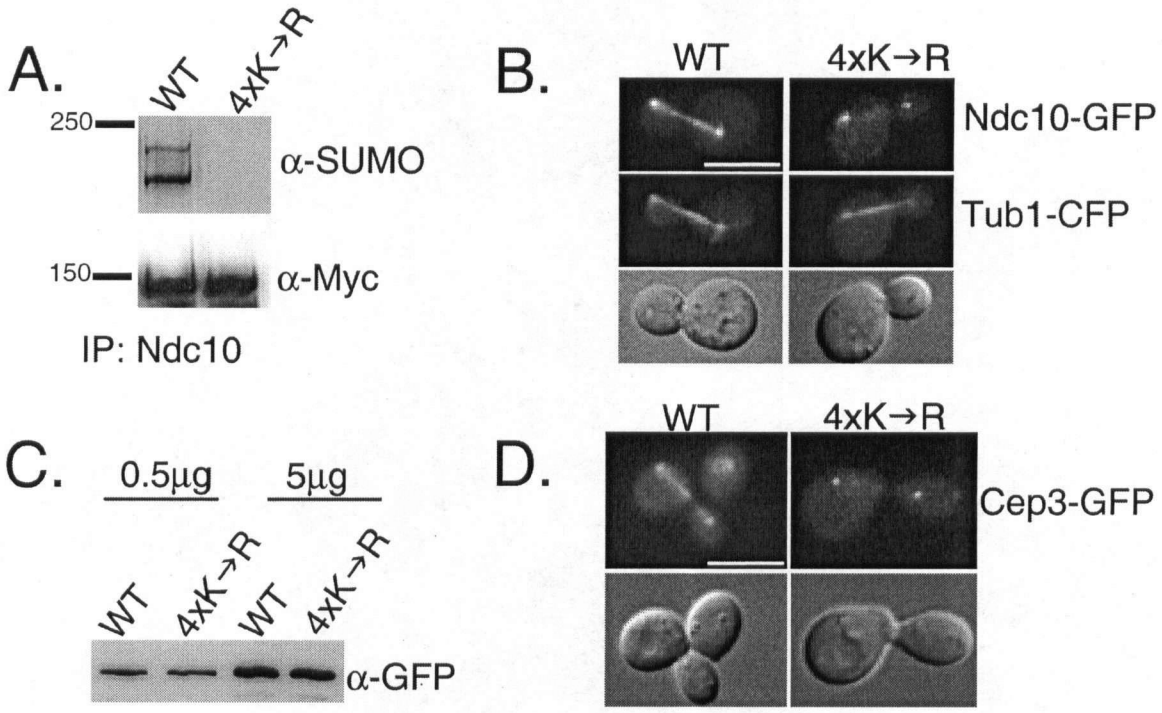


Figure 3-7. Loss of Ndc10 spindle localization. (A) Combining mutations that individually affect Ndc10 sumoylation results in loss of sumoylation *in vivo*. *NDC10* was mutated at the endogenous locus to encode K556R, 651,652,779R (4xK→R). (B) Ndc10 4xK→R mis-localizes from the mitotic spindle. Mutant and wild type protein was tagged with GFP and imaged in live cells. To visualize the mitotic spindle, Tub1-CFP was also introduced into each strain. (C) Ndc10 4xK→R is expressed at normal levels. Whole cell protein extracts were analyzed by Western blot using α-GFP antibodies (Roche). (D) Cep3 mis-localizes from the mitotic spindle in *ndc10 4xK→R* strains. Cep3 was tagged with GFP in a wild-type or *ndc10 4xK→R* strain and imaged in live cells. Scale bar represents 5μm.

recently shown to localize to the mitotic spindle (Bouck and Bloom, 2005). Cep3 was also mis-localized from mitotic spindles in *ndc10-4xK→R* mutants, supporting the idea that Ndc10 and Cep3 are present on the spindle as a complex (Figure 3-7d). The chromosomal passenger protein Sli15 and the microtubule-associated protein Ase1 still localized normally in the *ndc10-4xK→R* mutant (data not shown), as did Bir1 (Figure 3-11a), suggesting that the mis-localization of Ndc10 and Cep3 is not due to a gross defect in spindle structure. *ndc10-4xK→R* strains are also not Ts, and did not show changes in Ndc10's ability to dimerize (Russell et al., 1999), suggesting that these mutations have not altered the structure of the protein (data not shown).

Beyond Ndc10's canonical function at the kinetochore in chromosome segregation, recent reports have described roles for the CBF3 complex and Ndc10 in cytokinesis (Bouck and Bloom, 2005; Gillis et al., 2005). For this reason, *ndc10-4xK→R* mutants were tested for multi-budding, proper septin ring maturation, and for defects in the axial pattern of haploid budding. In these assays, no differences were observed between WT and *ndc10-4xK→R* (Figure 3-8).

K779 is the key sumoylation site

To further investigate the lysine residues being modified by SUMO, the pair wise combinations of lysine to arginine mutations were made in Ndc10 (*ndc10-2xK→R)*, which included K556R with K651/652R, K556R with K779R, and K651/652R with K779R. In each case, only those double mutants containing mutations in site K779R harbored a CTF and Ndc10 mis-localization phenotype (Fig 9). This suggests that the key site for modification is K779, which acts in concert with either of the other two sites to be functional with regards to CTF and spindle localization. However, the observation that

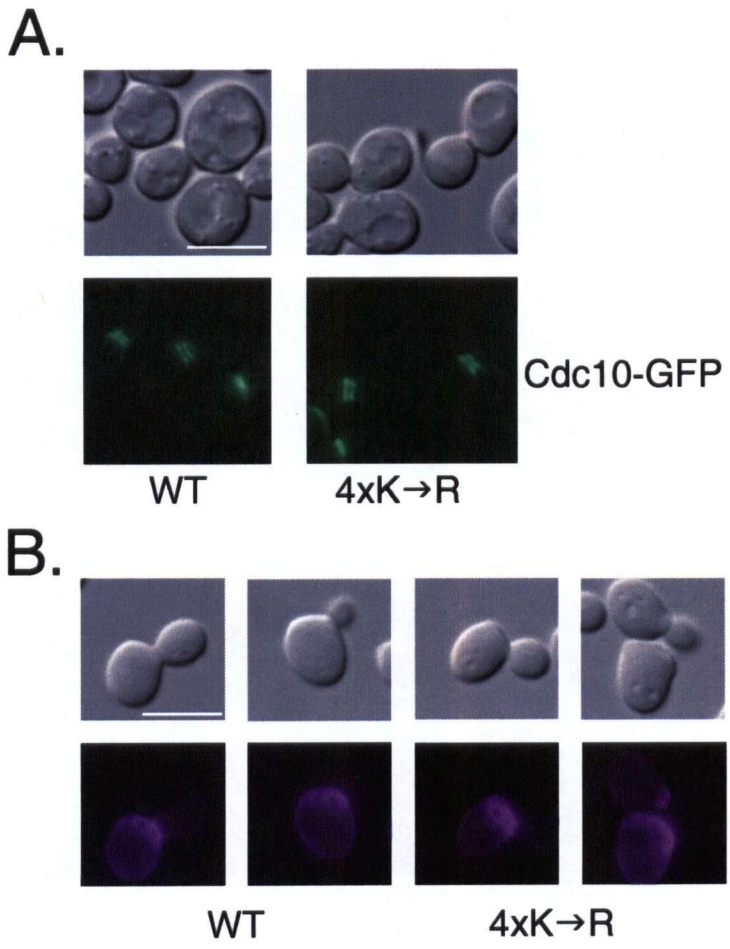


Figure 3-8. *ndc10 4xK→R* strains do not have defects related to cytokinesis. (A) Septin ring formation is normal in *ndc10 4xK→R* strains. Cdc10-GFP was used as a marker for the septin ring in wild type and *ndc10 4xK→R* strains, and unlike *ndc10-1* (Bouck and Bloom, 2005), septin rings mature and split normally. (B) Haploid axial bud pattern is unaffected in *ndc10 4xK→R* mutants. Wild type and *ndc10 4xK→R* strains were grown at 30°C and fixed. Bud scars were stained with calcofluor white. Scale bar represents 5μm.

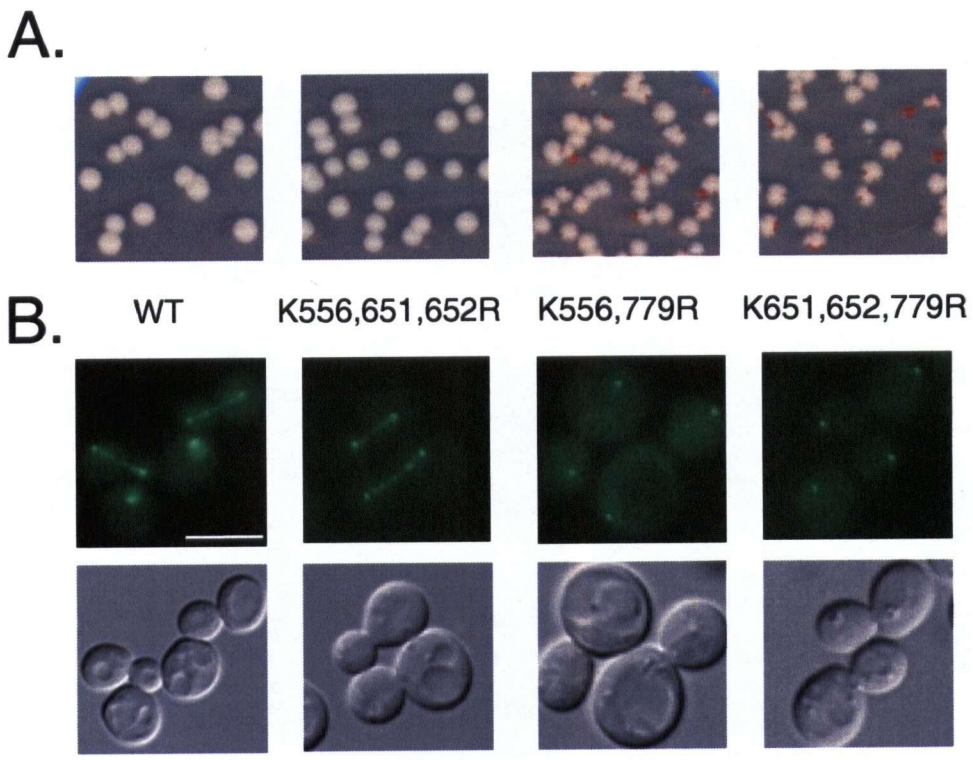


Figure 3-9. K779 is the key residue affecting chromosome segregation and spindle localization. (A) *ndc10* strains lose an artificial chromosome fragment at elevated rates when K779 is mutated in combination with one of the other modification sites. Formation of a colored sector indicates loss of the chromosome fragment. (B) Combining mutations with K779 also cause Ndc10 to mis-localizes from the mitotic spindle. Mutant and wild type protein was tagged with GFP and imaged in live cells. Scale bar represents 5μm.

the single K779R mutant does not have a CTF or localization defect suggests that when both K556 and K651/652 are present they can compensate for the loss of the K779 site with regards to these two functions.

***ndc10-4xK→R* mutants display chromosome instability**

In addition to the spindle localization defects, *ndc10-4xK→R* strains showed increased CIN in a color sector assay (Figure 3-10a). When quantified by half-sector analysis (Hyland et al., 1999; Koshland and Hieter, 1987), *ndc10-4xK→R* strains had rates of chromosome loss and chromosome nondisjunction 160X and 60X greater, respectively, than WT. In comparison, loss of checkpoint function in *bub1Δ* or *bub3Δ* strains causes a 50X increase in the rate of chromosome loss (Warren et al., 2002). These strains also lose endogenous chromosomes at an increased rate as determined by a diploid bi-mating assay (Spencer et al., 1990). In this assay, *ndc10-4xK→R* homozygous diploid strains formed mating colonies at a rate 10X that of WT diploids, presumably due to the loss of chromosome III (2N-1) (Figure 3-10b). Although *ndc10-4xK→R* strains showed increased rates of CIN, there is no observable delay in cell cycle progression of a αF-synchronized cell culture (Figure 3-10c). *ndc10-4xK→R* strains are also G2/M checkpoint proficient in both their ability to arrest and recovery from NZ exposure (data not shown). These results suggest that the defect causing chromosome missegregation is not eliciting a checkpoint response and frequent repair, but is more likely to be either a rare event that always leads to failure, or an event that does not trigger a G2/M checkpoint response at all.

***ndc10-4xK→R* mutants have mitotic spindle defects**

A common phenotype associated with mutations affecting kinetochore and/or

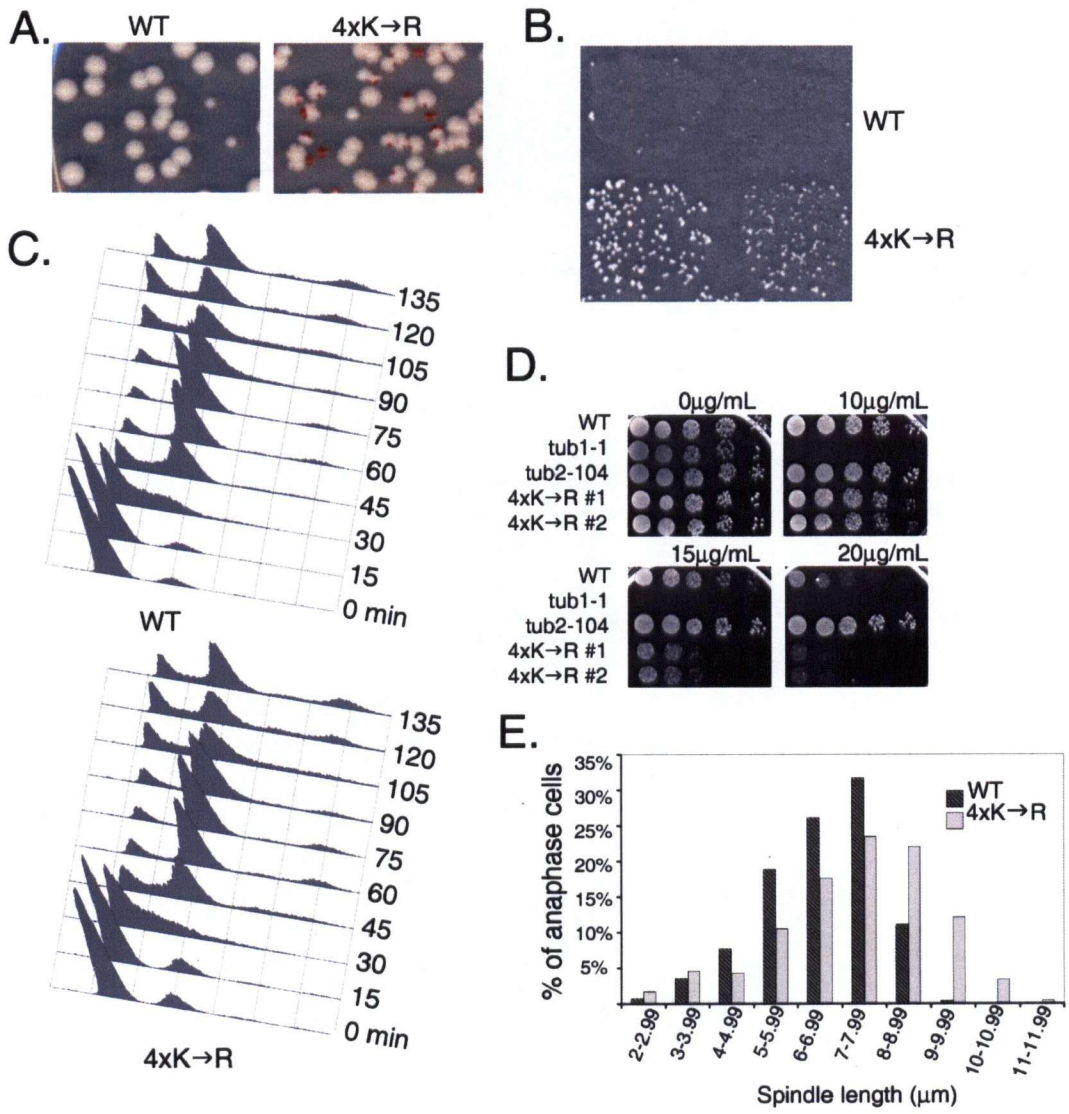


Figure 3-10. *ndc10 4xK→ R* strains have increased rates of chromosome mis-segregation and mitotic spindle defects. (A) *ndc10 4xK→ R* strains lose an artificial chromosome fragment at elevated rates. Formation of a colored sector indicates loss of the chromosome fragment. (B) *ndc10 4xK→ R* strains lose endogenous chromosomes. Independent isolates of wild type and *ndc10 4xK→ R* diploid strains were mated with haploid tester strains and mating products were selected. (C) Cell cycle progression is not delayed in ndc10 *4xK→ R* strains. Wild type and *ndc10 4xK→ R* strains were arrested in G1 with αF, and then released. DNA content was analyzed by flow cytometry to determine cell cycle progression. (D) *ndc10 4xK→ R* strains are benomyl sensitive. Serial dilutions of each strain were spotted plates containing 0, 10, 15, or 20μg/mL of benomyl and incubated at 30°C for 2 days. Benomyl hypersensitive *tub1-1* and resistant strain *tub2-104* are included as controls. (E) *ndc10 4xK→ R* strains have mitotic spindles of abnormal length. Anaphase mitotic spindle lengths were measured in asynchronous cultures of wild type and *ndc10 4xK→ R* strains containing Tub1-CFP.

spindle function is increased sensitivity to the microtubule-destabilizing drug benomyl. *ndc10-4xK→R* strains displayed benomyl sensitivity (Figure 3-10d), which taken together with the mitotic spindle mis-localization phenotype raise the possibility that the loss of Ndc10 sumoylation may be causing specific defects in spindle function. To investigate this possibility, the lengths of mitotic spindles in anaphase stage cells of an asynchronous culture were measured. In the *ndc10-4xK→R* mutant, anaphase spindles were of abnormal length, with spindles averaging 7.30 μm± 0.08 (n=483) compared to 6.62 μm ± 0.06 (n=443) for WT cells. In comparing the distribution of spindle lengths graphically, it was noted that the *ndc10-4xK→R* mutants had abnormally long anaphase spindles, ranging up to 10-12μm, lengths that are never seen in WT cells (Figure 3-10e). The observed spindle defect and CIN, which is associated with no observable G2/M delay, suggests that the defect(s) caused by loss of Ndc10 sumoylation is related to events in anaphase after the G2/M checkpoint has been silenced (e.g. spindle elongation).

Bir1 and Ndc10-4xK→R physically interact

In anaphase, Ndc10 and Bir1 co-localize on the mitotic spindle in a Bir1-dependent manner, suggesting that Ndc10 and Bir1 are part of a complex on the mitotic spindle (Bouck and Bloom, 2005; Widlund et al., 2006). In the *ndc10-4xK→R* strain, Ndc10 no longer localized to the mitotic spindle (Figure 3-7b), whereas Bir1 remained spindle-bound (Figure 3-11a). Therefore, the spindle length defect and mis-localization of Ndc10 may be due to a disruption of the Ndc10-Bir1 protein interaction. However, co-IP's demonstrated that the Ndc10-4xK→R interaction with Bir1 was comparable to that seen in WT cells (Figure 3-11b). The interaction between Ndc10 and Bir1 was not disrupted in cells arrested with αF or NZ, demonstrating that this interaction is not

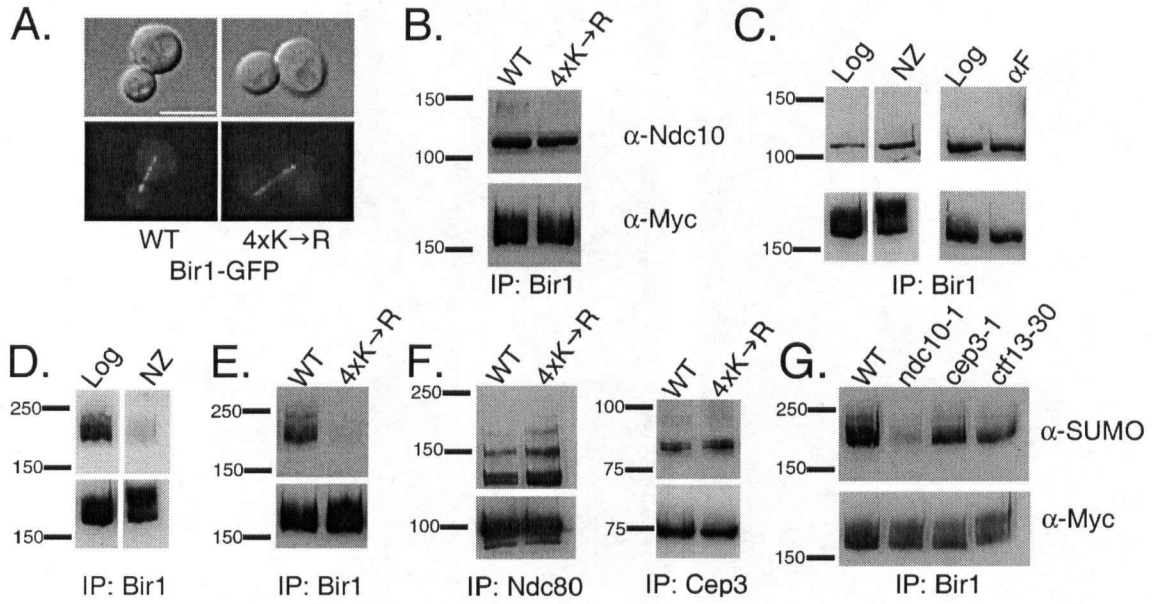


Figure 3-11. Bir1 is sumoylated in an Ndc10 dependent manner independent of CBF3 function. (A) Bir1-GFP remains spindle localized in *ndc10 4xK→R* anaphase cells. *BIR1* was tagged with GFP in a WT and *ndc10 4xK→R* strain and imaged in live cells. For panels B-E & G, Bir1was immunoprecipitated with α-Myc conjugated beads and used for Western blot analysis with α-Myc , α-Ndc10, or α-SUMO antibodies as indicated from wild type or *ndc10 4xK→R* strains. (B) Ndc10 and Bir1 interact *in vivo* independently of Ndc10 sumoylation. (C) The interaction between Ndc10 and Bir1 does not require microtubules (NZ) nor is it specific to mitotic cells (αF). (D) Bir1 is sumoylated *in vivo*, but reduced in response to NZ treatment. (E) Bir1 sumoylation is reduced in an *ndc10 4xK→R* strain. (F) Cep3 and Ndc80 sumoylation is unchanged in the *ndc10 4xK→R* mutant strain. (G) Bir1 sumoylation is not affected by mutations in CBF3 components other than Ndc10. Protein extracts from WT, *ndc10-1*, *cep3-1*, and *ctf13-30* were made after 3hrs at 37°C. Scale bar represents 5μm.

specific to mitosis and is not microtubule-dependent (Figure 3-11c). These results suggest that the observed interaction between Ndc10 and Bir1 occurs independently of the mitotic spindle, and that the spindle length defect in the *ndc10-4xK→R* strain is not due to the failure of Ndc10-4xK→R to interact with Bir1.

Bir1 is sumoylated in an Ndc10-dependent manner

Bir1 is a phosphorylated protein that was also recently identified as a sumoylated protein in proteomic studies (Widlund et al., 2006; Wohlschlegel et al., 2004; Zhou et al., 2004). By IP/western, it was confirmed that Bir1 is sumoylated, with an estimated 1% of Bir1 being SUMO conjugated in an asynchronous culture, and as seen for Ndc10, treatment of cells with NZ resulted in loss of Bir1 sumoylation (Figure 3-11d). Both the phosphorylation and sumoylation state of Bir1 were checked in the *ndc10-4xK→R* mutant strain, and westerns show that the overall appearance of Bir1 phosphorylation (as indicated by the diffuse western signal representing phosphorylated forms of Bir1) is unchanged in the mutant as compared to WT (Figure 3-11e, αMYC blot), but that sumoylation of Bir1 is reduced in the *ndc10-4xK→R* mutant to undetectable levels (Fig 7e, αSUMO blot). Cep3 and Ndc80 were also tested for changes in sumoylation state in the *ndc10-4xK→R* mutant (Figure 3-11f), but no significant differences were found compared to WT; thus, the loss of sumoylation in the *ndc10-4xK→R* mutant appears to be specific to the modification of Bir1. These data suggest that sumoylation of Ndc10 is required for Bir1 sumoylation.

Bir1 sumoylation is independent of CBF3 function

Ndc10's effect on Bir1 sumoylation may be dependent on the activity of the CBF3 complex or may occur via a CBF3-independent mechanism. To distinguish these

two possibilities, Bir1 sumoylation in strains carrying Ts mutations in the CBF3 component Cep3 (*cep3-1*) or Ctf13 (*ctf13-30*) were tested. In both cases, when cells were arrested at the non-permissive temperature, Bir1 sumoylation was detected; however, loss of sumoylation was observed when the *ndc10-1* Ts allele was used to abrogate Ndc10's function. This result indicates that Ndc10's effect on Bir1 sumoylation is not due to altered CBF3 activity (Figure 3-11g).

Discussion

These data demonstrate that the kinetochore proteins Ndc10, Cep3, Ndc80, and Bir1 are substrates for sumoylation. In the case of Ndc10, the effect of lysine mutations on Ndc10's localization demonstrates that sumoylation demarcates a specific subset of Ndc10 that associate with the mitotic spindle. This demarcation most likely occurs through an alteration of Ndc10's binding interactions with microtubules or microtubule-associated proteins, leading to the establishment or maintenance of spindle localization. This data also assigns a function to the fraction of Ndc10 protein on the mitotic spindle in controlling mitotic spindle dynamics.

Ndc10 contribution to spindle stability was previously demonstrated using the Ts allele ndc10-1 (Bouck and Bloom, 2005). At the non-permissive temperature the ndc10-1 protein becomes unstable resulting in protein degradation and loss of both kinetochore function and spindle checkpoint function (Fraschini et al., 2001; Gardner et al., 2001; Goh and Kilmartin, 1993). Interestingly, the phenotype observed using the ndc10-1 strain was an enrichment of cells with 2-4μm spindles that cycled through states of elongation, catastrophe, and rescue. This was attributed to Ndc10 having a role in promoting spindle stability during anaphase spindle elongation. However, this work demonstrated that when

Ndc10 mis-localizes specifically from the mitotic spindle the result was abnormally long spindles, which suggests Ndc10 has a role in destabilizing the mitotic spindle. These contradictory results, can be explained by the loss of kinetochore function in the ndc10-1 allele, since the kinetochore is known to have a stabilizing effect on microtubule dynamics (McIntosh et al., 2002). Moreover, Ndc10's localization to the mitotic spindle has been linked to a role in regulating cell separation during cytokinesis, using the ndc10-1 allele, strains at the non-permissive temperature were shown to form multi-cell clusters, and aberrant septin ring formations (Bouck and Bloom, 2005). In contrast, the *ndc10 4xK→R* strain shows no such defects, suggesting that the pleiotropic phenotypes observed in the ndc10-1 strain are likely due complete loss of Ndc10 protein in the cell and not just spindle localization. This interpretation of the data is support by recent work on Bir1, which when mutated causes mis-localization of Ndc10 from the mitotic spindle, but no defects observed in cytokinesis (Widlund et al., 2006). Overall, this supports a role for Ndc10 on the mitotic spindle in controlling spindle dynamics and suggests that there is no role for spindle bound Ndc10 in cytokinesis. The recent discovery of a checkpoint termed, NoCut, which halts cytokinesis in the presence of spindle damage supports this conclusion. The authors of this study found that NoCut was activated in response to spindle damage and when abolished, allows *ndc10-1* cells to complete cytokinesis (Norden et al., 2006). This suggests that the cytokinesis defects in *ndc10-1* cells are not due to a requirement of Ndc10 in cytokinesis, but due to the spindle damage that is present in this mutant causing activation of the NoCut checkpoint.

A number of other kinetochore proteins also localize to the spindle during anaphase including Bir1, Cep3, Cin8, Dam1, Duo1, Ipl1, Sli15, Slk19, and Stu2, most of

which have also been shown to play a role in controlling spindle dynamics (Biggins et al., 1999; Bouck and Bloom, 2005; Hofmann et al., 1998; Hoyt et al., 1992; Jones et al., 1999; Kosco et al., 2001; Widlund et al., 2006; Zeng et al., 1999). The localization of Ipl1, Sli15, and Ndc10 to the spindle requires the function of the phosphatase Cdc14 that is activated in anaphase to regulate mitotic exit (Bouck and Bloom, 2005; Pereira and Schiebel, 2003; Stegmeier and Amon, 2004). Moreover, Ndc10, Bir1, Cep3, and Sli15 are SUMO substrates (this work, (Wohlschlegel et al., 2004)) indicating that these spindle associated kinetochore proteins may be regulated via common mechanisms to control mitotic spindle dynamics during anaphase. Regulation of spindle dynamics likely includes delivery of these kinetochore proteins to the spindle mid-zone, which may be used as a signal to co-ordinate the collapse of the mitotic spindle and to initiate other late stage mitotic events once chromosome segregation has occurred. Given *ndc10-4xK→R's* long spindle phenotype, it is possible that the signal to commence spindle disassembly is delayed due to the absence of Ndc10 on the mitotic spindle, providing time for additional spindle elongation. It is currently unknown if the altered spindle dynamics are also responsible for the increase in CIN observed in *ndc10-4xK→R* mutants, but the lack of an observable cell cycle delay is suggestive of events that are invisible to the mitotic checkpoint machinery or occur during stages of the cell cycle when the checkpoint has already been satisfied (e.g. anaphase). Intriguingly, Ndc10 also localizes to MT's in telophase and into G_1 of the next cell cycle raising the possibility that MT associated Ndc10 may be required in non-mitotic stages of the cell cycle (Bouck and Bloom, 2005), which may include S phase when CEN DNA is replicated and kinetochore microtubule attachments are being established.

Ndc10's localization to the mitotic spindle is dependent on sumoylation and Bir1 (this study; (Bouck and Bloom, 2005; Widlund et al., 2006)). However, this work demonstrates that the interaction between Ndc10 and Bir1 occurs in non-mitotic cells and is independent of microtubules, Ndc10 spindle localization, and sumoylation of Ndc10 or Bir1. How then does Bir1 facilitate Ndc10's spindle association if not by physically interacting with Ndc10 on the mitotic spindle? Based on localization data, the other structure at which these two proteins co-localize is the kinetochore (Widlund et al., 2006). At the kinetochore, Bir1 could direct Ndc10 to microtubules in a manner dependent on the function of the Bir1-Sli15-Ipl1 kinase complex. In support of this possibility, Ndc10 has a weak two-hybrid interaction with Sli15 (Table 3-3). Moreover, Ndc10 has been shown to be an Ipl1 substrate for phosphorylation *in vitro*, and *ipl1* mutants, like *ndc10-4xK→R* mutants, contain spindles of abnormal length (Biggins et al., 1999; Buvelot et al., 2003). In experiments that use the *ipl1-321* temperature sensitive mutant, Ndc10 shows only slightly lower levels of mitotic spindle localization (Bouck and Bloom, 2005). It is known, however, that at the restrictive temperature, *ipl1-321* retains a low level of kinase activity, and a phosphorylation-dependent mechanism is therefore still possible (Pinsky et al., 2006).

In contrast to Ndc10, mutations that abolish Bir1 sumoylation (Figure 3-12a) do not result in a loss of Bir1 mitotic spindle localization (Widlund et al., 2006), implying that Bir1's association with the mitotic spindle does not rely on sumoylation. A clue to the function of Bir1's sumoylation may come from recent work on the mammalian homolog of Bir1, Survivin. In mammals, modification of Bir1/Survivin with ubiquitin appears to regulate dynamic protein-protein interactions important for chromosome

segregation at the centromere (Vong et al., 2005). Intriguingly, these modifications map to the IAP repeat region of mammalian Birl/Survivin (Vong et al., 2005), deletion of which in yeast Birl results in a loss of sumoylation (Figure 3-12a). Thus, the IAP repeat domain may be used to regulate both yeast and mammalian Birl/Survivin's function by lysine-directed modifications.

Once Ndc10 and Birl are on the mitotic spindle, it appears that these two proteins function to regulate spindle dynamics in an opposing manner given that the spindle length defect observed in *ndc10-4XK→R* mutants is in contrast to that seen in Birl mutants where spindles fail to fully elongate and are shorter than WT on average (this study; (Widlund et al., 2006)). The interplay between these two proteins is further illustrated by the fact that in the *ndc10-4xK→R* mutant, Birl sumoylation is undetectable, even though the Ndc10 mutant protein and Birl still interact normally. This dependence is not reciprocal in that Ndc10 sumoylation is not affected by mutations that block Birl's sumoylation or that disrupt the interaction between Birl and Ndc10 (Figure 3-12b). The reliance on Ndc10 being competent for sumoylation suggests that Ndc10 requires prior modification with SUMO before sumoylation of Birl can occur, and is indicative of a cascade of SUMO modification events where Ndc10 functions in *trans* to facilitate modification of Birl by SUMO.

Although Ndc10 is not conserved in higher eukaryotes, Birl and Ndc80 are well conserved. An important question is whether sumoylation of kinetochore proteins including Birl and Ndc80 function in an analogous manner in these evolutionarily diverse organisms. While neither Birl nor Ndc80 homologues have been shown to be sumoylated in higher eukaryotes, there is evidence linking sumoylation to kinetochore

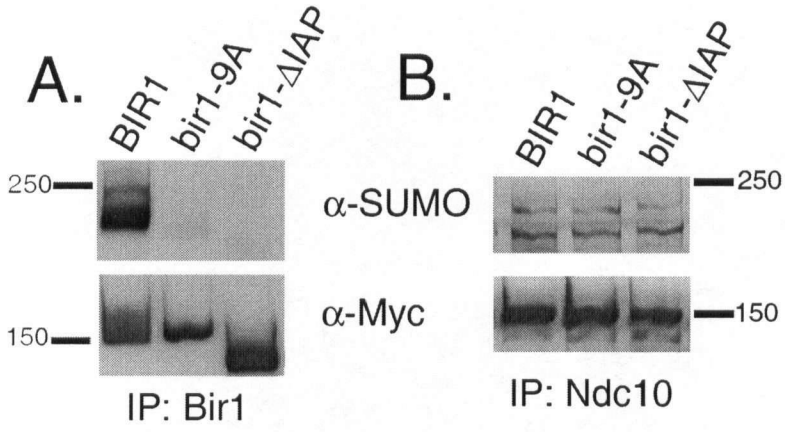


Figure 3-12. Bir1 phosphorylation and the IAP domain are required for Bir1 sumoylation. (A) Bir1 sumoylation is undetectable in the *bir1-9A* and *bir1-ΔIAP* mutants. Bir1-ΔIAP (lacks the inhibitor of apoptosis repeat) interacts with Ndc10 and the mitotic spindle while Bir1-9XA (lacking nine potential phosphorylation sites) does not interact with Ndc10 but remains spindle bound (Widlund et al., 2006). (B) Ndc10 sumoylation is not dependent on Bir1 sumoylation or a physical interaction with Bir1.

and mitotic spindle functions. For example, SUMO-2 modified proteins are enriched at the inner centromere of chromatids in *Xenopus* egg extracts, and alteration of SUMO modification of chromosomal substrates by SUMO-2 causes a block in the segregation of sister chromatids in anaphase (Azuma et al., 2005; Azuma et al., 2003). Moreover, RanGap1, the first identified sumoylated substrate, is targeted to mitotic spindles and kinetochores, and like Ndc10, mutations that abolish sumoylation lead to RanGAP1 mis-localization from the spindle (Joseph et al., 2004; Joseph et al., 2002; Mahajan et al., 1997; Matunis et al., 1996; Saitoh et al., 1997). Complexed with RanGAP1 is the SUMO E3 ligase RanBP2, which when depleted in mitotic cells results in mis-localization of RanGAP1, the spindle checkpoint proteins Mad1 and Mad2, and the kinetochore proteins CENP-E and CENP-F (Joseph et al., 2004). The phenotypic consequences of these effects include the accumulation of mitotic cells with multipolar spindles and unaligned chromosomes. The localization of RanBP2 to kinetochores places an E3 protein near the kinetochore, highlighting the possibility that there may be many proteins of the kinetochore targeted for sumoylation.

The number of sumoylated substrates in yeast and higher eukaryotes continues to grow rapidly, but the biological functions of the majority of these modifications remains elusive. The data presented here provides evidence for the regulation of chromosomal passenger proteins and kinetochore proteins by SUMO modification including the localization of Ndc10 to the mitotic spindle during anaphase. Findings in higher eukaryotes provide evidence that the mechanism for localizing proteins to microtubules via sumoylation may be conserved (e.g. RanGAP1). Overall, regulation of the kinetochore is still not well defined, and sumoylation of kinetochore proteins could

represent a novel mode of regulation that may be related to cell cycle checkpoint function. Recent publications provide evidence for sumoylation at mammalian kinetochores (Joseph et al. 2004, Chung et al. 2004) suggesting evolutionary conservation of this modification and possible mechanisms of action.

Chapter 4:

Future Directions

Future directions in the field of kinetochore research

In the past two decades the progress made in the field of chromosome segregation and kinetochore function has been astounding. This is highlighted by the discovery of more than 65 proteins that function at the budding yeast kinetochore alone (McAinsh et al., 2003). Despite these advances, for the majority of these proteins little more is known than the fact that these proteins localize at the kinetochore and are required for chromosome segregation. This presents a challenge to the kinetochore field in the coming years, which will be to shift from simple identification to thorough characterization of the biochemical activities and mechanisms of action of the large number of proteins comprising the kinetochore. These studies will be aimed at addressing a number of major questions still left to answer including: 1.) How does a kinetochore physically attach to a microtubule? 2.) How does the attachment of kinetochores to microtubules allow for the generation of a motive force to move sister chromatids during anaphase? 3.) How is the mechanical signal of attachment and tension converted into a biochemical signal that is processed by the spindle assembly checkpoint? 4.) What are the underlying processes that regulate the formation and function of a kinetochore? To answer these questions the field will likely have to move in the direction of developing an *in vitro* system of kinetochore assembly and function, coupled with solving the structure of many of these proteins and characterizing the structural characteristics of the complexes they reside in.

Beyond understanding how a kinetochore is built and regulated as an isolated biochemical system, the kinetochore field will also be challenged with understanding how other cellular processes impact on the function of this supermolecular complex. For example, a critical role for chaperones (proteins that prevent non-specific aggregation of

nascent polypeptides and promote their correct folding) was recently demonstrated for the activation of the CBF3 complex components Skp1 and Ctf13, which is required for proper CBF3-*CEN* complex formation (Stemmann et al., 2002). Tubulin chaperones also show genetic interactions with many of the non-essential kinetochore components (Tong et al., 2004), and several genes involved in tubulin folding in yeast were first identified in a screen for mutants that displayed a CIN phenotype (Hoyt et al., 1997; Hoyt et al., 1990; Stearns et al., 1990). As discussed in Chapter 2, the kinetochore also interacts with components of the nuclear envelope. Examples include spindle assembly checkpoint proteins that bind to the nuclear pore, where they are sequestered until activation of checkpoint function (Campbell et al., 2001; Iouk et al., 2002), and nuclear pore components that reloclalize to the kinetochore and SPB in mitosis (Salina et al., 2003). Furthermore, mutants in a component of the Mtw1 complex, Nnf1, was originally described to have phenotypes similar to nuclear pore and nucleocytoplasmic transport mutants suggesting there are shared subunits and/or functions between these complexes (Shan et al., 1997).

These examples highlight the highly complex and integrated pathways that function to control chromosome segregation. While a good start has been made in identifying the building blocks of a kinetochore, a mechanistic and biochemical understanding of this process will require much more work. In relation to the work presented in this thesis, future research directions are discussed below.

Spc24, Spc34 and Apq12 in chromosome segregation

The work presented in Chapter 2 provides the groundwork for a variety of future research opportunities. First, the analysis of Ts alleles of both Spc24 and Spc34 that

differ in their terminal arrest phenotypes suggests that these two proteins are multifunctional and provide hints as to the location of specific functional domains in each protein. For instance, *spc24-9*, which carries a mutation in the C-terminus of Spc24, was SS or SL with the *chl4*, *ctf3*, *ctf19*, *iml3* and *mcm21* central kinetochore mutants. *spc24-8* and *spc24-10*, which carry mutations in the N-terminal region of Spc24 that contains two coiled-coil domains, did not display genetic interactions with central kinetochore mutants. *spc24-9* mutants also have a much higher rate of chromosome loss than *spc24-8* and *spc24-10* mutants. Thus, it is likely that the C-terminal mutation in *spc24-9* affects a different Spc24 function or protein-protein interaction than the *spc24-8* and *spc24-10* mutants. These data are consistent with a recently published structural analysis of the Ndc80 complex which demonstrates that the C-terminus of Spc24 is a globular domain that likely interacts with the kinetochore (Wei et al., 2005). In the case of Spc34, although *spc34-6* and *spc34-7* have a common amino acid mutation (S18P) they do not display similar genetic interactions. This may be due to the K198E mutation present in *spc34-6*, which is directly adjacent to an Ipl1 phosphorylation site (T199) (Cheeseman et al., 2002a). It will be of interest to investigate this site further for possible phosphorylation events given that the Dam1 complex is a critical target of the Ipl1 kinase in regulating kinetochore microtubule attachments, of which Spc34 is a component (Cheeseman et al., 2002a). Therefore, future work focused on determining the alterations caused by each individual mutation on protein-protein interactions and post-translational modifications of Spc24 and Spc34 could be very informative, especially in light of the recent structural information published for both the Ndc80 complex and Dam1 complex (Miranda et al., 2005; Wei et al., 2005; Westermann et al., 2005).

A second opportunity would involve the characterization of Apq12's role in cell cycle control and CIN, since it is unclear how the deletion of APQ12 affects these processes. Deciphering the role of a protein in a given process is often aided by determining its physical interaction partners, but in the case of Apq12, very little is known. Therefore, a first step in the characterization of Apq12 could involve a mass spectrometry or two-hybrid based screen for binding partners. Because Apq12 is a nuclear envelope protein and contains a transmembrane domain, it may be beneficial to perform these screens with the N- and C-terminal domains as well as full length protein. Expressing different domains of Apq12 to attempt rescue of particular defects in a null mutant could also be used to map function onto different domains of the protein. This is important because of the possibility that the observed phenotypes in the apq12 deletion are a secondary consequence of wide scale perturbation of mRNA export. It may also be interesting to use the benomyl resistance phenotype of apq12Δ strains as a basis for a suppressor screen to define other processes that work in concert with Apq12.

Finally, the SL screens using Spc24 and Spc34 identified many genetic interactions that were not investigated, but offer interesting possibilities for further investigation. This includes the SL interactions observed between two negative regulators of the cAMP pathway, *ira2* and *pde2,* in the *spc24-9* screen. *PDE2* was also recently identified as a high copy suppressor of Dam1 kinetochore complex mutants (Li et al., 2005), and five negative regulators of the cAMP pathway, including *ira2* and *pde2*, were also identified as benomyl sensitive mutants in genome-wide screens (Pan et al., 2004). Therefore, upregulation of the cAMP pathway by mutation of its negative regulators

appears to have a deleterious effect on kinetochore function, and may hint at a novel mode of regulation that involves cAMP signaling.

Sumoylation: Future bouts with chromosome segregation

The original identification of SUMO in budding yeast was through a high copy suppressor screen of a mutation in the kinetochore protein Mif2 (Meluh and Koshland, 1995). Since then, SUMO and the process of sumoylation has been linked to chromosome segregation in numerous ways. This includes the observation that sumoylation is required for passage through mitosis, and more specifically that the SUMO machinery is required for checkpoint function, sister chromatid cohesion and chromosome stability (Bachant et al., 2002; Li and Hochstrasser, 1999; Li and Hochstrasser, 2000; Seufert et al., 1995; Stead et al., 2003). The work presented in Chapter 3 adds to these findings by demonstrating that the kinetochore proteins Ndc10, Ndc80, Cep3, and Bir1 are modified by SUMO. However, with the possible exception of Ndc10, the purpose of these modifications remains unclear and will require further investigation to wrestle the meaning of these modifications into the spot light.

In the case of Cep3, mutation of lysine residues found in SUMO consensus sites failed to identify a modification site, which leaves another 35 lysine residues that could be targeted by this modification. Without the structure of Cep3 to prioritize surface lysines, possible approaches to tackling this problem are performing mutagenesis on all remaining lysine residues or using mass spectrometry to identify SUMO modifications on Cep3 purified from yeast cell lysate or modified *in vitro*. Identification of the modification site will be important not only in characterizing the role of Cep3

sumoylation, but in combination with the *ndc10-4xK→R* mutant may shed light on the role sumoylation plays in regulating CBF3 function.

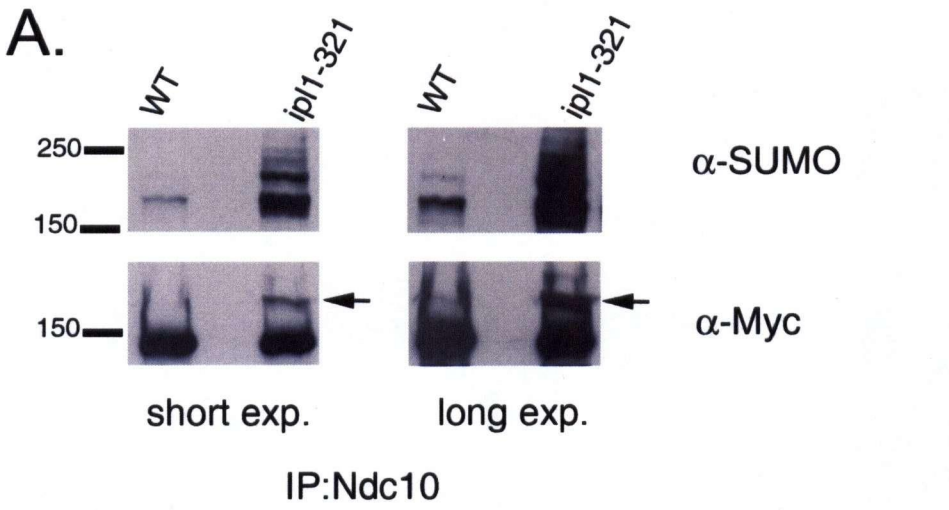
Mutations in Ndc80 and Bir1 that effectively abolish sumoylation were both identified in this work (ndc80 K231R and bir1-ΔIAP), but no phenotypes related to chromosome segregation were observed. It is almost certain that these modifications are important for the function of these proteins, and it is likely that the relevant phenotypes are not being tested. One approach that could be used to place sumoylation of these two substrates into a biological context is to perform genome-wide synthetic lethal screening with these alleles. The usefulness of identifying synthetic lethal genetic interactions is that they can be used to identify genes whose products buffer one another or impinge on the same essential pathway. SL screens also provide a list of genetic interactions that can be used as a fingerprint to identify genes and gene products functioning in a similar manner. It is known that the number of common interactions observed between two query genes correlates with known protein-protein interactions between the corresponding gene products. In other words, each allele that is screened gives a pattern of interactions that would be similar to other genes that function in the same process. For example, the CTF8 and CTF18 gene products physically interact in the same complex, and although these two genes are not connected in genetic networks, they share a large number of common interactions (Tong et al., 2004). This is further illustrated by the fact that 28 of 333 gene pairs with more than 16 common interactions encode physically interacting proteins, which is an 11-fold enrichment over those proteins that do not share a similar frequency of common interactions (Tong et al., 2004). The predictive nature of this approach is limited by the size of the genetic network, but genes involved in chromosome biology are

over-represented in the SL data produced to date, so it is likely that both the individual SL interactions and overall pattern of interactions would provide clues to the function of Ndc80 and Bir1 sumoylation.

SUMO modification of Ndc10 can be linked to both Ndc10 and Cep3 spindle localization, regulation of mitotic spindle dynamics and chromosome segregation. Exactly how the SUMO modification mediates these events is unknown, but since neither Ndc10 nor Cep3 have microtubule binding or motor protein properties it seems likely that SUMO is responsible for establishing the interaction between Ndc10 and the protein(s) required to bridge Ndc10 and Cep3 to the spindle. This would predict that in the SUMO bound state, Ndc10 or SUMO itself is bound by other factors to properly localize. Given the capacity to generate *in vitro* sumoylated Ndc10, one strategy to identify such factors could be to generate an affinity column with immobilized Ndc10-SUMO, on to which, whole cell yeast lysate from various conditions (e.g. anaphase) could be loaded. After extensive washing, proteins bound to the column could be eluted and identified by mass spectrometry. Alternatively, two-hybrid screening systems have been designed to detect interactions that are dependent on phosphorylation (Guo et al., 2004), which could be similarly designed to detect SUMO dependent interactions. This has the added benefit of detecting these interactions *in vivo*. The identification of a spindle localizing factor that recognizes sumoylated substrates could be very exciting in light of the fact that Ndc10, Cep3, and human RanGap1 all localize to the mitotic spindle, and that the localization of Ndc10 and RanGap1 is SUMO dependent (Matunis et al., 1996).

Finally, data in Chapter 3 suggests that Ndc10's spindle localization may rely on the interaction of Ndc10 with the Ipl1/Bir1/Sli15 complex. This is reflected in the

observation that Ndc10 has a two-hybrid interaction with Bir1 and Sli15, is a substrate for Ipl1 phosphorylation in vitro, and is mis-localized from the spindle when Bir1 is mutated. Furthermore, the data suggests that this interaction occurs prior to spindle loading since the Bir1 interaction can still be observed in the *ndc10-4xK→R* mutant. To investigate this further, it will be necessary to investigate the effect of Sli15 and Ipl1 mutants on the localization of Ndc10. Interestingly, this has already been done in ipl1-321 mutants and there appears to be only a slight decrease in the levels of mitotic spindle localization (Bouck and Bloom, 2005). It is known, however, that at the restrictive temperature, *ipl1-321* retains a low level of kinase activity, and a phosphorylation-dependent mechanism is therefore still possible (Pinsky et al., 2006). In fact, evidence of an interaction between phosphorylation and sumoylation has been shown for many substrates, and has been termed a phospho-SUMO switch. It has been shown for these substrates that these modifications can inhibit or promote one another (Yang and Gregoire, 2006). In preliminary experiments using *ndc10-4xK→R* mutant, it can be observed that reduction of Ipl1 kinase activity in an ipl1-321 allele results in hyper-sumoylation of Ndc10, but not Cep3 or Ndc80, suggesting that this effect is specific to Ndc10 and does not reflect whole cell changes in modification levels (Figure 4-1a and data not shown). This argues for the presence of a phospho-SUMO switch in Ndc10. Inspection of the Ndc10 sequence reveals a number of potential phosphorylation sites near two of the lysine residues in Ndc10 that are critical for sumoylation (Figure 4-1b). Mutational analysis of these sites will be critical in understanding the function of such a switch, since phenotypes arising in the ipl1-321 allele are not specific to loss of Ndc10 phosphorylation and subsequent hyper-sumoylation given the number of known Ipl1



B.

K556 LTFASSHNPDTHPTQKQESEGPLQMSQLDTT

K779 IEQKLGSHTGKFSTLKRPQLYMTEEHNVGFD

Figure 4-1. Ndc10 is hypersumoylated when Ipl1 kinase activity is abolished. (A) IP/western blots were performed on protein extracts from WT or ipl1-321 strains grown at 37°C for 3hrs. A short and long exposure are shown of the same westerns. Note that in the ipl1-321 lane a sumoylated form of Ndc10 can be seen in the MYC blots, which is normally unobservable in WT (arrow). (B) Inspection of the Ndc10 sequence reveals a number of potential phosphorylation sites (blue) near lysine residues K556 and K779 (red), which are critical for sumoylation in Ndc10.

targets (Cheeseman et al., 2002a). One intriguing possibility is that phosphorylation of Ndc10 may be used to regulate Ndc10 sumoylation in response to checkpoint activation, since Ndc10 sumoylation is decreased in NZ challenged cells. If this is the case, inactivating this switch by mutation should provide some insight into the function of Ndc10 in spindle checkpoint function (Fraschini et al., 2001).

In conclusion, sumoylation of various kinetochore proteins provides evidence for a novel means of regulating chromosome segregation. It remains to be seen if these SUMO modifications also occur on the human homologs of these proteins, but given the severe defects in chromosome segregation and nuclear architecture that is seen in SUMO knockout mice, it is certain that sumoylation plays a central role in chromosome segregation in mammalian systems (Nacerddine et al., 2005).

Conclusion

The work presented in this thesis set out to further characterize the function(s) and regulation of a few essential kinetochore proteins in budding yeast to better understand their roles in chromosome segregation. Like most research, the results obtained in these studies led down a winding path that ultimately raised more questions than answers. In this work, I have been able to highlight a connection between kinetochore proteins and the nuclear envelope and a role for sumoylation in mediating chromosome segregation. Together with data from other groups, I believe this work allows for a more detailed description of the process of chromosome segregation to be synthesized. It is my hope that this work may contribute to a better understanding of normal cell cycle controls and the process of chormosome segregation.

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