

**IDENTIFICATION OF NOVEL FACTORS REQUIRED
FOR CHROMOSOME SEGREGATION IN BUDDING
YEAST**

BY

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Abstract

During the course of the mitotic cell cycle, the genetic material must be faithfully replicated and segregated to daughter cells. After DNA replication when chromosomes have been duplicated, each pair of identical sister chromatids must remain tethered together until all pairs of sister centromeres have attached to the mitotic spindle in a bi-oriented manner, a state termed metaphase. Once metaphase has been successfully achieved, the initiation of anaphase can take place, and sister chromatids are pulled apart to the two daughter cells. Errors in this process lead to chromosome missegregation (chromosome loss or non-disjunction) and result in aneuploidy, which may have deleterious effects. Processes important in chromosome segregation fidelity include kinetochore attachment to the spindle and sister chromatid cohesion.

A genome wide two hybrid screen using *SGT1* as the "bait" identified a previously uncharacterized open reading frame, YDR014W (*RAD61*) that, when deleted, missegregated a chromosome fragment. YDR014W corresponded to the gene encoding the complementation group, *CTF6*, and was also recently characterized as *RAD61* in a screen for deletion mutants sensitive to ionizing radiation. *rad61Δ* diploid mutant strains displayed a G2/M progression delay dependent on Mad2p and were hypersensitive to DNA damaging agents. Rad61p localizes to the nucleus and a fraction binds chromatin. Rad61p is not a core component of the yeast kinetochore and is not required for homologous recombination repair of DNA damage, but is important for sister chromatid cohesion.

Using co-immunoprecipitation and mass spectrometry analysis, we identified a protein-protein interaction between Rad61p and Ded1p, an RNA helicase of the DEAD

box family that has important roles in initiation of translation and mRNA splicing. Ded1p binds chromatin and may have direct roles in chromosome biology. A temperature sensitive allele of the essential *DED1* gene causes an increased rate of chromosome missegregation. *rad61Δ* and *ded1* temperature sensitive alleles displayed conditional synthetic lethality, indicating that the interaction is functionally significant within yeast cells. Taken together, these results suggests that Rad61p and Ded1p function together in the nucleus for processes that are important for sister chromatid cohesion and for chromosome segregation.

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LIST OF ABBREVIATIONS

APC/C –	Anaphase Promoting Complex / Cyclosome
bp –	base pairs
CEN –	centromere
CIN –	Chromosome Instability
ChIP –	Chromatin Immunoprecipitation
CDK –	Cyclin Dependent Kinase
CF –	Chromosome Fragment
CFP –	Cyan Fluorescent Protein
CKI –	CDK Inhibitor
CTF –	Chromosome Transmission Fidelity
DMSO -	Dimethylsulphoxide
DSB –	Double Strand Break
EMS -	Ethylmethanesulfonate
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)
GAP -	GTPase activating protein
GEF -	Guanine nucleotide Exchange Factor
GFP –	Green Fluorescent Protein
GDP –	Guanine Di-Phosphate
GTP -	Guanine Tri-Phosphate
HR –	Homologous Recombination

IP –	Immunoprecipitation
LAC -	Lactose
LOH –	Loss Of Heterozygosity
M –	Mitosis
Mbp -	Mega base pairs
MIN –	Microsatellite Instability
MMS –	Methylmethanesulfonate
MRX –	Mre11p / Rad50p / Xrs2p
NHEJ –	Non-Homologous End Joining
ORC –	Origin Replication Complex
ORF –	Open Reading Frame
PCNA –	Proliferating Cell Nuclear Antigen
PIKK -	phosphoinositide 3-kinase related kinases
pre-RC –	pre-Replicative Complex
RFC –	Replication Factor C
RPA –	Replication Protein A
RSC –	Remodels the Structure of Chromatin
S –	Synapsis (DNA replication phase of cell cycle)
SCF –	Skp1p / Cullin / F-box protein
SDL –	Synthetic Dosage Lethality
SLAM –	Synthetic Lethal Analysis on Microarrays
SPB –	Spindle Pole Body
ssDNA –	single stranded DNA

TET -	Tetracycline
UBC -	Ubiquitin Carrying Enzyme
UTR -	Untranslated Region
VFP -	“Venus” yellow fluorescent protein
YFP -	Yellow Fluorescent Protein
YPD -	Yeast Proteome Database
YPD -	Yeast Extract Peptone Dextrose

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CHAPTER 1: INTRODUCTION

**CHROMOSOME SEGREGATION IN YEAST AND
HUMANS**

Introduction

The proper replication and segregation of the genetic material in dividing cells is a fundamental process in biology. Cells have evolved mechanisms to ensure that chromosomal DNA is replicated faithfully, packaged into chromatin (consisting of DNA, histones, and associated proteins), held together as sister chromatids, and then properly segregated, one copy to each of the daughter cells. Errors in this process can lead to aneuploidy, a state in which a cell does not have the proper complement of chromosomes. In aneuploid cells, one or more chromosomes are missing or there is an excess number of chromosomes. Aneuploidy can cause the uncovering of recessive mutations, or result in cell death or adverse growth effects. Aneuploid cells and genomic instability are also important factors that contribute to human diseases such as Down Syndrome and cancer. Studying the factors that govern genomic stability is thus important not only for understanding basic cell processes, but also for understanding human disease and for developing potential therapeutics.

Consequences of Aneuploidy in Humans

Genomic instability and aneuploidy are important factors in tumourigenesis. The majority of solid tumours contain aneuploid cells. Tumour cell lines have an increased rate of aneuploidy generation and not just an increased number of cells that are aneuploid. The vast majority of colon tumours (>80%) are classified as having a chromosome instability (CIN) phenotype (Nowak et al. 2002). One model for how aneuploidy could contribute to tumourigenesis is that an increased rate of aneuploidy could act to increase the rate of loss of heterozygosity (LOH) thereby increasing the

chances of uncovering recessive mutations (e.g. in tumour suppressor genes) or by leading to higher rates of dosage imbalances of genes (Knudson, 2001). Approximately 13% of solid tumours are not aneuploid, but contain mutations that cause a mutator phenotype (Nowak et al., 2002). In colon cancer, the mutator phenotype is caused by mutations in the mismatch repair genes (e.g. *hMLH1* and *hMSH2*) and is characterized by microsatellite instability (MIN). The MIN phenotype and hereditary pre-dispositions to colon cancer arising from mutations in the mismatch repair genes were discovered based on analysis of the MIN phenotype in yeast (Strand et al., 1993; Fishel et al., 1993; Leach et al., 1993; Papadopoulos et al., 1993) and subsequent screening of human candidate genes based on that work. MIN and CIN phenotypes are thought to increase the chance that multiple genetic changes necessary for tumorigenesis will occur; MIN by causing mutations in key genes and CIN by LOH and/or dosage imbalance of genes.

A common model from mathematical extrapolations indicates that six to ten genetic alterations are required for cancer (Nowak et al., 2002; Knudson, 2001; Rajagopalan and Lengauer, 2004). These genetic alterations typically confer a growth advantage and become fixed in the population through selection and clonal expansion. After the genetic alteration is fixed in the cell population, the next genetic event would occur in one of the cells in the population, and a similar process of selection and clonal expansion would occur. Accumulating six to ten of these genetic events would lead to cancer and MIN and CIN phenotypes could accelerate the rate at which independent “hits” accumulate. There is also evidence linking the amount of aneuploidy of a tumour with the severity of the disease, although using aneuploidy to adjust prognosis and treatment has not been attempted (Rajagopalan and Lengauer, 2004).

What can cause this increased rate of aneuploidy due to increased rates of chromosome missegregation in tumour cells? Clearly, a very large number of genes are known that could be responsible and many more are yet to be discovered. Researchers in recent years have screened for somatic mutations in candidate CIN genes implicated in genome stability based on sequence similarity to genes identified in model organisms such as the budding yeast *Saccharomyces cerevisiae*. Mutations in mitotic checkpoint genes such as *hBUB1* and *hBUBR1* have been found in colorectal tumour cell lines and expression of *hBUB1* mutant genes in non-CIN cell lines causes a dominant negative effect leading to chromosome instability (Cahill et al., 1998). A recent report showed that biallelic mutations in *hBUB1B* were associated with an inherited predisposition to cancer (Hanks et al., 2004), providing strong support for the model that CIN is a predisposing factor in cancer initiation/progression. Another spindle assembly checkpoint protein first characterized in budding yeast, *MAD2*, has also been found to be transcriptionally silenced in breast and other cancers (Li and Benezra, 1996). *MAD2*^{+/-} mice were found to develop lung tumours at a high rate after long latencies, further implicating the mitotic checkpoint in tumourigenesis. In a recent survey screening for mutations in 100 candidate genes in a panel of colorectal tumours, the Vogelstein group identified five new CIN genes (*MRE11*, *hZW10*, *hZwilch*, *hRod*, *Ding* (*PDS1*/securin homolog) (Wang et al., 2004) mutated in cancer. These mutations account for <20% of the colon tumours (Wang et al., 2004) and, therefore, the genetic basis for chromosome instability remains unknown in the majority of colon tumours. Finding additional CIN genes in model organisms in order to sequence their human homologues as candidate genes in tumour cell lines will help to identify the complete spectrum of CIN genes

responsible for aneuploidy in cancer. The success in identifying the genes responsible for the MIN phenotype and finding mutations in candidate CIN genes provides examples that research with budding yeast can lead to insights in cancer.

Characterizing the genes that are involved in CIN tumours can provide practical applications in cancer treatment. Recent experiments have shown that in CIN cell lines that do not have mutations in the spindle assembly checkpoint, introducing a mutation or lowering the expression of one of the spindle assembly checkpoint proteins (*hMAD2*) can cause massive chromosome loss and non-disjunction, leading to apoptotic cell death in these aneuploid cancer cell lines (Kops et al., 2004). Knowing the underlying mutations involved in aneuploidy in a particular tumour can help us to develop therapies that selectively target the cancer cells for death. For example, finding gene mutations that are synthetic lethal in budding yeast with the cancer gene mutation that is causing the aneuploidy can help identify candidate proteins in tumour cell lines that when targeted by a drug can cause death to the cancer cell without killing adjacent normal cells. In addition, understanding the CIN mutational spectrum can lead to sub-classification of tumours based on the gene mutated and this could lead to improved diagnostics, prognosis and predictions of response to different treatments (Rajagopalan and Lengauer, 2004). Finding genes that are involved in the CIN phenotype in tumour cell lines will provide a rich resource for both clinical and basic research and may also provide targets for gene therapy that will prevent the CIN phenotype in people pre-disposed to inherited forms of cancer.

Many of the factors that contribute to genomic stability in mitosis are also important during meiosis and understanding their function could lead to insights into the

generation of trisomies in humans such as trisomy 21 in Down Syndrome. Non-disjunction events leading to aneuploidy in the first meiotic division in female oocytes account for the majority of trisomies (Hassold and Sherman, 2000). Trisomies occur in approximately 0.3% to 0.5% of live births (Antonarakis et al., 2004) and a maternal age effect is associated with trisomies (Reeves et al., 2001). A two hit hypothesis has emerged to explain the dynamics of trisomy generation. Defects in the spindle assembly checkpoint or sister chromatid cohesion have been postulated to be one of the hits required for generating trisomies. The generation of bivalents that are achiasmatic (homologous chromosomes that do not undergo a recombination event and do not have chiasmata) or have a chiasma located too far or too near to centromeres can lead to an increased chance of non-disjunction and is postulated to be the other hit required for generating trisomies. The combination of these two hits contributes to non-disjunction when the first meiotic division is completed shortly before ovulation (Hassold and Sherman, 2000). Investigating factors involved in chromosome segregation in mitotic cells and investigating their roles in meiosis could lead to understanding the mechanisms of trisomy generation.

Budding yeast as a model organism

Saccharomyces cerevisiae, a budding yeast, has served as a model organism for understanding essential and important processes in the mitotic cell cycle. Components and pathways involved in basic intracellular processes such as DNA replication, cell cycle checkpoints, DNA repair, sister chromatid cohesion, kinetochore function and cell cycle machinery are conserved from yeast to man (Kitagawa and Hieter, 2001).

Saccharomyces cerevisiae is a unicellular eukaryote that has both a haploid and a diploid life cycle. $2n$ diploids can be induced to sporulate by nitrogen starvation on a poor carbon source and can form four $1n$ haploid spores that are encased in a durable spore wall. The spores can be germinated and each of the haploid offspring can be recovered, making budding yeast especially useful for genetic manipulation. As well, haploid yeast cells of opposite mating types can mate and form diploids, and following meiosis, hybrid spore products can be detected and analyzed with novel combinations of mutations (reviewed in Rose et al., 1990). One of the greatest assets in using yeast as a model organism is the ease in which the genome can be manipulated, i.e. recombinant genetics through homologous recombination. Deletions, point mutations, and epitope tagged versions of genes can be introduced at their endogenous loci facilitating genetic, phenotypic, cell biological, and biochemical characterization in cells (Longtine et al., 1998).

Budding yeast was used to identify many of the key components of the cell cycle machinery which are highly conserved in eukaryotic cells. Morphology of individual yeast cells can be correlated with a particular stage of the cell cycle as shown in Figure 1. Unbudded cells are in G1 and have recently exited mitosis and undergone cytokinesis. Small budded cells are entering S phase and undergoing spindle pole body duplication (site of microtubule nucleation, similar to the centrosome in human cells). As the bud grows larger, the yeast cell passes through S phase into G2. Large budded cells are generally classified as G2/M cells and are approaching anaphase. This correlation between morphology and the cell cycle was used to isolate mutants that arrested budding yeast cells at particular stages in the cell cycle. They helped to isolate proteins that were

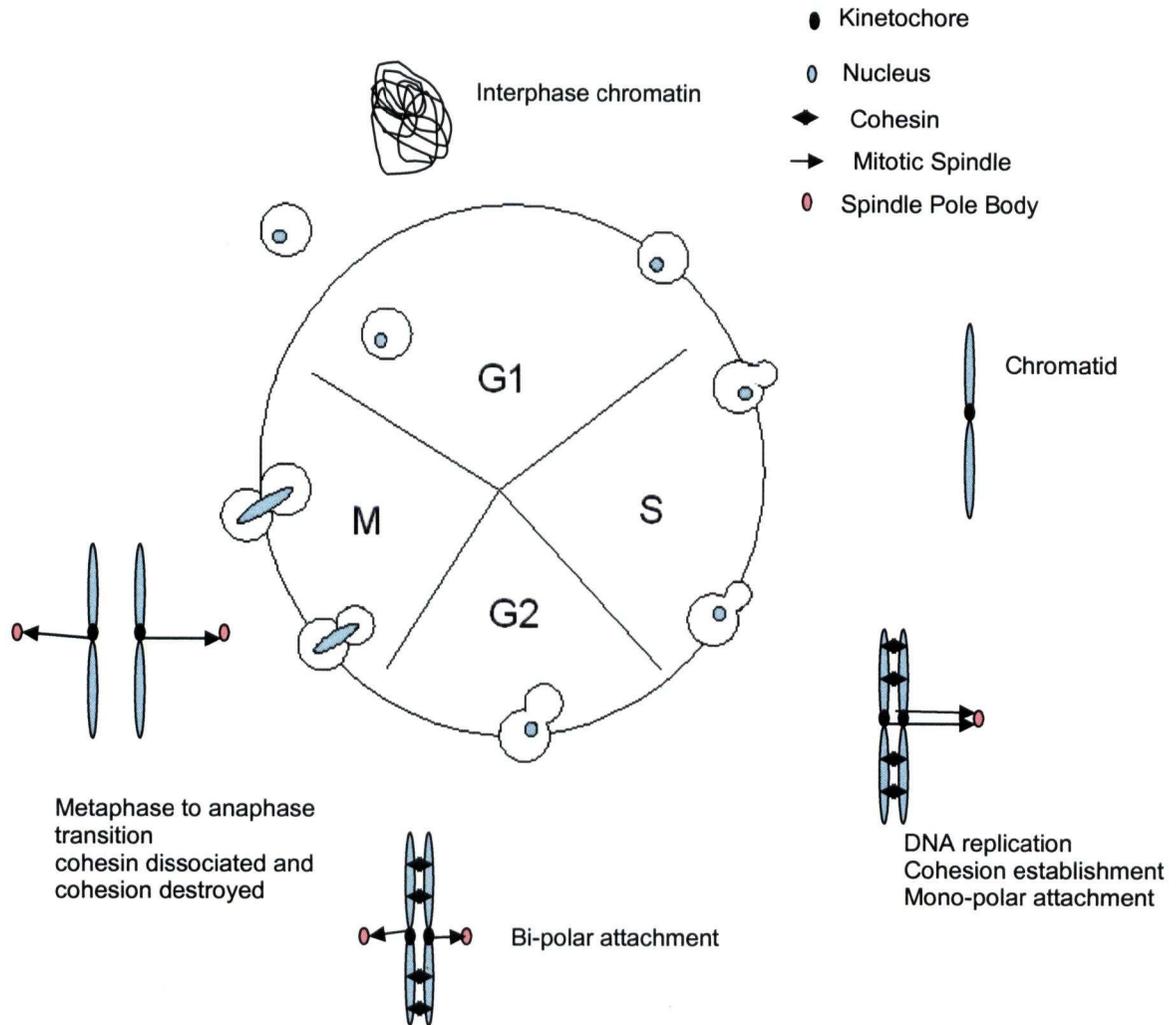


Figure 1-1 – The budding yeast cell and chromosome cycle.

Cellular morphology can be correlated to cell cycle progression. The chromosome cycle (condensation, replication, sister chromatid cohesion and separation) is shown with cell cycle progression.

important for cell cycle progression in budding yeast, and by homology, other eukaryotes (Hartwell, 1980; Wood and Hartwell, 1982). Dr. Leland Hartwell won the Nobel Prize in Physiology and Medicine in 2001 for his pioneering work on “key regulators of the cell cycle”.

Budding yeast was the first eukaryote to have its genome sequenced (Goffeau et al., 1996), and many technologies, such as micro-array analysis of gene expression, SAGE (Serial Analysis of Gene Expression) (Velculescu et al., 1997), and SGA (Synthetic Genetic Array) (Tong et al., 2001) screens were first developed in budding yeast. The experimental tractability of budding yeast facilitates the testing of the validity of novel genomic and proteomic data sets. There are ~6000 genes in budding yeast, ~1100 of which are essential for viability; this has led to the generation of gene deletion sets that contain deletion mutants of the non-essential genes (the haploid sets and the homozygous diploid set) as well as all the genes in yeast (the heterozygous diploid deletion set) (Giaever et al., 2002).

In studying factors that are important for chromosome segregation fidelity, there are several differences that should be pointed out between budding yeast and mammalian cells. Budding yeast chromosomes and centromere DNA are much smaller than their mammalian counterparts. The average yeast chromosome is ~ 1 Mega base pairs (Mbp), and the centromere is approximately 125 base pairs (bp), whereas human chromosomes are approximately 150 Mbp and centromeres in human cells can be as large as 5 Mbp (Loidl, 2003). One microtubule is bound to each yeast kinetochore whereas dozens of microtubules are attached to human kinetochores. Budding yeast kinetochores remain bound to the spindle pole body through interphase and tend to cluster together (Jin et al.,

2000) and microtubules bind kinetochores soon after replication. In human cells microtubules are not connected to kinetochores immediately after replication (Gadde and Heald, 2004). Budding yeast undergoes a “closed mitosis” in which the nuclear envelope does not breakdown during the course of mitosis (Loidl, 2003), and the spindle pole bodies remain anchored in the nuclear membrane during mitosis. In contrast, mammalian cells undergo an “open mitosis” in which the nuclear envelope breaks down and chromosomes and centrosomes become part of the cytoplasm.

The Cell Cycle and Genome Stability

Genome stability in dividing cells is intimately tied to cell cycle control. Cell cycle control is crucial to ensure that events during the cell cycle take place with high fidelity (for a review see Murray and Hunt (1993)). This involves ensuring that certain events happen only after other events have occurred (e.g. sister chromatids cannot be segregated until they have been duplicated and until they are attached to opposite spindle pole bodies) and that certain events can only happen once during the cell cycle (e.g. the firing of origins of replication, the duplication of the spindle pole body). There are points during the cell cycle at which cells will commit to completing events. For example, the Start point in the budding yeast cell cycle is the point in G1 when the cell begins the vegetative cell cycle and cannot thereafter be affected by mating pheromone. Another example would be the metaphase to anaphase transition when sister chromatids are pulled apart. A breakdown in cell cycle control could lead to errors that contribute to chromosome missegregation and aneuploidy. Two CIN genes mutated in colorectal

tumours are *hCDC4* (Rajagopalan et al., 2004) and *DING/SECURIN* (Wang et al., 2004); two genes important for cell cycle control.

The cell cycle control machinery is conserved among all eukaryotes. Cyclin Dependent Kinases (CDKs) serve as the regulators of the cell cycle and, in concert with a bound cyclin subunit (which serves to activate its kinase activity), can drive the cell cycle through phosphorylating substrates. Cyclins were first discovered to be proteins that accumulated and then disappeared in a cell cycle dependent manner (Evans et al., 1983; Cross, 1988; Hadwiger et al., 1989; Nash et al., 1988; Murray, 1989). This periodic accumulation and degradation allows CDK activity to be regulated in a periodic manner and drives the cell cycle through phosphorylating substrates at the right time during the cell cycle. CDKs can also be regulated by phosphorylation and the binding of inhibitors (CDK Inhibitors, CKIs) such as Sic1p in budding yeast.

Cdc28p and Pho85p are the CDKs involved in cell cycle progression in budding yeast with Cdc28p being the main regulator (Mendenhall and Hodge, 1998). Cdc28p is expressed and maintained at a high level throughout the cell cycle and cyclin binding controls its activity. In budding yeast, G1 cyclins (Cln1p, Cln2p and Cln3p) promote events after mitotic exit and cytokinesis and are important for passage through Start during G1 (Cross, 1990; Hadwiger et al., 1989; Richardson et al., 1989). S phase cyclins (Clb5p, Clb6p, Clb3p, and Clb4p) promote entry into S phase (Dahmann et al., 1995; Richardson et al., 1990). Mitotic cyclins (Clb1p, Clb2p) drive the cell cycle through mitosis and past the metaphase to anaphase transition (Fitch et al., 1992; Ghiara et al., 1991).

Coordinated expression and translation of mRNAs and proteins during the cell cycle is controlled by phosphorylation of transcription factors by CDKs (Wittenberg and Reed, 2005). These proteins are important for events that are coordinated in a cell cycle dependent manner such as bud morphogenesis, spore wall deposition, and DNA duplication. Proper cell cycle control is also dependent on protein degradation by regulated proteolysis (Vodermaier, 2004). For example, Sic1p is a CKI that must be degraded during G1 in order for the G1/S phase transition to occur (Schwob et al., 1994; Donovan et al., 1994; Bai et al., 1996). Proteolysis of target proteins is a mechanism for cells to proceed through a “gate” in the cell cycle whereby the cell cannot return to an earlier stage of the cell cycle, thus preventing events from re-occurring that must occur only once in the cell cycle such as DNA replication or spindle pole body duplication.

Much of the cell cycle regulated proteolysis is accomplished by targeting proteins for degradation by the 26S proteasome (Hershko, 1997; Chun et al., 1996). This targeting is accomplished by attaching ubiquitin, a highly conserved polypeptide, onto the lysine residues of target proteins. Ubiquitin on proteins is then extended and this polyubiquitin chain serves as a tag for degradation by the 26S proteasome. Ubiquitin mediated proteolysis is well conserved in eukaryotes and the process of attaching ubiquitin to target proteins is accomplished by a well conserved cascade of enzymes. E1 ubiquitin activating enzymes bind to ubiquitin and prepare the moiety for subsequent binding to an E2, a ubiquitin conjugating enzyme (or Ubiquitin Carrying Enzyme, UBC). An E3 complex, ubiquitin ligase, will bring substrate proteins and the UBC-ubiquitin together and E3 complexes provide the specificity in the targeting of proteins.

The Anaphase Promoting Complex / Cyclosome (APC/C) and the SCF complex (Skp1p / Cullin / F-box protein) are E3 complexes that are important for cell cycle proteolysis (for reviews see Peters, 1998; Vodermaier, 2004; Page and Hieter, 1999; Willems et al., 1999; Zachariae and Nasmyth, 1999). The two complexes each have a cullin family protein (Apc2p in the APC/C and Cdc53p in the SCF) that acts as a scaffold and structural protein aiding in E2 recruitment (Ohta et al., 1999; Tang et al., 2001; Zheng et al., 2002). Both complexes depend on a RING finger protein (Apc11p in the APC/C and Rbx1p in the SCF complex) for UBC-ubiquitin recruitment (Ohta et al., 1999; Levenson et al., 2000; Gmachl et al., 2000). The complexes contain multiple subunits and they depend on substrate specificity factors that can bind onto the core APC/C or SCF complex and target specific proteins for degradation. In the case of the APC/C the specificity factors are Cdc20p and Cdh1p (Visintin et al., 1997) and a meiosis specific factor, Ama1p (Cooper et al., 2000), and for the SCF complex the specificity factors are the different F-box proteins that bind the SCF (e.g. Cdc4p, Grr1p, Met30p) (Skowyra et al., 1997). The specificity factors contain protein-protein interaction domains such as WD-40 repeats, which will contribute to protein-protein binding and are responsible for bringing substrates together with the UBC-ubiquitin. The APC/C (Cdc20p) is responsible for targeting proteins that are important for the metaphase to anaphase transition (such as Pds1p, discussed below) and also passage through mitosis. APC/C (Cdh1p) is responsible for proteolysis later in mitosis and during early G1 and targets substrates such as the mitotic cyclin, Clb5p, as well as Cdc20p (Visintin et al., 1997; Pflieger et al., 2001). The SCF is active at many stages of the cell cycle and has proteolytic functions unrelated to cell cycle progression but it is especially important for

the G1/S phase transition, with one of its main substrates being the CKI Sic1p (Bai et al., 1996). The SCF complex also targets the G1 cyclins (Cln1p and Cln2p) and Cdc6p, a protein involved in pre-replication complex formation (discussed below) for degradation (Willems et al., 1999).

DNA Replication and Genome Stability

Eukaryotic genomes are much larger than prokaryotic genomes and in order to replicate the entire genome in the time frame of the typical cell cycle, multiple origins of replication are needed (for reviews see Bell and Dutta, 2002; Diffley, 2004). Origins of replication are chromosomal elements that are necessary to initiate DNA replication and establish bi-directional replication forks (Bell, 1995). Origins of replication direct the binding of the pre-Replicative Complex (pre-RC) onto DNA and subsequent loading of the other proteins involved at replication forks (e.g. DNA polymerases, processivity factors such as Proliferating Cell Nuclear Antigen, PCNA) (Bell and Dutta, 2002). In budding yeast, these origins of replication are approximately 100 to 150 base pairs and include highly conserved A elements and less well conserved B elements that may aid in unwinding of the DNA double helix (Bell, 1995). The sequence of origins of replication in other eukaryotes is less well established (Bielinsky and Gerbi, 2001; Blow et al. 2001) and the surrounding chromatin domain appears to be important.

The pre-RC is assembled onto chromosomes and directs the placement of factors that will be used to replicate DNA. The pre-RC consists of the Origin Recognition Complex (ORC, made up of Orc1 – 6), Cdc6p, Cdt1p and Mcm2 – 7 complex. ORC contains DNA binding motifs and interacts directly with origins of replication. In

budding yeast the binding of ORC to DNA is constitutive and does not appear to be cell cycle regulated (Ogawa et al., 1999; Aparicio et al., 1997; Santocanale and Diffley, 1996; Tanaka et al., 1997). Cdc6p requires ORC to bind DNA (Neuwald et al., 1999; Coleman et al., 1996) and is a substrate of the SCF (Cdc4p) and is targeted for degradation in late G1 and early S phase (Drury et al., 1997; Elsasser et al., 1999; Perkins et al., 2001; Piatti et al., 1996). Cdc6p is required for the loading of the Mcm2 – 7 complex and Cdc6p degradation (which is dependent on its phosphorylation by CDK) is one mechanism to ensure that DNA is not re-replicated (Coleman et al., 1996). The Mcm2 – 7 complex binds chromatin in a Cdc6p and ORC dependent manner and this binding of Mcm2 – 7 may be one of the key functions of Cdc6p and ORC (Hua and Newport., 1998). Mcm2 – 7 is regulated also in a cell cycle manner but not by proteolysis. Phosphorylation of subunits in the Mcm2 – 7 complex that are not chromatin bound targets the complex for export from the nucleus to the cytoplasm (Labib et al., 1999; Nguyen et al., 2000; Pasion and Forsburg, 1999). The pre-RC may exist to provide a chromatin framework for binding of the subsequent proteins that are needed for DNA replication and to provide a mechanism for establishing origins of replication that will replicate only once in the cell cycle (Bell and Dutta, 2002). There is some evidence that Mcm2 – 7 functions as a DNA helicase but this has not been completely proven (You et al., 2002; Lee and Hurwitz, 2002).

Once the pre-RC is formed at origins of replication in late G1, high levels of CDK activity will also induce the subsequent binding of other proteins that will begin DNA replication. Another kinase, Cdc7p / Dbf4p (Dbf4p Dependent Kinase, DDK), is also needed along with CDK to initiate S phase (Pasero et al., 1999). Mcm10p and Cdc45p

are loaded onto the origins of replication by the pre-RC and direct the initiation of replication by recruiting other proteins such as replication protein A (RPA), a single stranded DNA (ssDNA) binding protein that protects and stabilizes ssDNA generated by origin firing, and also the DNA polymerase α that is needed to generate the RNA primers required to initiate DNA replication (Bell and Dutta, 2002). Once the RNA primers are generated and DNA polymerase α extends the fragment by 20 bps, the Replication Factor C complex (RFC, consisting of Rfc1 – 5) displaces the polymerase, and acts to load Proliferating Cell Nuclear Antigen (PCNA), a ring like “clamp” that encircles DNA and provides processivity to DNA polymerase δ , which replaces DNA polymerase α , and acts as the main DNA polymerase that replicates DNA (Majka and Burgers, 2004; Hubscher et al., 2002). This polymerase switch between the primase and the main processing polymerase is not well understood.

Proper timing and control of DNA replication plays an important role in genome stability. Mis-regulation of origins of replication can lead to increased genomic instability (Lengronne and Schwob, 2002; Huang and Koshland, 2003; Kolodner et al., 2002). During DNA replication, lesions that are either spontaneously generated or caused by DNA damaging agents can lead to the replication fork being stalled, broken, or displaced from DNA (Longhese et al., 2003). The stabilization of replication forks and processing and repair of DNA lesions are important in maintaining genome stability. The checkpoint mechanisms and repair processes will be discussed in sections below.

The Mitotic Spindle and Chromosome Segregation

The mitotic spindle is the specialized structure assembled during mitosis in order to physically pull apart sister chromatids during anaphase (Gadde and Heald, 2004). It is composed of microtubules, one of the key components of the cytoskeleton providing structure to cells. Microtubules are non-covalent polymers of tubulin. The tubulin subunits of microtubules are made up of a hetero-dimer of α -tubulin (Tub1p and Tub3p in budding yeast) and β -tubulin (Tub2p in budding yeast), two proteins that share approximately 50% identity (Desai and Mitchison, 1997). The hetero-dimers of α -tubulin and β -tubulin are arranged in a head to tail conformation giving the microtubule structural polarity. Microtubule plus ends are more dynamic and form the growing head of the structure, while their minus ends remain anchored and nucleated at microtubule organizing centres (centrosomes in human cells and spindle pole bodies in budding yeast). The head to tail interactions of the hetero-dimers are arranged into linear protofilaments that will associate to form a hollow cylindrical tube of a microtubule (Kline-Smith and Walczak, 2004).

Microtubules are dynamic structures and the prevailing model for their action is that of dynamic instability driven by four factors: 1) the rate of polymerization (growth) 2) the rate of de-polymerization (shrinkage) 3) the frequency and occurrence of catastrophes (the change from growth to shrinkage) 4) the frequency and occurrence of rescues (the change from shrinkage to growth) (Gadde and Heald, 2004; Desai and Mitchison, 1997; Kline-Smith and Walczak, 2004). While both α and β -tubulin can bind GTP, GTP bound β -tubulin is more important in terms of regulation and dynamic instability. GTP bound β -tubulin is incorporated at the growing ends of microtubules

during growth. During or shortly after polymerization, the GTP bound form of β -tubulin is hydrolyzed and this GDP bound form of β -tubulin does not exchange and forms the majority of the β -tubulin in the microtubule complex. There is thought to be a lag between incorporating the GTP bound tubulin and GTP hydrolysis, and this lag provides a GTP cap on growing ends of microtubules that is thought to provide some stability to the growing plus end. When a catastrophe event occurs, de-polymerizing GDP bound β -tubulin is released rapidly and the plus end undergoes rapid shrinkage. GTP is exchanged for GDP on the free β -tubulin, allowing this subunit to undergo polymerization when a rescue event occurs. This dynamic instability is thought to allow rapid re-organization of microtubules and allow a process such as “search and capture” (Kline-Smith and Walczak, 2004).

The mitotic spindle in budding yeast consists of microtubules that emanate from spindle pole bodies that either bind to kinetochores (kinetochore microtubules), or that form the central spindle where microtubules from the opposite spindle pole bodies interact in an anti-parallel array (interpolar microtubules). The spindle pole body is also the site of nucleation of the astral microtubules that will project out into the cytoplasm. Microtubules are dynamic and unstable but microtubule associated proteins as well as attachment to kinetochores can stabilize microtubules (Saxton et al., 1984; Zhai et al., 1996). Kinetochore-microtubule attachments, once attached to opposite spindle pole bodies and stabilized, provide the signal for the metaphase to anaphase transition to occur.

The mechanism by which microtubules will bind to kinetochores is termed “search and capture” and consists of microtubules at the plus end alternately growing and

shrinking, probing for kinetochores. Once bound, the kinetochore microtubule attachments are stable. Because of sister chromatid cohesion the sister kinetochores are oriented away from each other allowing attachments of the sister kinetochores to occur with microtubules from opposite spindle poles (Tanaka et al., 2000). Kinetochore regulating factors such as Ip11p/Aurora Kinase (discussed below) will destabilize kinetochore microtubule attachments that are mono-polar (Biggins and Murray, 2001; Dewar et al., 2004; Pinsky et al., 2003; Tanaka et al., 2002). Tension that is produced by bi-oriented sister kinetochore microtubule attachments are thought to further stabilize these attachments (Kline-Smith et al., 2005). 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.

Once sister chromatids have formed stable kinetochore microtubule attachments (bi-oriented to opposite spindle poles), a process that is monitored by the spindle assembly checkpoint (discussed below), sister chromatid cohesion is dissolved and the mitotic spindle pulls sister chromatids towards the poles. This segregation of sister chromatids is dependent on microtubule dynamics and also microtubule motor proteins. Microtubule de-polymerization can serve to drag sister chromatids towards the poles by either of two mechanisms. One mechanism is that kinetochores induce microtubule disassembly at plus ends while maintaining their attachments, “chewing” their way towards the spindle pole. The second mechanism is kinetochore microtubule attachment and microtubule stability at the plus end remaining unaffected, and an increased rate of de-polymerization occurs at the minus end, pulling the chromosomes to the poles (Gadde

and Heald, 2004). Microtubule motor proteins are also involved in generating force that can pull chromosomes apart. Microtubule motor proteins exist either as microtubule minus end or microtubule plus end directed motors. One example is the kinesin Kar3p, which is a minus end directed motor that can increase de-polymerization of microtubules at the minus end (Desai and Mitchison, 1997; Biggins and Walczak, 2003). Movement of chromosomes towards poles represents anaphase A, and after this movement, poles will separate by elongation of the mitotic spindle (anaphase B), a process that is also driven by stabilization of microtubule dynamics. Finally, before cytokinesis, the mitotic spindle must be disassembled and this disassembly is driven by de-polymerization (Seshan and Amon, 2004; Cheeseman and Desai, 2004).

The Centromere and Kinetochore in Budding Yeast

The kinetochore, which consists of the centromere DNA and associated proteins, is essential as an attachment point for microtubules emanating from opposite spindle pole bodies and also as a platform for sensing proper bi-polar attachment. The budding yeast centromere is a “point” centromere and consists of three conserved elements (CDE I, CDE II, and CDE III) that span 125 base pairs (Loidl, 2003). The associated kinetochore proteins have been categorized into inner kinetochore proteins that have direct connections with the centromere DNA (or are in a complex with proteins that directly interact with CEN DNA), central kinetochore proteins that serve as scaffolding and link the inner kinetochore to the outer kinetochore proteins, and outer kinetochore proteins that link the kinetochore to microtubules (for reviews see Cheeseman et al., 2002; Measday and Hieter, 2004; McAinsh et al., 2003; Skibbens and Hieter, 1998; Kitagawa

and Hieter, 2001). The budding yeast kinetochore is assembled in a hierarchical manner with distinct sub-complexes (De Wulf et al., 2003).

The budding yeast inner kinetochore consists of a complex called CBF3 (which is made up of Ndc10p, Ctf13p, Cep3p and Skp1p) that binds to the CDEIII region of the centromere. All of the components of the CBF3 complex are essential for viability. Cbf1p protein is also at the yeast inner kinetochore and it binds at the CDEI region of the centromere. Cbf1p is not essential for viability. Cse4p is a well conserved histone H3 variant that replaces H3 in the histone octamer in and around the centromere region and is essential for viability (Cheeseman et al., 2002). Within the CBF3 complex, only Skp1p is well conserved from yeast to man (Kitagawa and Hieter, 2001) and the role of human Skp1p at the kinetochore is unclear (Gstaiger et al., 1999). The lack of conservation of the other members of the CBF3 complex may reflect the difference in centromere sequence between yeast and humans.

Skp1p is an especially interesting protein as it plays roles in two distinct processes. Skp1p was originally identified in our laboratory as a high copy suppressor of a temperature sensitive mutant, *ctf13-30* (Connelly and Hieter, 1996), and characterized as a component of the kinetochore. Skp1p was also found as a suppressor of *cdc4* mutants (Bai et al., 1996) and characterized as a subunit of the SCF. F-box proteins bind to the SCF through Skp1p and their F-box domains; Ctf13p is also an F-box protein and binding of Skp1p to Ctf13p requires Ctf13p's F-box domain (Russell et al., 1999). Temperature sensitive alleles of *SKP1* fall into two different classes; mutants that arrest at the restrictive temperature with a G1/S profile with a defect in SCF function, e.g. *skp1-3*, and mutants that arrest at the restrictive temperature with a G2/M profile and a defect

in kinetochore function, e.g. *skp1-4* (Connelly and Hieter, 1996). *SGT1* was identified as a high copy suppressor of *skp1-4* (Kitagawa et al., 1999) and Sgt1p was found to be a protein that directly interacted with Skp1p and functioned with Skp1p at the kinetochore and the SCF. *SGT1* is an essential gene and temperature sensitive alleles of *SGT1* can be distinguished in the same way as *SKP1* temperature sensitive alleles can, into mutants with an SCF defect (*sgt1-5*) or mutants with a kinetochore defect (*sgt1-3*). At the kinetochore, Sgt1p interaction with Skp1p is mediated by the HSP90 chaperones and their activity is required to activate Ctf13p and promote CBF3 assembly (Bansal et al., 2004; Lingelbach and Kaplan, 2004).

The central kinetochore consists of at least 3 different sub-complexes, the NDC80 complex, the MIND complex and the COMA complex. There are also other proteins of the central kinetochore that have been isolated that have not been placed into these sub-complexes but together they form a large macro-molecular complex at the central kinetochore that can be co-purified by biochemical assays such as co-immunoprecipitation (Pot et al., 2003; Measday et al., 2002; De Wulf et al., 2003; Measday and Hieter, 2004). All of the central kinetochore proteins depend on the CBF3 inner kinetochore complex for localization to the kinetochore but the question of whether or not these sub-complexes interact with the inner kinetochore independently of each other or whether there is a higher level of regulation and interdependency is still unknown (Measday and Hieter, 2004). 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.

The outer kinetochore consists of proteins that bind to microtubules. Members of the DAM1 complex have been shown to bind the mitotic spindle and Dam1p has been shown to bind microtubules *in vitro* (Kang et al., 2001; Li et al., 2002; Cheeseman et al., 2002). The localization of the DAM1 complex to the kinetochore is dependent on an intact mitotic spindle. Recent work has shown that the DAM1 complex forms a ring around microtubules (Miranda et al., 2005; Westermann et al., 2005). There are also other proteins that have been localized to the kinetochore with microtubule binding capabilities, such as Stu2p, Bik1p, Bim1p and kinesin related proteins (microtubule motors) Cin8p, Kip1p and Kip3p (He et al., 2001; Cheeseman et al., 2002).

Researchers have also begun to investigate proteins that regulate the budding yeast kinetochore. Sister kinetochores initially are attached in a mono-polar manner to the old SPB. Kinetochore microtubule capture and release is not well understood but the mechanisms for regulating proper bi-polar attachment have begun to be elucidated. The current model involves a kinase complex consisting of Sli15p / Ipl1p that senses the lack of tension generated by mono-polar attachment and phosphorylates Dam1p, Ndc80p and Ndc10p (Biggins and Murray, 2001; Dewar et al., 2004; Pinsky et al., 2003; Tanaka et al., 2002). This phosphorylation is hypothesized to remove the kinetochore-microtubule attachment, and new attachments would have to be established. As well as this mechanism to ensure bi-polar attachment, the spindle assembly checkpoint, which in budding yeast consists of Mps1p, Mad1p, Mad2p, Mad3p, Bub1p and Bub3p, generates a wait anaphase signal that inhibits the metaphase to anaphase transition until all sister chromatids have achieved bi-polar attachment (discussed below).

Sister Chromatid Cohesion

Eukaryotic chromosome replication is separated in time from chromosome segregation. After S phase each chromosome will consist of two identical sister chromatids and in order to ensure that sister chromatids segregate equally into separate cells during mitosis, they must be kept attached until mitosis. This attachment, or sister chromatid cohesion, ensures that sister chromatids stay together and promotes bi-orientation of the sister chromatids such that amphitelic attachments (sister kinetochores binding to microtubules from opposite spindle pole bodies) can occur (Tanaka et al., 2000). Defective sister chromatid cohesion or defective regulation of cohesion can lead to precocious sister chromatid separation leading to chromosome loss or non-disjunction.

The mechanism of sister chromatid cohesion is well conserved in eukaryotes and depends on a chromosomal protein complex called cohesin (for reviews see Nasymth, 2001; Uhlmann, 2003; Uhlmann, 2004; Cohen-Fix, 2001). Cohesin is a complex made up of at least four subunits, a heterodimer of two SMC (Structural Maintenance of Chromosomes) proteins, Smc1p and Smc3p, bound to two other proteins, Scc1p and Scc3p (Michaelis et al., 1997; Guacci et al., 1997; Losada et al., 1998). Structural studies have shown that cohesin is a ring link structure (Anderson et al., 2002; Haering et al., 2002; Gruber et al., 2003). Smc1p and Smc3p are dimerized in a head to head and a tail to tail conformation with the circumference of the cohesin ring consisting of the flexible coiled coil regions of Smc1p and Smc3p. The tails of the Smc1p and Smc3p interact to form a hinge region (see Figure 2 for a model). The head regions of Smc1p and Smc3p have similarity to the ATPase domains of ABC transporters and it is thought that their binding is dependent on ATP binding. Scc1p binds the head region of Smc1p and

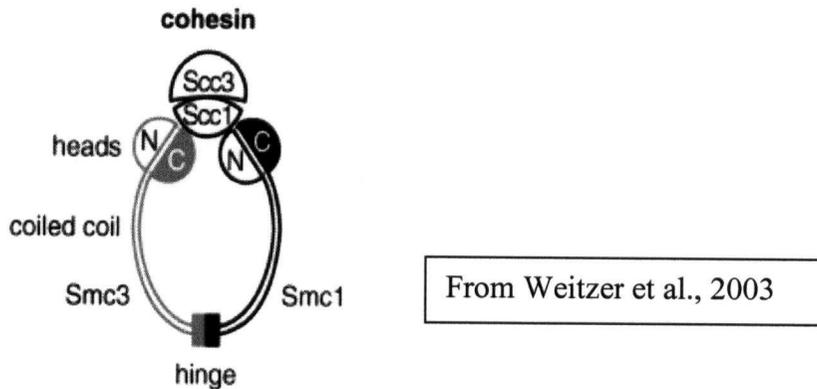


Figure 1-2 – The cohesin complex forms a ring like structure.

The coiled coil regions of Smc1p and Smc3p form the circumference of the ring, with their tails forming a hinge region and their heads associating to close the ring. Scc1p binds to the head region with Scc3p associating with Smc1p and Smc3p through Scc1p.

Smc3p, with Scc3p binding to Scc1p (Haering et al., 2002). Scc1p can bind both ATP and ADP bound forms of Smc1p and Smc3p, while ADP bound Smc1p and Smc3p heads bind each other with less affinity than ATP bound Smc1p and Smc3p. ATP hydrolysis by Smc1p and Smc3p is needed for cohesin binding to DNA which supports the idea that ATP bound Smc1p and Smc3p will hydrolyze ATP and bind DNA and then have the ADP bound Smc1p and Smc3p heads stabilized by Scc1p and Scc3p (Arumugam et al., 2003; Weitzer et al., 2003). All four subunits of the cohesin complex are essential for viability in budding yeast (*Saccharomyces Genome Database, SGD*).

The cohesin complex associates with discrete chromosomal regions prior to S phase and will stay bound to chromatin until its dissolution at the metaphase to anaphase transition (Michaelis et al., 1997). There is no consensus sequence of DNA that cohesin will bind to, although preferred cohesin binding sites exist. The centromere is the site of the most deposition of cohesin (Blat and Kleckner, 1999; Tanaka et al., 1999; Megee et al., 1999) and along the chromosome arms cohesin deposition generally occurs in intergenic regions with high AT content. Cohesion attachment sites are spaced roughly

10 to 15 kbases apart (Blat and Kleckner, 1999; Tanaka et al., 1999). The cohesin complex can be moved along chromatin and appears to localize to sites of convergent transcription (Glynn et al., 2004; Lengronne et al., 2004). Cohesin is loaded onto unreplicated chromatin by a protein complex consisting of Scc2p and Scc4p (both of which are essential for viability in budding yeast) (Ciosk et al., 2000) and this loading occurs in late G1. During DNA replication, sister chromatid cohesion will be established using the cohesin that has been loaded.

The establishment of sister chromatid cohesion is not well understood but is known to require replication and the acetyltransferase activity of Eco1p / Ctf7p (Skibbens et al., 1999; Toth et al., 1999). The proteinaceous ring structure of cohesin has given rise to the idea that cohesin encircles the two sister chromatids and that the connection between the sister chromatids is a topological one (Campbell and Cohen-Fix, 2002). The replication forks may slide through the cohesin complex and leave both sister chromatids circled by the cohesin complex. There is no clear structure that has been isolated showing a cohesin ring encircling sister chromatids and the idea that cohesin forms a ring around DNA remains unproven. Cohesin could form the “glue” that sisters bind to in order to remain associated with one another (Huang et al., 2005; Campbell and Cohen-Fix, 2002).

Sister chromatid cohesion must be maintained through G2 and into mitosis when all sister kinetochores have achieved bipolar attachment to the mitotic spindle. When bipolar attachment occurs cohesion must be dissolved between the sister chromatids in order to allow the sister chromatids to be pulled apart. This dissolution of sister chromatid cohesion is dependent on the cleavage of the Scc1p subunit of cohesin by

Separase (Uhlmann et al., 2000), and triggers the metaphase to anaphase transition. The Separase, Esp1p in budding yeast, is tightly regulated by a protein called Securin, Pds1p in budding yeast, in order to ensure that Scc1p cleavage and the dissolving of sister chromatid cohesion does not occur too early (reviewed in Yanagida, 2000). Securin is a protease inhibitor and binds to Separase to inhibit its activity. Pds1p is a target of the APC/C and its targeted degradation and subsequent activation of Esp1p is checkpoint dependent (discussed below). Inhibition of the APC/C (Cdc20p) induced by DNA damage or improper attachment of mitotic spindles and kinetochores can lead to high Pds1p levels that will generate a wait anaphase signal. In unperturbed cell cycles, once all sister kinetochores are properly attached, Pds1p is ubiquitinated by the APC and is degraded by the 26S proteasome. Its degradation triggers Esp1p protease activity, which will then cleave Scc1p leading to sister chromatid segregation. Pds1p is also required for the nuclear localization of Esp1p and in that manner also plays a role in activating Separase (Jensen et al., 2001). Scc1's cleavage by Esp1p releases the cohesin complex from sister chromatids by making an opening in the cohesin ring allowing the cohesin complex to dissociate from chromatin (Uhlmann et al., 2000). A cleavage product of Scc1p also prevents the association of Smc1p and Smc3p's head regions, providing another mechanism to ensure that sister chromatid cohesion is eliminated rapidly (Weitzer et al., 2003). This cleavage product is subsequently degraded in order not to interfere with cohesin in the next cell cycle (Rao et al., 2001). After mitotic exit and cytokinesis, the new daughter cells in G1 will begin preparing for a new cycle of sister chromatid cohesion by forming the Smc1p / Smc3p heterodimer in anticipation of Scc1p's transcription and translation in late G1 (Uhlmann, 2004).

One important difference exists between sister chromatid cohesion in budding yeast and sister chromatid cohesion in human cells. In budding yeast, the cohesin complex remains associated with centromeres and chromosome arms until anaphase whereas in human cells 95% of the cohesin (along the chromosome arms) is released during prophase (Morrison et al., 2003). Cohesin released during prophase is not dependent on Scc1p cleavage and the cohesin complex remains largely intact after being released from the chromosome arms. This release is dependent on the mitotic kinase, Polo like kinase (Polo, Cdc5p in budding yeast) but the mechanism for this release and why centromeric cohesion is unaffected is unclear (Sumara et al., 2002).

Additional proteins required for sister chromatid cohesion

There are other proteins that are required for sister chromatid cohesion. Pds5p is an essential protein that binds to the same chromosomal locations as the cohesin complex. *PDS5* mutants can establish cohesion but exhibit precocious sister chromatid separation and have a maintenance of cohesion defect (Hartman et al., 2000; Panizza et al., 2000). Pds5p is sumoylated (SUMO is a small protein conjugate similar to ubiquitin) and its sumoylation is required for the efficient dissolution of sister chromatid cohesion (Stead et al., 2003).

Characterization and isolation of the proteins involved in sister chromatid cohesion has employed a precocious sister chromatid separation assay (Guacci et al., 1994; Straight et al., 1996; Michaelis et al., 1997). This assay measured the percentage of cells that had precociously separated sister chromatids after DNA replication as measured in cells arrested in G2/M by a microtubule depolymerizing agent. With no

microtubules to pull sister chromatids apart, wild type cells should have sister chromatids in close proximity, whereas cells with defective cohesion show precocious separation of sister chromatids. In the most commonly used approach, budding yeast strains are constructed with tetracycline (TET) or lactose (LAC) operator sequence arrays integrated at different chromosome locations. Green Fluorescent Protein (GFP) tagged TET or LAC repressor proteins are expressed in these strains and visualization of the GFP signal is used to assess the proximity of sister chromatids containing the operator arrays. Defects in sister chromatid cohesion increase the percentage of cells that exhibit two GFP signals (indicating that there was precocious sister chromatid separation) as opposed to one signal (no precocious separation). Mutations in genes coding for subunits of the cohesin complex (*SCC1*, *SCC3*, *SMC1*, *SMC3*), or in the cohesin loading complex (*SCC2*, *SCC4*), caused a high increase in the percentage of cells that exhibit two GFP signals (up to 60% of mutant cells exhibited 2 GFP signals compared to 8% to 10% in wild type cells). An increasing number of non-essential gene deletion mutants (strains that contain a deletion of a non-essential gene) have been tested using this assay and intermediate values for precocious sister chromatid separation have been found (ranging from 15% to 30% compared to the 8% to 10% for wild type cells). The function of many of these proteins is unclear as they do not appear to be part of the cohesin complex or part of the chromatin loading process. Many of the proteins have been categorized as factors needed for establishment of cohesion during S phase, although with non-essential proteins, the exact time that they are needed is difficult to determine (Mayer et al., 2001; Mayer et al., 2004; Warren et al., 2004).

An interesting subset of the non-essential genes involved in cohesion appear to work together in an alternative RFC complex. Ctf18p replaces Rfc1p in the RFC complex and is found in a complex with the other four subunits (Rfc2,3,4 and 5) as well as Ctf8p and Dcc1p (Mayer et al., 2001). This alternative RFC complex plays a role in the establishment of sister chromatid cohesion and deletion mutants of *CTF8*, *CTF18* and *DCC1*, as well as mutants of *RFC4* show cohesion defects when tested by the precocious sister chromatid separation assay (Mayer et al., 2001). One model for the mechanism of action of this alternative RFC complex is that it is responsible for loading or unloading PCNA at replication forks in order to carry out a polymerase switch. The alternative RFC complex can load PCNA weakly onto DNA as well as unloading PCNA from DNA *in vitro* (Bylund and Burgers, 2005). This polymerase switch would facilitate DNA replication through sites of cohesion containing the cohesin complex (Mayer et al., 2001). Initially this idea looked promising as a new DNA Polymerase, Polymerase σ , was found and mutations in its subunits (*TRF4* and *TRF5*) displayed cohesion defects. Recently it has been shown however that polymerase σ may be a poly(A) polymerase instead of a DNA polymerase, and the pleiotropic effects of mutations may be an indirect consequence of altered protein expression (Saitoh et al., 2002; Read et al., 2002).

There are many intriguing connections between the replication fork machinery and sister chromatid cohesion. Temperature sensitive mutations in Eco1p / Ctf7p, required for establishing cohesion at S phase, can be suppressed by overexpression of PCNA (Skibbens et al., 1999) and Eco1p / Ctf7p can associate with RFC components (Kenna and Skibbens, 2003). Cohesion establishment may also occur shortly after the replication fork has passed by using PCNA that has stayed bound to DNA. Other genes

that have been implicated in sister chromatid cohesion include Chl1p, a DNA helicase; Mre11p involved in DNA damage checkpoint signaling (discussed below), Ctf4p, and Tof1p. Tof1p is a topoisomerase I interacting protein that is involved in the DNA damage response pathway in S phase. Proteins involved in microtubule dynamics such as Kar3p and Bim1p also have cohesion defects and the mechanism for this defect is unclear (Mayer et al., 2004; Warren et al., 2004).

Recent work has implicated the RSC (remodel the structure of chromatin) nucleosome remodeling complex in sister chromatid cohesion (Baetz et al., 2004; Hsu et al., 2003; Huang et al., 2004; Huang and Laurent, 2004). RSC is a multi-subunit complex that is part of the SWI/SNF family of ATP-dependent chromatin remodelers (Wang, 2003). Their main function is to reposition nucleosome arrays and change the underlying chromatin structure of chromosomes in order to facilitate or repress transcription. RSC contains distinct sub-complexes containing either Rsc1p or Rsc2p and these sub-complexes have different effects on sister chromatid cohesion and chromosome segregation. *rsc2Δ* mutants have a high rate of chromosome missegregation and more severe sister chromatid cohesion defects than *rsc1Δ* mutants. There are conflicting reports of whether or not the RSC complex is necessary for cohesin loading with one group claiming that RSC mutants have defective loading of Scc1p onto chromosome arms (with centromere loading unaffected) (Huang et al., 2004) while other groups have not seen this defect (Baetz et al., 2004). The RSC mutants do not appear to be involved in maintaining cohesion in G2/M. Transcription of genes involved in sister chromatid cohesion is not down-regulated in transcriptional profiling experiments performed on RSC mutants so the effect of RSC on sister chromatid cohesion is likely not due to RSC

remodeling chromatin to allow access of transcription factors to such genes. The RSC complex may play a role in the positioning of nucleosomes during replication and in that manner help to establish sister chromatid cohesion during replication.

Meiotic sister chromatid cohesion

Sister chromatid cohesion and kinetochore dynamics must be modified during meiosis (Petronczki et al., 2003; Watanabe, 2004). Meiotic division couples two rounds of chromosome segregation to one round of DNA replication, thereby forming $1n$ haploid progeny from a $2n$ diploid parent. In the first round of meiosis, homologous chromosomes are paired, bivalents are formed and homologous chromosomes undergo recombination facilitated by the synaptonemal complex. Sister kinetochores must attach to the same spindle pole body, and this attachment is promoted by a meiosis specific kinetochore protein, monopolin, Mam1 in budding yeast (Toth et al., 2000). Rec8p, a variant of Scc1p, replaces Scc1p in the cohesin complex during meiosis (Buonomo et al., 2000). Rec8p that is part of cohesin on the chromosomal arms, but not Rec8p at the centromere, is cleaved by Esp1p during meiosis I (after Pds1p is ubiquitinated and degraded by the APC, as in mitosis), and Rec8p containing cohesin is released from chromosome arms leading to loss of sister chromatid cohesion and the resolution of chiasmata. Rec8p containing cohesin on chromosome arms provides the attachment between homologous chromosomes, and once dissolved, homologous chromosomes (each consisting of two sister chromatids) are pulled to opposite spindle poles (Buonomo et al., 2000). Rec8p is protected from cleavage at the kinetochore by Sgo1p and this residual cohesin complex provides cohesion until the second round of meiosis (Kitajima

et al., 2004; Marston et al., 2004; Katis et al., 2004). During the second round of meiosis, Rec8p is cleaved at the centromere, Rec8p containing cohesin is released from the centromere, and centromeric cohesion is dissolved, allowing sister chromatids to separate (Klein et al., 1999; Buonomo et al., 2000). Sgo1p was initially characterized as a protein important for meiosis and Rec8p function but recent work has shown that it is also necessary for proper mitotic chromosome segregation and is involved in mitotic kinetochore and checkpoint functions sensing tension (Watanabe and Kitajima, 2005; Indjeian et al., 2005). Sgo1p's roles both at meiosis and mitosis highlights the need to study both processes for a clearer understanding of chromosome segregation.

Sister Chromatid Cohesion and DNA damage checkpoint and repair

The detection of, response to, and repair of DNA damage is of fundamental importance to genome stability. Double strand breaks (DSB) are particularly dangerous as they cause a disruption to both strands of DNA (Aylon and Kupiec, 2004). DSBs that are not dealt with correctly can lead to genome instability. Mechanisms have therefore evolved to respond to DNA damage that occurs spontaneously in the cell cycle (e.g. replication fork stalling and collapsing) as well as from environmental insults (e.g. ionizing radiation). There are two major pathways for the repair of DSBs, non-homologous end joining (NHEJ) and homologous recombination (HR) (Aylon and Kupiec, 2004). HR does not result in the loss of genetic material (i.e. DNA sequences surrounding the DSB) and is the primary pathway of repair of DSBs in budding yeast (Friedl et al., 1998; Lisby et al., 2004). It requires the presence of a template, either a sister chromatid or a homologous chromosome, in order to repair the DSB and therefore

much of this repair process occurs after replication when there is a sister chromatid as a template. Sister chromatid cohesion is important for this process and in budding yeast it has been shown that cohesion established during DNA replication is necessary for proper repair of DNA DSBs (Sjogren and Nasmyth, 2001). There has been some debate about whether the cohesion established during S phase, per se, is the requirement for post-replicative repair or if there was some other direct function of the cohesin complex in DSB repair. In human cells, cohesin has been shown to accumulate and localize at sites of DSBs (Kim et al., 2002) and a cohesin subunit (Smc1) is phosphorylated by ATM (Tel1) in response to DNA damage induced by ionizing radiation (Kitagawa et al., 2004; Kim et al., 2002b; Yazdi et al., 2002). This phosphorylation is necessary for the checkpoint response. Recent work in budding yeast has shown that cohesin does in fact localize to sites of DNA DSBs and that this localized concentration of cohesin is necessary for efficient repair of DSBs.

Through a series of elegant experiments using the HO endonuclease (a site specific nuclease that will generate a DSB) and Chromatin Immunoprecipitation (ChIP) two groups were able to show that in budding yeast the cohesin complex is recruited to flanking regions of a DSB (up to 100 kbases, 50 kbases each side of the DSB) (Unal et al., 2004; Strom et al., 2004). This recruitment of cohesin is required for efficient repair of DSBs and is dependent on Scc2p and Scc4p complex, the complex that loads cohesin onto chromatin. Cohesin that is recruited during the DNA damage response is functional and can prevent sister chromatid separation (Strom et al., 2004). This recruitment of cohesin is also dependent on the phosphorylation of Histone H2AX (the major Histone H2A variant in budding yeast) at sites of recruitment by Mec1p and Tel1p (Unal et al.,

2004). Mec1p and Tel1p are phosphoinositide 3-kinase-like kinases involved in the DNA damage checkpoint response pathway and recognition of DNA damage sensors (discussed below). Rad53p and Mre11p were also shown to be necessary for cohesin recruitment to sites of DSB. Mre11p is in a complex (Mre11p/Rad50p/Xrs2p, MRX complex) that localizes to DSBs and may serve as the early sensor and indicator of DNA damage and Rad53p is a kinase involved in transducing the DNA damage checkpoint signal (see below). The likely function of cohesin recruited to DSBs is to provide sister chromatid cohesion and ensure that the right sequences are used as donor templates in HR repair (Sjogren and Nasmyth, 2001; Strom et al., 2004; Unal et al., 2004). Are there other functions that cohesin is playing at sites of DSBs and DNA damage? In human cells the two SMC subunits of cohesin have also been shown to be required for the S phase checkpoint signaling pathway and it is not clear if this activity is independent of their functions in the cohesin complex (Lehmann, 2005).

Many of the deletion mutants of the non-essential genes coding for factors involved in sister chromatid cohesion are also hyper-sensitive to DNA damaging agents. *CTF8*, *CTF18*, *CHL1*, *DCC1*, and *CTF4* deletion mutants are hyper-sensitive to DNA damaging agents such as ionizing radiation, bleomycin, and methylmethanesulfonate (MMS) (Game et al., 2003). While these factors are non-essential for viability in budding yeast, their sensitivity to DNA damaging agents could represent their action in dealing with DNA damage. On the other hand, the sister chromatid cohesion defect may contribute to inefficient repair of damage caused by DNA damaging agents and the hyper-sensitivity is simply the result of the sister chromatid cohesion defect. Understanding the biochemical function of non-essential genes and the time that they are

needed represents further challenges in understanding all the different aspects of sister chromatid cohesion.

Checkpoints that Monitor Events and Damage during the Cell Cycle

Checkpoints are mechanisms that ensure that events in the cell cycle occur in the proper order and that the cell cycle is proceeding with no defects that may cause problems (Hartwell and Weinert, 1989). Checkpoint mechanisms will halt progress through the cell cycle to allow cells time to repair defects (Kastan and Bartek, 2004). There are irreversible events during the cell cycle and executing these events before the cell is ready can have detrimental effects on survival. Checkpoint mechanisms generally consist of three levels or steps; first there must be a mechanism for sensing the damage or defect, secondly the checkpoint must transduce or mediate this signal usually through protein kinases that phosphorylate substrates, and finally the signal must trigger responses through effector proteins such as arresting the cell cycle until repair is completed and also mobilizing factors that will repair the damage (Nyberg et al., 2002). The checkpoint mechanisms that will be discussed next are important for chromosome segregation and genome stability and include the DNA damage / S phase checkpoint and the spindle assembly checkpoint. These pathways are well conserved from budding yeast to humans and the medical importance of many of the proteins involved in these checkpoint functions is demonstrated by the mutations in genes encoding these proteins that have been found in tumour cell lines.

DNA Damage Checkpoint / S phase checkpoint

Damage to DNA can be caused by sources external to the cell such as ionizing radiation or sources that are the result of cell processes, such as free oxygen radicals that arise from normal cellular metabolism. During S phase replication forks can also become stalled if the nucleotide pool is disrupted or if there is a physical impediment to the replication forks such as DNA adducts caused by alkylating agents. A stalled replication fork that collapses would cause a DSB that requires repair. The DNA damage / S phase checkpoint is responsible for monitoring DNA for damage and responding to the damage (for reviews see Nyberg et al., 2002; Longhese et al., 2003; Murakami and Nurse, 2000).

The signals that trigger the DNA damage checkpoint have not been completely elucidated. Because there are many forms of DNA damage it is generally assumed that processing of the DNA damage into specific forms is a part of the process of detecting and signaling damaged DNA. Single stranded DNA and DSBs have both been postulated to be important structures that constitute the signal that the DNA damage checkpoint is responding to (Nyberg et al., 2002). The Rpa heterotrimer that binds single stranded DNA and is important for DNA replication is also important for DNA damage detection and response (Longhese et al., 1996; Santocanale et al., 1995). As well, an alternative RFC complex with Rad24p replacing Rfc1p can load a PCNA-like complex consisting of Rad17p/Ddc1p/Mec3p onto sites of DNA damage (Majka and Burgers, 2003). Also, the MRX complex localizes to DSBs and acts as a signal at DSBs (D'Amours and Jackson, 2002). These proteins are all involved in signaling DNA damage and are upstream of proteins involved in cell cycle arrest and DNA repair.

The transducers that serve to amplify the DNA damage signal and help to mediate a response consist of kinases that phosphorylate substrates in response to the initial DNA damage signal. The two chief mediators are phosphoinositide 3-kinase related kinases (PIKKs), Mec1p (ATR in human cells) and Tel1p (ATM in human cells) (Abraham, 2001). Biochemical evidence has suggested that Mec1p is important for amplifying the signal from single stranded DNA while Tel1p is more important for DSB signaling (Nyberg et al., 2002). Downstream kinases that are phosphorylated by Mec1p and Tel1p include Chk1p and Rad53p, both of which are important for inducing cell cycle arrest (Longhese et al., 2003).

DNA damage at various stages of the cell cycle can cause a halt in cell cycle progression until there has been time to repair the damage. G1-, S- and G2-arrest can all occur in response to DNA damage. The G1 arrest in response to DNA damage in budding yeast is weak and lasts for approximately an hour. The response is mediated by Rad53p phosphorylation of Swi4p/6p transcription factors thereby inhibiting and delaying the production of G1 cyclins (Sidorova and Breeden, 1997). Budding yeast cells can proceed through this temporary block into S phase even with DSBs. The likely reason for this weaker G1 DNA damage response is that homologous recombination from sister chromatid templates is the major repair mechanism in budding yeast (Nyberg et al., 2002). DNA damage that is encountered in S phase, such as DNA adducts caused by alkylating agents, result in stalled replication forks due to the physical impediment of the adduct as both checkpoint proficient and deficient cells have stalled replication forks (Tercero and Diffley, 2001). The DNA damage checkpoint in S phase (dependent on many of the factors involved in the replication fork, as well as Rad9p, Mec1p and

Rad53p) has three functions; it prevents firing of late origins of replication, it maintains the stalled replication fork so that DNA replication can proceed once damage is fixed, and it prevents entry into mitosis (Tercero and Diffley, 2001).

G2 DNA damage in budding yeast also causes arrest of cells, preventing entry into mitosis using the same machinery that is used by the spindle assembly checkpoint (discussed below). The checkpoint prevents the metaphase to anaphase transition by maintaining high levels of the securin, Pds1p, and preventing the release of Esp1p and the subsequent degradation and dissociation of cohesins from sister chromatids (Cohen-Fix and Koshland, 1997; Tinker-Kulberg and Morgan, 1999). The homologous recombination machinery (the MRX complex, the Rad52p epistasis group) is active in G2 to repair DSBs that have been generated directly or indirectly from processing of other types of DNA damage (Lisby and Rothstein, 2005). As well as arresting cells and allowing time for repair, effector responses can mediate transcriptional responses that are required for repair or adaptation to DNA damage. Genes that have been shown to be transcriptionally regulated by DNA damage include genes involved in DNA metabolism such as *RAD2*, *RAD18*, *RAD54*, *CDC9*, *RNR2* and *RNR3*. (Gasch et al., 2001)

The Spindle Assembly Checkpoint

The spindle assembly checkpoint monitors proper bi-polar attachment of the mitotic spindle to the kinetochores of sister chromatids. Spindle damage (for example, caused by microtubule depolymerizing drugs such as nocodazole or benomyl) will also trigger the spindle assembly checkpoint. The checkpoint will arrest cells in G2/M by preventing the metaphase to anaphase transition. Many of the proteins involved in the

spindle assembly checkpoint were first isolated in budding yeast and subsequently found to be conserved in multicellular eukaryotes. In budding yeast, the majority of the spindle assembly checkpoint genes are not essential and were isolated in random mutagenesis screens identifying mutants hypersensitive to microtubule depolymerizing drugs (for reviews see Lew and Burke, 2003; Skibbens and Hieter, 1998; Amon, 1999).

The signal that the spindle assembly checkpoint responds to has been proposed to be either lack of tension exerted at kinetochores or the lack of microtubule binding at the kinetochores (Lew and Burke, 2003). The difficulty in distinguishing between the two possibilities is that both processes are tied together and tension may regulate microtubule binding and unattached kinetochores are not under tension. For example, Ipl1p may destabilize kinetochore microtubule attachments that lack tension and signal this lack of tension. Alternatively, the transient unattached kinetochores that have been caused by Ipl1p activity may be the signal (Tanaka, 2005). Both processes are likely important as cellular localization of spindle assembly checkpoint components has shown differences dependent on the status of kinetochore microtubule attachments and tension exerted on kinetochores (Lew and Burke, 2003).

The components that are involved in the spindle assembly checkpoint have been extensively characterized in budding yeast. An intact kinetochore is considered to be necessary for the checkpoint response as it provides a platform to allow detection of impaired kinetochore microtubule attachments (Gardner et al., 2001; Goh and Kilmartin, 1993) and mutations in members of the CBF3 complex have an effect on the checkpoint because the kinetochore does not assemble properly. There is a direct connection between the CBF3 complex and the spindle assembly checkpoint as Skp1p has been

found to associate with Bub1p, and the association is necessary for signaling kinetochore tension defects (Kitagawa et al., 2003). The NDC80 complex also appears to play a role in checkpoint response. The kinetochore remains largely intact in mutants of the NDCc80 complex and this complex may play a role in recruiting and regulating the proteins involved in the checkpoint (Janke et al., 2001; McClelland et al., 2003). Mad1p, Mad2p, Mad3p, Bub1p (a protein kinase) and Bub3p, as well as Mps1p (involved also in SPB duplication and a protein kinase) are proteins important for the spindle assembly checkpoint (Lew and Burke, 2003; Amon, 1999). The prevailing hypothesis on how the checkpoint induces cell cycle arrest is that in the event of kinetochore microtubule defects, Mad2p is exchanged from a Mad1p/Mad2p complex to a Cdc20p/Mad2p complex. Cdc20p is one of the adaptors for the APC/C (APC/C (Cdc20p) is responsible for the ubiquitination and degradation of Pds1p securin) and Mad2p binding of Cdc20p prevents this activity, thus maintaining high levels of Pds1p and preventing the release of Esp1p, thus preventing the degradation and dissociation of cohesins from sister chromatids (Luo et al., 2000; Sironi et al., 2002; Yu, 2002).

Chromosome Transmission Fidelity Mutants and Characterizing Novel Genes Involved in Chromosome Segregation

In order to isolate and characterize genes involved in chromosome segregation, different screens have been performed in the past by monitoring loss of a chromosome marker on minichromosomes, chromosome fragments, or endogenous chromosomes. The strategy has been to use these screens as a primary screen to isolate mutants that have reduced chromosome transmission fidelity as the sole criterion and to subsequently

characterize the collection using secondary screens. The chromosome transmission fidelity (ctf) mutant collection takes advantage of an artificial chromosome fragment that was engineered to contain the *SUP11* suppressor gene (Spencer et al., 1990). The chromosome fragment was introduced into yeast strains that contained the *ade2* mutation. The *ade2* mutation causes a red pigment to accumulate in yeast colonies while the *SUP11* suppressor will suppress this mutation and the yeast colony will be white. This provides a visual assay for marker loss by observing the rate of sectoring (red sectors in a white yeast colony) and was the basis for isolating mutants that increased the rates of sectoring (and by extension, the rate of loss and non-disjunction of endogenous chromosomes) compared to wild type yeast cells. The initial set of ctf mutants were isolated using ethylmethanesulfonate (EMS) mutagenesis and led to the identification of mutants that were involved in different aspects of chromosome segregation such as kinetochore function, sister chromatid cohesion, and DNA metabolism.

Early work in the Hieter laboratory focused on finding the ctf mutants that were involved in kinetochore function. To identify the relevant mutants, secondary assays were developed. One example of a secondary assay used is the centromere transcriptional readthrough assay assessing kinetochore proteins binding to CEN in ctf mutants. It employed a reporter gene under the GAL promoter introduced near the CEN sequence. Ctf mutants defective for kinetochore function allow expression of the reporter gene while other mutants or wild type strains did not allow expression (Doheny et al., 1993). Another example is Synthetic Dosage Lethality (SDL) whereby overexpressing a gene involved in kinetochore function may have no effect in wild type cells but may cause lethality in a kinetochore defective mutant (Kroll et al., 1996; Hyland et al., 1999).

The lethality is most likely due to compromising what few proper kinetochores have assembled by further upsetting the stoichiometric balance in the cell. Using these secondary assays, several *ctf* genes have been identified and subsequently characterized as members of the inner and central kinetochore (such as Ctf13p, Ctf14p (Ndc10p), Ctf19p, Ctf3p, Chl4p) (Hyland et al., 1999; Measday et al., 2002; Pot et al., 2003).

An alternative approach is to employ genetic interaction screens using mutations in genes known to be important components of the segregation machinery as genetic entry points for identifying additional genes important for chromosome segregation. High copy suppression occurs when having multiple copies of a gene that is functionally related to a second gene, rescues a phenotype of mutants in the second gene (typically temperature sensitivity). High copy suppression screens have been used to identify interacting proteins as well as proteins involved in similar biochemical pathways. *SKP1* (Suppressor of Kinetochore Protein) was isolated as a high copy suppressor of the temperature sensitive mutation *ctf13-30* and was subsequently characterized to be a member of the CBF3 complex (Connelly and Hieter, 1996) as well as being a subunit of the SCF complex (Bai et al., 1996). *SGT1* (Suppressor of the G2 allele of *SKP1*) was found as a high copy suppressor of *skp1-4* and was subsequently characterized as a protein partner of Skp1 at both the kinetochore and the SCF complex (Kitagawa et al., 1999). Further characterization of the budding yeast kinetochore and other components of the chromosome segregation machinery could therefore be undertaken with *SGT1* as the potential springboard for genetic screens that may isolate novel interactors and regulators.

Overview and Scope of the Thesis

When this work began there were relatively few components of the budding yeast kinetochore identified and characterized. In the last five years there has been a large increase in the number of proteins that have been identified and placed at the kinetochore. Many of the proteins that have been placed in the central kinetochore have functions that are largely unclear. Finding regulators of these proteins and the yeast kinetochore could shed new light on many of these proteins. Work in the yeast community in the last few years has also elucidated much of the mechanism of sister chromatid cohesion and how it affects bi-orientation of sister kinetochores and also how it affects post-replicative DNA repair.

The goal of this work was to further characterize components important for chromosome segregation, initially at the budding yeast kinetochore but also at sister chromatid cohesion as the work continued to progress. We began this work using *SGT1* as a starting point. A class of temperature sensitive mutations in *SGT1* display defects in kinetochore function and we were interested in identifying high copy suppressors of the temperature sensitivity. We reasoned that this may identify novel components or regulators of the yeast kinetochore. To identify potential protein partners of Sgt1p, we carried out a two-hybrid screen using *SGT1* as the “bait” against the genome wide collection of ORFs in collaboration with Stan Fields. Phenotypic analysis of deletion mutants corresponding to genes that were identified in the two screens focused our interest on an uncharacterized ORF, YDR014W. Much of the work in the second half of Chapter 2 and in the majority of Chapter 3 is focused on characterizing the role of

YDR014W (*RAD61*) in chromosome segregation and the function of a Rad61p protein interaction partner, Ded1p.

Chapter 2:

Analysis of *SGT1* genetic and physical interactions and identification of a factor involved in chromosome segregation, *RAD61* / *CTF6*

Introduction

Two approaches were undertaken to identify genes that could be important for chromosome segregation and in particular, kinetochore function. Both approaches attempted to identify proteins that were playing a role at the budding yeast kinetochore in concert with Sgt1p. The first approach utilized strains that contained the *sgt1-3* mutation that causes arrest in G2/M at non-permissive temperature. We looked for genes that when present in multiple copies, could suppress the temperature sensitive phenotype of *sgt1-3* mutant strains. This high copy suppression screen identified four ORFs, that when present in multiple copies, rescued the lethality of *sgt1-3* strains at 37°C. The second approach was to identify protein partners of Sgt1p by using *SGT1* as the “bait” in a genome wide two-hybrid screen in collaboration with Stan Fields. We focused on YDR014W / *RAD61* / *CTF6* because the deletion mutant displayed a chromosome missegregation phenotype. The experiments that we performed in the second half of the chapter were designed to characterize Rad61p’s role in chromosome segregation.

Materials and Methods

Yeast strains, growth conditions and media

Yeast strains used in this study are listed in Table 2-1. Strains were grown at 25°C unless otherwise indicated. Temperature sensitivity and lethality was assessed at 37°C. Media for growth and sporulation were described previously (Rose et al., 1990). To visualize the loss of the non-essential chromosome fragment (CF), the strains were first grown in SC media lacking either uracil or tryptophan (selecting for the CF), then either plated (200 to 300 cells per plate) or streaked to single colonies onto SC complete media with 65 μ M adenine concentration. Colonies were visualized after growth at 25°C or the temperature indicated for 3 to 4 days followed by 1 or 2 days at 4°C to increase the resolution of the red versus white sectors. Cells that contain the CF will appear as white colonies while cells that have lost the CF will appear as red colonies. For assays testing sensitivity to microtubule depolymerizing drugs, benomyl from DuPont (Wilmington, DE) was dissolved in dimethylsulphoxide (DMSO) at 10 mg/mL and added to the indicated concentrations to YPD plates, with addition of DMSO lacking drug as a control. Methylnmethanesulfonate (MMS) in liquid form (99% pure, from Sigma) or bleomycin (10 Units/mL dissolved in distilled water) was added to YPD plates at the indicated concentrations. Epitope tagging and deletion of genes were performed at the endogenous loci based on Longtine et al. (1998). Transformations of yeast cells was performed according to Gietz and Schiestl (1995).

High Copy Suppressor Screen and Confirmation

A 2 μ URA genomic library (Connelly and Hieter, unpublished) was transformed into YKK 66 strain carrying the *sgt1-3* mutation, and transformants were first selected at 25°C for 3 days before replica plating to 37°C. We identified ~300 yeast colonies that grew at 37°C from 4 x 10⁵ yeast transformants and pooled them into 6 fractions of approximately 50 colonies each. We purified plasmid DNA from the pooled colonies (containing plasmid DNA that rescued the conditional lethality at 37°C of *sgt1-3*). Purified plasmid DNA was transformed into *E. coli* and the bacterial transformants were screened by colony hybridization for *SGT1*, *SKP1* and *CTF13* genomic sequences. We found 18% of the bacterial colonies contained an insert with *SGT1*, 1% *SKP1*, and 2% *CTF13*. Of the remaining transformants, we purified plasmid DNA from 70 colonies and retransformed the plasmid DNA into YKK 66 to confirm the suppression of the temperature sensitivity phenotype. We found four transformants that were able to suppress the conditional lethality of *sgt1-3* at 37°C. Sub-cloning using restriction sites present in the genomic fragments was used to identify the ORFs that were responsible for suppression. Putative ORFs were cloned into GAL promoter containing vectors and transformed into YKK 66. YKK 66 strains containing the GAL vectors were allowed to grow on galactose or glucose plates at 25°C or 37°C to check that suppression was based on expression of the ORF.

Co-immunoprecipitation experiments from yeast extracts

Co-immunoprecipitations were performed as described previously in Measday et al., (2002). In brief, yeast extracts were generated using glass bead lysis, equal amounts of extracts (as measured by Bradford Assay) were incubated with conjugated Anti-MYC or Anti-HA beads (Covance). Co-IPs were performed overnight at 4°C, washed in extract buffer for a minimum of 4 times, and eluted with sample buffer at 100°C for 4 minutes.

Genome wide two-hybrid assay

SGT1 was cloned into pOBD2 as described by Cagney et al., (2000). The pOBD2-*SGT1* was functional based on complementation of *sgt1-3* temperature sensitive mutants. The two-hybrid screen was performed as described by Uetz et al., (2000). Putative two hybrid positives were retested and confirmed by transforming the pOAD vectors containing the putative positives with pOBD2-*SGT1* and testing for interaction.

Fluorescence Activated Cell Sorting (FACS) Analysis

FACS analysis was performed as previously described in Hyland et al., (1999).

Chromosome Spreads and Chromatin Binding Assays

Chromosome spreads were performed as previously described in Michaelis et al., (1997). 9E10 Anti-MYC antibodies from Covance were used as the primary antibody with a CY3-GAM secondary used to visualize Rad61-13MYCp staining. DAPI was used as the marker for chromatin DNA. Chromatin Binding was performed as previously

described in Donovan et al., (1997) with histone H4 and Carboxypeptidase-Y used as controls for a chromatin binding protein and a cytoplasmic protein respectively.

Stability Assays on Rad61p

The yeast mating pheromone, alpha-factor, was added to logarithmically growing cell cultures at concentrations of 5 $\mu\text{g}/\text{mL}$ and strains were arrested for 3 hours at 25°C. Cells were washed with YPD and released into YPD media. Protein extracts were made as previously described in Measday et al., (2002) and western blots performed using anti-Cdc28 antibodies and the 9E10 Anti-MYC antibody from Covance at the time points indicated as well as preparing FACS samples. For assays investigating the half life of Rad61p, we added cyclohexamide to a concentration of 10 $\mu\text{g}/\text{mL}$ and protein extracts were made at the time points indicated. For testing the stability of Rad61p in *sgt1-5* mutants compared to wild type, we cloned HA-Rad61 into a GAL expression vector and transformed it into YKK 57 (*sgt1-5*) and wild type strains. Cultures were grown in liquid media selecting for the plasmid vector with glucose as the carbon source. Cells were subsequently harvested, washed and allowed to grow in raffinose for 2 hours. Galactose was added to a concentration of 2% for 2 hours to induce *HA-RAD61* transcription, time point 0 was upon subsequent addition of glucose to repress transcription of *HA-RAD61*.

Microscopy Experiments and Indirect Immunofluorescence

Strains used for microscopy were grown in FPM (Synthetic Complete medium supplemented with adenine and 6.5 g/L sodium citrate) in order to reduce auto-fluorescence. Unless otherwise indicated microscope pictures are of live cells harvested

during log growth, and resuspended in an equal mixture of FPM and 1.2% low melting point agarose to immobilize cells on the microscope slide. Cells were fixed by addition of 4% paraformaldehyde to equal amounts of liquid culture (to a final concentration of 2% paraformaldehyde) for 10 minutes at room temperature and washed with SK (1 M Sorbitol, 50 mM KPO₄ pH 7.5) media. Microscope images were taken with a Zeiss Axioplan microscope equipped with a CoolSNAP HQ camera (Photometrics) and Metamorph (Universal Imaging). Stacks or one focal plane of images were taken as indicated in the figure legends. Indirect immunofluorescence was performed as previously described in Hyland et al., (1999). Anti-MYC (9E10) antibodies were applied at a dilution of 1/1000 for 1 hour at 37°C, and secondary fluorescent antibody GAN-F or GAN-R was applied at a dilution of 1/1000 at 37°C for 1 hour also. DAPI was contained in the mounting media.

GAL-HO experiments and Double Strand Break induction experiments

Plasmids containing the HO endonuclease under the control of the GAL promoter (plasmid 132) were transformed into wild type, *rad52*Δ, and *rad61*Δ haploid strains and lethality was investigated on YEP plates with galactose as the carbon source instead of glucose (YPG plates). Experiments involving the induction of the HO endonuclease from the GAL-HO plasmid in order to generate a double strand break and investigation of the co-localization of Rad52p to that double strand break site were performed as previously described in Lisby et al., (2003).

Table 2-1 List of yeast strains used in this chapter

Strain	Genotype	Reference
YPH499	Mata <i>ura3-52 lys2-801 ade2-101 his3Δ200 leu2Δ1 trp1Δ63</i>	Sikorski and Hieter., 1989
YPH500	Mata <i>ura3-52 lys2-801 ade2-101 his3Δ200 leu2Δ1 trp1Δ63</i>	Sikorski and Hieter., 1989
YPH501	Mata/α <i>ura3-52/ura3-52 lys2-801/lys2-801 ade2-101/ade2-101</i>	Sikorski and Hieter., 1989
YBC15	<i>his3Δ200/his3Δ200 leu2Δ1/leu2Δ1 trp1Δ63/trp1Δ63</i>	This study
YBC16	Mata <i>ura3-52 lys2-801 ade2-101 his3Δ200 leu2Δ1 trp1Δ63 sog2Δ::HIS3</i>	This study
YBC17	Mata <i>ura3-52 lys2-801 ade2-101 his3Δ200 leu2Δ1 trp1Δ63 sog2Δ::HIS3</i>	This study
YKK560	Mata <i>ura3-52 lys2-801 ade2-101 his3Δ200 leu2Δ1 trp1Δ63 sog2Δ::HIS3 CFIII (CEN3.L.) TRP1 SUP11</i>	K. Kitagawa
YKK556	Mata <i>ura3-52 lys2-801 ade2-101 his3Δ200 leu2Δ1 trp1Δ63 sog3Δ::HIS3</i>	K. Kitagawa
YKK529	Mata <i>ura3-52 lys2-801 ade2-101 his3Δ200 leu2Δ1 trp1Δ63 sog3Δ::HIS3 CFIII (CEN3.L.) TRP1 SUP11</i>	K. Kitagawa
YBC108	Mata <i>ura3-52 lys2-801 ade2-101 his3Δ200 leu2Δ1 trp1Δ63 sgt1Δ::HIS3 6HA-SGT1-LEU2 @ Ch.III</i>	This study
YBC109	Mata <i>ura3-52 lys2-801 ade2-101 his3Δ200 leu2Δ1 trp1Δ63 SOG2-13MYC-TRP1 sgt1Δ::HIS3 6HA-SGT1-LEU2 @ Ch.III</i>	This study
YKK57	Mata <i>ura3-52 lys2-801 ade2-101 his3Δ200 leu2Δ1 trp1Δ63 SOG3-13MYC-TRP1 sgt1Δ::HIS3 6HA-SGT1-LEU2 @ Ch.III</i>	K. Kitagawa
YKK190	Mata <i>ura3-52 lys2-801 ade2-101 his3Δ200 leu2Δ1 trp1Δ63 sgt1-5:LEU2</i>	K. Kitagawa
YCTF7	Mata <i>ura3-52 lys2-801 ade2-101 his3Δ200 leu2Δ1 trp1Δ63 sgt1-3:LEU2</i>	Spencer et al., 1990
YCTF40	Mata <i>ura3-52 lys2-801 ade2-101 his3Δ200 leu2Δ1 trp1Δ63 CFIII (CEN3.L.) URA3 SUP11 ctf6-7</i>	Spencer et al., 1990
YCTF60	Mata <i>ura3-52 lys2-801 ade2-101 his3Δ200 leu2Δ1 trp1Δ63 CFIII (CEN3.L.) URA3 SUP11 ctf6-40</i>	Spencer et al., 1990
YCTF134	Mata <i>ura3-52 lys2-801 ade2-101 his3Δ200 leu2Δ1 trp1Δ63 CFIII (CEN3.L.) URA3 SUP11 ctf6-60</i>	Spencer et al., 1990
YPH311	Mata <i>ade2-101 his3Δ200 ura3-52 tub1-1</i>	Spencer et al., 1990
YPH312	Mata <i>ade2-101 ura3-52 tub2-104</i>	Spencer et al., 1990
YBC197	Mata <i>ura3-52 lys2-801 ade2-101 his3Δ200 leu2Δ1 trp1Δ63</i>	This Study
YBC198	Mata <i>ura3-52 lys2-801 ade2-101 his3Δ200 leu2Δ1 trp1Δ63 RAD61-13MYC-HIS3</i>	This study

Table 2-1 List of yeast strains used in this chapter, continued

YBC204	Mata <i>ura3-52 lys2-801 ade2-101 his3Δ200 leu2Δ1 trp1Δ63</i>	This study
YBC205	<i>rad61Δ::HIS3</i> CFIII (CEN3.L) <i>TRP1 SUP11</i>	This study
YBC207	Mataα <i>ura3-52 lys2-801 ade2-101 his3Δ200 leu2Δ1 trp1Δ63</i> <i>rad61Δ::HIS3</i> CFIII (CEN3.L) <i>TRP1 SUP11</i>	This study
YBC309	Mata/α <i>ura3-52/ura3-52 lys2-801/lys2-801 ade2-101/ade2-101</i> <i>his3Δ200/his3Δ200 leu2Δ1/leu2Δ1 trp1Δ63/trp1Δ63</i> <i>rad61Δ::HIS3/rad61Δ::HIS3</i>	This study
YBC200	Mata/α <i>ura3-52/ura3-52 lys2-801/lys2-801 ade2-101/ade2-101</i> <i>his3Δ200/his3Δ200 leu2Δ1/leu2Δ1 trp1Δ63/trp1Δ63</i> <i>rad61Δ::HIS3/rad61Δ::HIS3 mad2Δ::HIS3/mad2Δ::HIS3</i>	This study
YBC201	Mata <i>ura3-52 lys2-801 ade2-101 his3Δ200 leu2Δ1 trp1Δ63</i> <i>sgt1Δ::HIS3 6HA-SGT1-LEU2 @ Ch.III RAD61-13MYC-TRP1</i>	This study
YPH1555	Mata <i>ura3-52 lys2-801 ade2-101 his3Δ200 leu2Δ1 trp1Δ63</i> <i>sgt1Δ::HIS3 6HA-SGT1-LEU2 @ Ch.III RAD61-13MYC-TRP1</i>	Pot et al., 2003
YBC671	Mata <i>ura3-52 lys2-801 ade2-101 his3Δ200 leu2Δ1 trp1Δ63</i> <i>NDC10-13MYC-kanMX6</i>	This study
YBC672	Mata <i>ura3-52 lys2-801 ade2-101 his3Δ200 leu2Δ1 trp1Δ63</i> <i>RAD61-VFP-kanMX6</i>	This study
YBC673	Mataα <i>ura3-52 lys2-801 ade2-101 his3Δ200 leu2Δ1 trp1Δ63</i> <i>RAD61-VFP-kanMX6</i>	This study
YBC674	Mata <i>ura3-52 lys2-801 ade2-101 his3Δ200 leu2Δ1 trp1Δ63</i> <i>RAD61-VFP-kanMX6 NIC96-CFP-hphMX</i>	This study
YBC658	Mataα <i>ura3-52 lys2-801 ade2-101 his3Δ200 leu2Δ1 trp1Δ63</i> <i>RAD61-VFP-kanMX6 NIC96-CFP-hphMX</i>	Lisby et al., 2003
YBC661	Mata <i>ade2-1 bar1::LEU2 trp1-1 LYS2 RAD5 RAD52-CFP</i> <i>ura3::3xURA3-tetOx112 I-SceI(ura3-1) his3-11,15::YFP-LacI-</i> <i>his3-x leu2-3,112::LacO-LEU2 HO-iYCL018W(leu2-3,112)</i> <i>TetR-RFP(iYGL119W)</i> (W303 background)	This study
YBC675	Mata <i>ade2-1 bar1::LEU2 trp1-1 LYS2 RAD5 RAD52-CFP</i> <i>ura3::3xURA3-tetOx112 I-SceI(ura3-1) his3-11,15::YFP-LacI-</i> <i>his3-x leu2-3,112::LacO-LEU2 HO-iYCL018W(leu2-3,112)</i> <i>TetR-RFP(iYGL119W) rad61Δ::HIS3</i> (W303 background)	Giaever et al., 2002
YPH1474	Mata <i>his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 rad52Δ::kanMX6</i> <i>CTF8-13MYC-HIS3</i>	Mayer et al., 2001
YPH1020	Mataα <i>ura3-52 lys2-801 ade2-101 his3Δ200 leu2Δ1 trp1Δ63</i> <i>CFIII (CEN3.L) TRP1 SUP11</i>	Spencer et al., 1990

Results

Identification of High Copy Suppressors of *sgt1-3* (Suppressors of the G2 allele of *SGT1*, *SOGs*)

Sgt1p is a protein that, together with its direct binding partner Skp1p, is essential for two distinct functions: 1) in the assembly of the budding yeast kinetochore, 2) and as a subunit of the SCF E3 ubiquitin ligase complex (Kitagawa et al., 1999). *SGT1* is an essential gene and, consistent with the two distinct functions of the Skp1p/Sgt1p complex, temperature sensitive mutants carrying mutations in *SGT1* fall into either of two classes; at the restrictive temperature one class arrests at G1/S with small budded cells (*sgt1-5*), while the other class arrests at G2/M with large budded cells (*sgt1-3*) (Kitagawa et al., 1999). The G1/S alleles are defective in SCF function while the G2/M alleles are defective in kinetochore function. High copy suppressor screens led to the initial identification of *SKP1* as a suppressor of *ctf13-30*, and *SGT1* as a suppressor of *skp1-4* (Connelly and Hieter, 1996; Kitagawa et al., 1999). In an attempt to identify additional novel genes important for budding yeast kinetochore function, we performed a multi-copy suppressor screen of an *sgt1-3* mutant.

A genomic library carried in a high copy plasmid was transformed into a yeast strain that harbored the *sgt1-3* mutation and a screen was performed for genomic segments that could rescue the lethality of the strain when grown at 37°C (see Materials and Methods for the details of the screen). Genomic fragments that could suppress the temperature sensitivity of *sgt1-3* were sub-cloned to find the minimal sequences that could still suppress the temperature sensitivity. Four Open Reading Frames (ORFs) that were not *SGT1*, *SKP1*, or *CTF13* were identified that could suppress the temperature

sensitivity of *sgt1-3*. The four ORFs identified were YLR388W (*RPS29A*), YLR073C, YDR266C, and YDL190C (*UFD2*). We provisionally named YLR073C and YDR266C *SOG2* and *SOG3* for Suppressors of the G2 allele of *SGT1*. To confirm that expression of these ORFs could indeed suppress the temperature sensitivity phenotype of *sgt1-3*, we cloned each ORF into a vector that contained a galactose inducible promoter and showed that in each case the suppression of the temperature sensitivity depended on the expression of the ORFs. An example suppression of the temperature sensitivity phenotype of *sgt1-3* by *SOG2* or *RPS29A*, as well as a negative control, YLR387C, and vector alone, is shown in Figure 2-1. Expression of Sog2p and Rps29ap in *sgt1-3* strains rescued the temperature sensitivity phenotype of the *sgt1-3* strain and allowed growth at 35°C whereas vector alone or expressing the protein product of YLR387C did not rescue the temperature sensitivity phenotype and the strains did not grow at 35°C.

***SOG2* and *SOG3* do not encode Sgt1p binding proteins and are not required for chromosome segregation**

RPS29A is a ribosomal structural subunit (91% identical to *RPS29B* in budding yeast and 67% identical to human *RPS29*) and its function is well characterized in mRNA binding and translation. *UFD2* is a gene involved in assembly of multiubiquitin chains on ubiquitin-protein conjugates. The deletion mutants of *RPS29A* and *UFD2* are viable and do not have chromosome missegregation phenotypes as measured by three different screens, the sectoring assay (loss of a non-essential chromosome fragment), the a – like faker mating screen, and the bi-mater screen (Karen Yuen, personal communication) and,

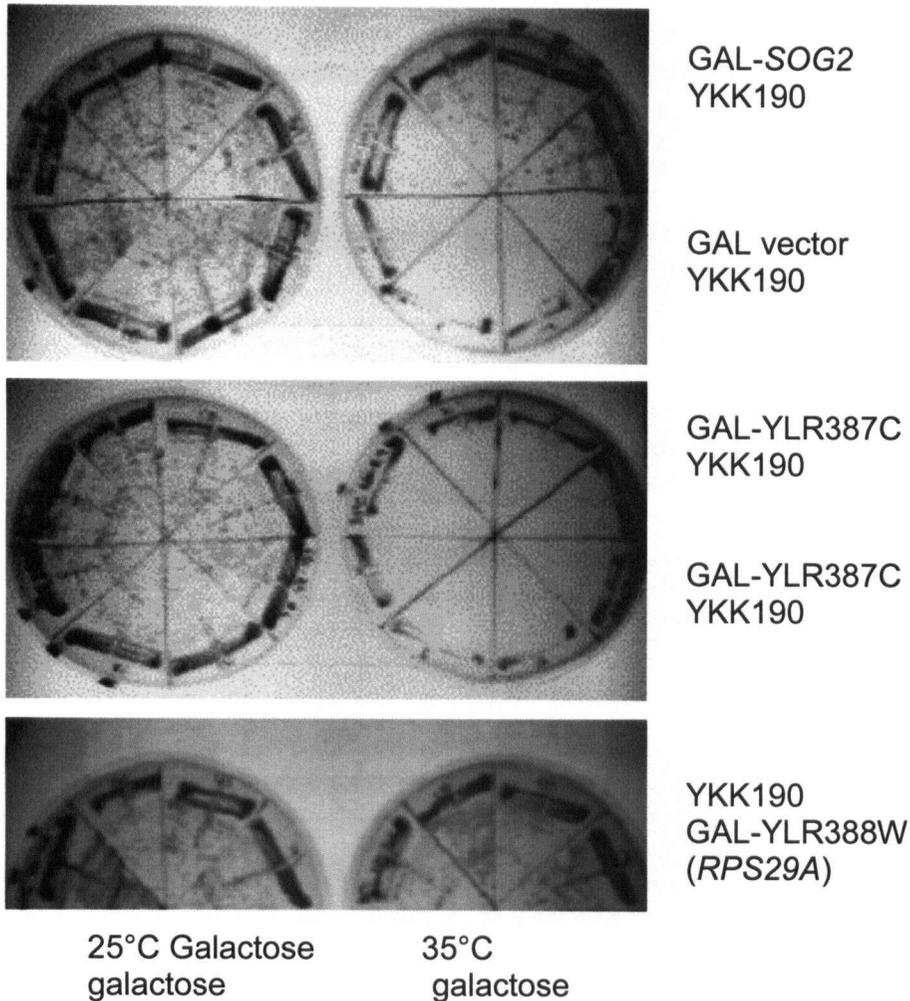


Figure 2-1 - Suppression of the temperature sensitivity of *sgt1-3* by overexpression of *SOG2* and *RPS29A*.

An example of suppression. Vectors carrying the *SOG2* (YLR073C), *RPS29A* (YLR388W), and a candidate gene (YLR387C – negative) with a Galactose inducible promoter, were transformed into strains carrying the temperature sensitive allele *sgt1-3*. Strains were then streaked and allowed to grow at 25°C or 35°C for 4 days on galactose media. *SOG2* and *RPS29A* when overexpressed were able to suppress the lethality of *sgt1-3*, but not the vector alone or YLR387C.

therefore, these two genes were not further characterized. *SOG2* and *SOG3* were previously uncharacterized ORFs that coded for proteins of unknown function.

We performed phenotypic assays on the deletion mutants of *SOG2* and *SOG3* in order to investigate any potential role of Sog2p or Sog3p in chromosome segregation. Neither *sog2Δ* nor *sog3Δ* strains exhibited increased rates of missegregation of a marker chromosome fragment relative to wild type (data not shown). The strains also did not show sensitivity to the microtubule depolymerizing drug benomyl (Figure 2-2). Benomyl sensitivity is a hallmark of genes that are involved in kinetochore or sister chromatid cohesion function. In addition, *SOG2* and *SOG3* did not display genetic interactions such as SDL with components of the inner and central kinetochore. For example, when we overexpressed *CTF13*, *NDC10*, *SKP1*, *SGT1*, and *CTF19* from galactose inducible promoters in *sog2Δ* and *sog3Δ* strains, there was no effect on growth of the deletion mutants, as compared to wild type strains (data not shown).

We performed co-immunoprecipitation experiments using yeast extracts to determine if either Sog2p or Sog3p interacted physically with Sgt1p. Epitope tagged constructs of Sog2p, Sog3p and Sgt1p were made at their endogenous loci. Yeast extracts were prepared from strains containing *SOG2-13MYC* and *SGT1-HA* (lanes 1 to 3 in Figure 2-3a and 2-3b), *SOG3-13MYC* and *SGT1-6HA* (lanes 4 to 6 in Figure 2-3a and 2-3b), and *SGT1-6HA* alone as a negative control (lanes 7 to 9 in Figure 2-3a and 2-3b). An anti-MYC IP was performed on the extracts and we did not detect any interaction between Sgt1-6HAp and Sog2-13MYCp or Sog3-13MYCp (Figure 2-3b, see lanes 3 and 6). From this co-immunoprecipitation experiment it appears that neither Sog2p nor Sog3p physically interact with Sgt1p.

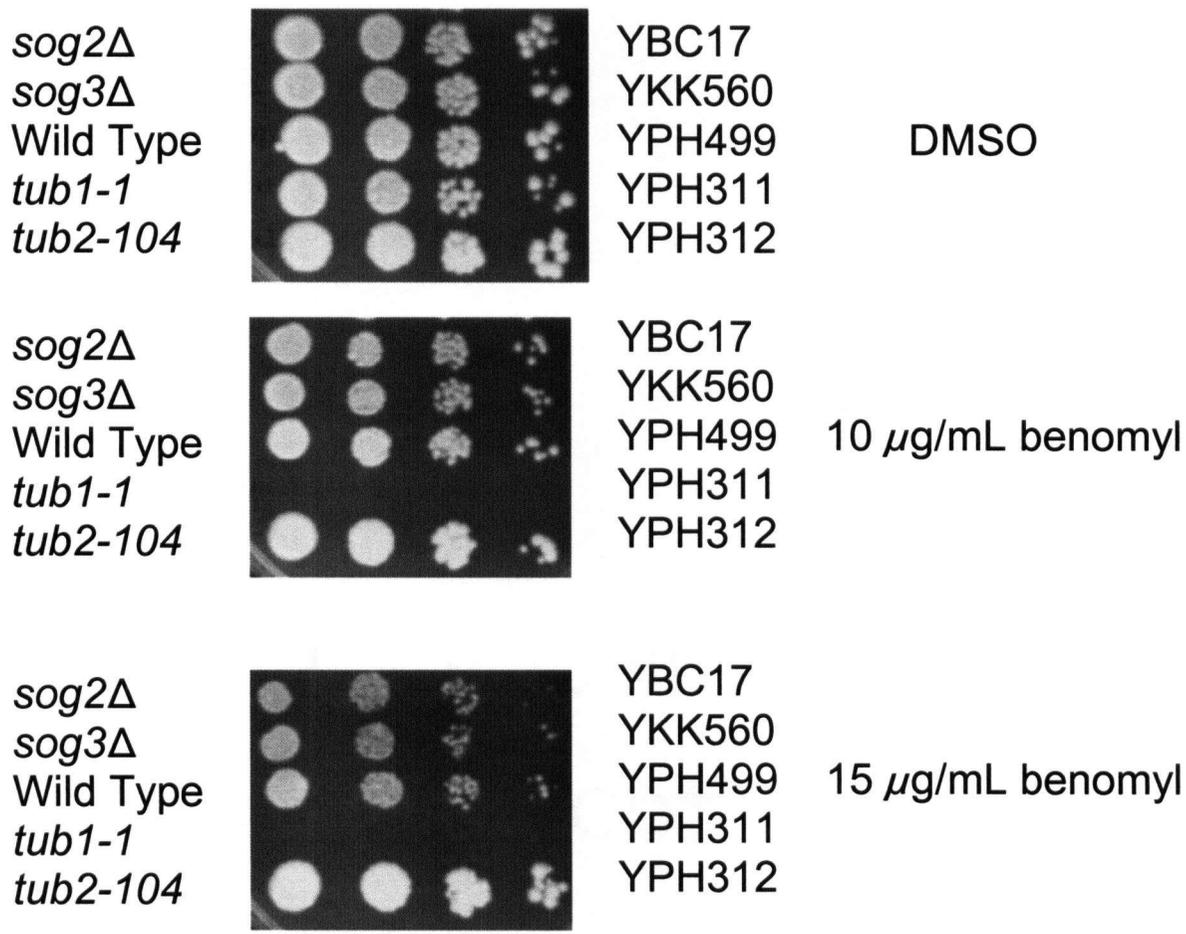


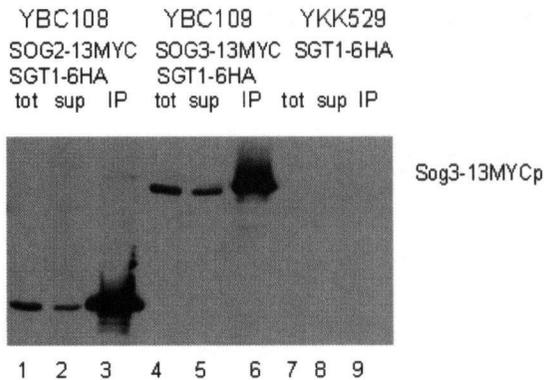
Figure 2-2 - *sog2* and *sog3* deletion mutants show similar sensitivity to benomyl as wild type strains.

Serial dilutions (10x) were plated on media containing increasing amounts of benomyl. *tub1-1* (sensitive to benomyl) and *tub2* mutants (resistant to benomyl) were used as controls. Cells were grown at 25°C for 5 days.

By phenotypic analysis of the deletion mutants and co-immunoprecipitation tests for protein-protein interaction in whole cell extracts, we determined that Sog2p and Sog3p were not important for chromosome segregation and do not interact with Sgt1p. We therefore did not pursue further analysis of the suppressor genes.

Figure 3 Sog2p and Sog3p do not co-IP with Sgt1p in yeast extracts

a) Anti-MYC IP, Anti-MYC Western



b) Anti-HA Western



Figure 2-3 – Sog2p and Sog3p do not co-IP with Sgt1p in yeast extracts.

Yeast extracts from strains expressing SOG2-13MYCp and SGT1-6HAp (lanes 1 to 3), SOG3-13MYCp and SGT1-6HAp (lanes 4 to 6), or SGT1-6HAp (lanes 7 to 9) were used for immunoprecipitation using Anti-MYC beads (9E10). A) Anti-MYC (9E10) Western to show the IP of the MYC tagged constructs, we loaded an excess amount of IP sample to detect any interaction thus the large amounts seen in the IP lanes (3 and 6). B) Anti-HA (12CA5) Western to detect Sgt16HAp in the IPs. Sgt1-6HAp appears as a doublet. Lanes 7 to 9 were used as a negative control for non-specific binding of Sgt1-6HAp to the Anti-MYC beads.

Identification of an ORF, YDR014W, as an SGT1 two hybrid interacting gene required for chromosome transmission fidelity

As an alternative approach to identifying novel genes involved in chromosome transmission fidelity, we performed a genome wide two hybrid screen using *SGT1* as the protein partner “bait”. *SGT1* was cloned into a DNA binding domain vector (pOBD2) that created a fusion protein containing the GAL4 DNA binding domain fused to *SGT1*. The construct was then screened against the entire set of yeast open reading frames fused to the GAL4 activating domain. The two-hybrid screen identified several expected

interactors, including *SKP1* and several F-box containing genes (*CDC4*, *GRR1*, and *MET30*) that are also members of the SCF complex (Table 2-2). YDR014W, an ORF of unknown function, was also identified. When the YDR014W gene was deleted, the deletion mutant missegregated an artificial marker chromosome fragment at a rate higher than wild type (Figure 2-4). We performed a half-sector analysis by counting colonies

Table 2-2 Genome wide two hybrid interactions with *SGT1*

Bait	Target	ORF	Function
<i>SGT1</i>	<i>SKP1</i>	YDR328 C	Kinetochore complex and component of SCF
	<i>CDC4</i>	YFL009 W	F-box protein and component of SCF complex
	<i>GRR1</i>	YJR090 C	F-box protein and component of SCF complex, glucose repression and glucose and cation transport
	<i>MET30</i>	YIL046 W	F-box protein and component of SCF complex, regulates sulfur assimilation genes
	<i>YLR368 W</i>	YLR368 W	F-box protein, unknown function
	<i>RPN7</i>	YPR108 W	Proteasome
	<i>BDF1</i>	YLR399 C	Required for sporulation, has two bromodomain motifs and one ET domain
	<i>YMR111 C</i>	YMR111 C	Unknown
	<i>RAD61</i>	YDR014 W	Unknown

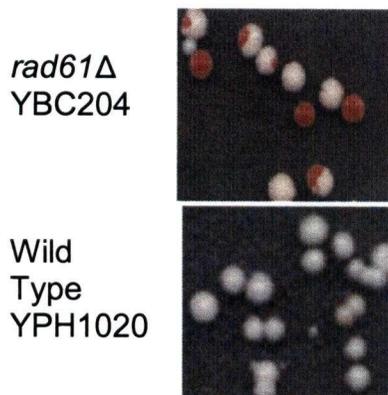


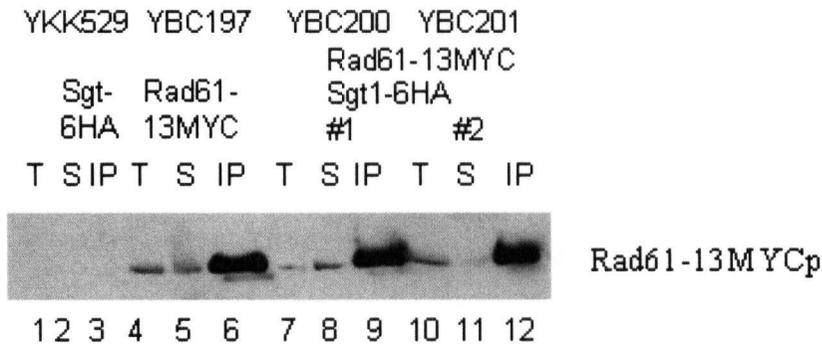
Figure 2-4 – *rad61Δ* mutants sector.

Haploid *rad61Δ* cells were plated onto Synthetic Complete media containing 65 μ M adenine and compared to wild type strains. Both strains contain a Chromosome Fragment III that is marked with *SUP11* and *TRP1*. Red indicates loss of chromosome fragment and white indicates retention of the chromosome fragment.

from wild type or *rad61Δ* strains that contained half-sectors (or more) of red pigment in order to quantify the chromosome missegregation rate of *rad61Δ* strains and found that *rad61Δ* strains had a $\sim 20x$ elevated rate of chromosome missegregation compared to the wild type (data not shown).

To test if the two-hybrid interaction identified a protein-protein interaction in yeast cells we performed a co-immunoprecipitation experiment on yeast extracts expressing epitope tagged Sgt1p and Rad61p (YDR014W, see below). We used strains expressing only *SGT1-6HA* (lanes 1 to 3 in Figure 2-5a, and lanes 1 and 2 in Figure 2-5b) or *RAD61-13MYC* (lanes 4 to 6 in Figure 2-5a, and lanes 3 and 4 in Figure 2-5b) as negative controls and we used two strains expressing *SGT1-6HA* and *RAD61-13MYC* (lanes 7 to 12 in Figure 2-5a, and lanes 5 to 8 in Figure 2-5b) to test for interaction. We could not detect an interaction between the two proteins (see lanes 6 and 8 in Figure 2-5b) implying that the interaction may be transient and sub-stoichiometric, or that the initial two-hybrid result was a false positive.

a) Anti-MYC IP, Anti-MYC Western



b) Anti-HA Western

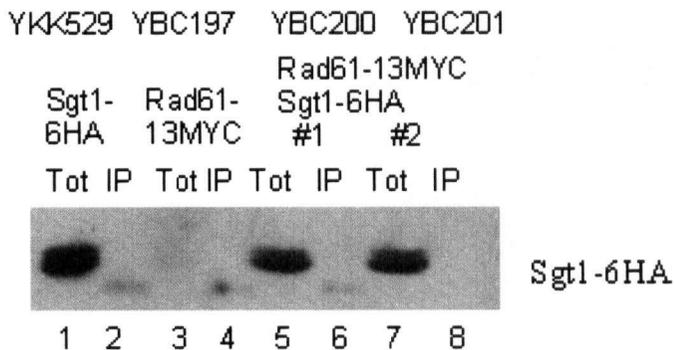


Figure 2-5 – Co-immunoprecipitation from yeast extracts does not indicate an interaction between Rad61-13MYCp and Sgt1-6HAp.

A) Yeast extract from strains expressing Sgt1-6HAp (lanes 1 to 3), Rad61-13MYCp (lanes 4 to 6), and two strains expressing Sgt1-6HAp and Rad61-13MYCp (lanes 7 to 12) were prepared and Anti-MYC IPs performed. The single tagged strains were used as negative controls. B) Samples from Figure 5a were rerun (only total yeast extracts and IP samples). Lanes 1 to 4 are the single tagged control strains, total and IP samples, lanes 5 to 8 are the two strains co-expressing Sgt1-6HAp and Rad61-13MYCp, total and IP samples.

YDR014W corresponds to a *ctf* mutant (*CTF6*) as well as a *rad* mutant (*RAD61*)

In a parallel study, YDR014W was isolated as an ORF able to complement the sectoring phenotype caused by mutant alleles within the *CTF6* complementation group (Karen Yuen, personal communication). To determine whether YDR014W was indeed *CTF6*, the entire YDR014W ORF was sequenced for each of four independent isolates within the *CTF6* complementation group. Single point mutations were identified in the coding region of YDR014W in each case. The mutations were nonsense mutations that produced truncated protein products that were therefore likely non-functional (Table 2-3). The mutations fell into two classes; *ctf6-7* and *ctf6-60* were identical and *ctf6-40* and *ctf6-134* were identical, suggesting that the pairs of alleles were “siblings” derived from outgrowth of the mutagenized cells in the original mutagenesis protocol that generated the *ctf* collection. In a diploid mating test assay, *ctf6-40* / *ctf6-134* (homozygous G1227A, TRP to STOP) mutant diploids exhibited chromosome III missegregation at a rate 100x higher than wild type strains (Spencer et al., 1990). We conclude that YDR014W encodes the gene that corresponds to the *CTF6* complementation group and is important for chromosome segregation.

Table 2-3 *RAD61* mutant alleles in the *CTF* mutant collection

<i>RAD61</i> allele	DNA Mutation	Amino Acid
<i>ctf6-7</i>	Base pair 1226 G to A	409 TRP to STOP
<i>ctf6-40</i>	Base pair 1227 G to A	409 TRP to STOP
<i>ctf6-60</i>	Base pair 1226 G to A	409 TRP to STOP
<i>ctf6-134</i>	Base pair 1227 G to A	409 TRP to STOP
<i>ctf6-53</i>	Not Sequenced	Not Sequenced

1 Mutations that were found were seen in a minimum of two sequencing reactions

YDR014W was also independently identified as a deletion mutant that is sensitive to ionizing radiation in a screen performed on the yeast genome wide non-essential gene deletion set and named *RAD61* (Bennett et al., 2001; Game et al, 2003). *rad61Δ* strains are sensitive to bleomycin (a gamma radiation mimetic causing double strand breaks) as well as MMS (a methylating agent), phenotypes that we were able to confirm in our laboratory strain background (Figure 2-6a). To be consistent with published nomenclature, YDR014W will be referred to as *RAD61*.

***rad61Δ* mutants exhibit a cell cycle delay in G2/M that is dependent on the spindle assembly checkpoint**

rad61Δ haploid mutants displayed a sectoring phenotype indicating a much higher rate of chromosome loss than wild type strains, but there were no growth effects at various temperatures tested (16°C to 37°C) (data not shown.) The haploid deletion mutants had approximately equal populations of G1 and G2 cells in logarithmically growing cell populations as measured by FACS, similar to wild type strains, and did not display increased sensitivity to benomyl, a microtubule depolymerizing drug (data not shown). However, *rad61Δ / rad61Δ* homozygous diploid deletion mutants displayed a G2 accumulation (Figure 2-6b, left hand panels). When we investigated cellular morphology of *rad61Δ / rad61Δ* compared to wild type diploids, we found a higher percentage of cells in a logarithmically growing population as short spindle pre-anaphase cells (29% in *rad61Δ / rad61Δ* versus 13% in wild type diploid cells). The G2 accumulation was

dependent on Mad2p (Figure 2-6b, right hand panels). We also found that *rad61Δ / rad61Δ* strains were resistant to intermediate levels of benomyl, compared to wild type diploids (Figure 2-6c). Mad2p is a member of the spindle assembly checkpoint that monitors and assures that sister kinetochores achieve bi-polar attachment to the spindle before allowing the metaphase to anaphase transition to occur. It has previously been shown that strains that have a defective kinetochore or a defect in sister chromatid cohesion may display a

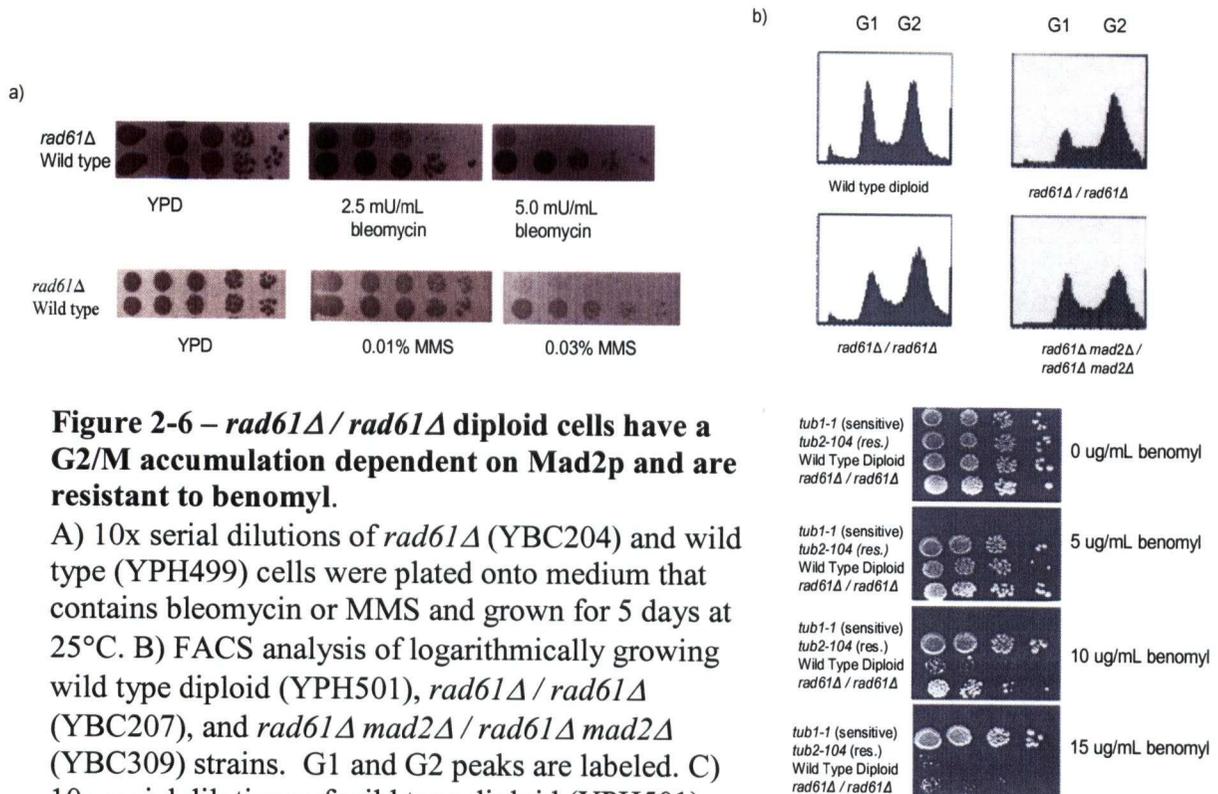


Figure 2-6 – *rad61Δ / rad61Δ* diploid cells have a G2/M accumulation dependent on Mad2p and are resistant to benomyl.

A) 10x serial dilutions of *rad61Δ* (YBC204) and wild type (YPH499) cells were plated onto medium that contains bleomycin or MMS and grown for 5 days at 25°C. B) FACS analysis of logarithmically growing wild type diploid (YPH501), *rad61Δ / rad61Δ* (YBC207), and *rad61Δ mad2Δ / rad61Δ mad2Δ* (YBC309) strains. G1 and G2 peaks are labeled. C) 10x serial dilutions of wild type diploid (YPH501), *rad61Δ / rad61Δ* (YBC207), *tub1-1* (YPH311), and *tub2* mutant (YPH312) cells were plated onto medium containing 0 to 15 ug/mL of benomyl (in DMSO) and grown for 5 days at 25°C.

G2 accumulation that is dependent on the spindle assembly checkpoint (Hyland et al., 1999; Mayer et al., 2001); Rad61p could potentially function in one of these two processes. Strains with defective kinetochores or sister chromatid cohesion may also show benomyl sensitivity presumably due to the synergistic effects of having perturbed microtubules and a defect in kinetochore or cohesion function. The resistance of the homozygous diploid deletion mutant to the drug benomyl is harder to explain and may be due to the slower progression through G2/M allowing cells more time to deal with depolymerized microtubules.

Rad61p is a stable nuclear protein

RAD61 was identified as having a two-hybrid interaction with *SGT1*, but this interaction was not confirmed by a co-immunoprecipitation experiment performed with yeast extracts. A possible explanation for this discrepancy could be that Rad61p is a substrate of the SCF complex and the interaction is transient. To address this we investigated the stability of Rad61p by quantifying the levels of Rad61p at various stages in the cell cycle. Previous experiments by others indicated that *RAD61* mRNA levels are stable through the cell cycle (Saccharomyces Genome Database, SGD). We synchronized yeast cell cultures with the addition of α -factor mating pheromone (see Materials and Methods) causing a G1 arrest. We then released the cells from G1 arrest by washing and removing the mating pheromone. Consistent with the RNA results, Rad61p protein levels were also constant throughout the cell cycle, (Figure 2-7a) as determined by analysis of yeast protein extracts by western blot using antibodies to an epitope tagged Rad61 protein and the FACS analysis indicating cell cycle progression.

Anti-Cdc28p antibodies were used as a control for protein loading. The Rad61-13MYCp fusion was functional as determined by complementation of a *rad61* Δ mutation using the sectoring assay (data not shown).

The constant level of the Rad61 protein could be due to a long half life of the protein or to constant expression of Rad61p with a short half life. We investigated the two possibilities by treating cells with cyclohexamide (a translation inhibitor). After treatment with cyclohexamide we prepared yeast extracts every 15 minutes. We found that Rad61-13MYCp appeared stable and had a half life much greater than that of Clb2p, a mitotic cyclin targeted for degradation by the APC/C (Anaphase Promoting Complex) (Figure 2-7b) with a high turnover rate. We concluded that Rad61p is a stable protein with a long half life.

We also tested to see if overexpressed Rad61p could be a substrate for the SCF complex. We cloned an HA-Rad61 fusion protein into a vector with a galactose inducible promoter, and transformed the Gal-HA-RAD61 vector into wild type and *sgt1-5* strains. Upon induction with galactose and subsequent repression of expression with glucose, we did not see stabilization of HA-Rad61p in the SCF mutant strains (Figure 2-7c) compared to wild type indicating that turnover of excess Rad61p was not dependent on the SCF complex. We conclude that Rad61p is not a substrate of the SCF complex and its expression and levels are constant through the cell cycle.

The phenotypic studies on *rad61* Δ mutants suggested that Rad61p could potentially function at the budding yeast kinetochore or in sister chromatid cohesion. To further investigate these possibilities, the cellular localization of Rad61p was determined by indirect immunofluorescence microscopy using antibodies to epitope tagged Rad61p

(Rad61-13MYCp). In logarithmically growing cell cultures that were then fixed, Rad61-13MYCp showed a nuclear localization as determined by colocalization with DAPI stained DNA in all the cells examined (~150 cells in various stages of the cell cycle) (Figure 2-8a). Live cell imaging of logarithmically growing cell cultures was also undertaken using Rad61p tagged at the C-terminus with VFP (a GFP variant, Venus Fluorescent Protein) and integrated at the endogenous locus. The localization of

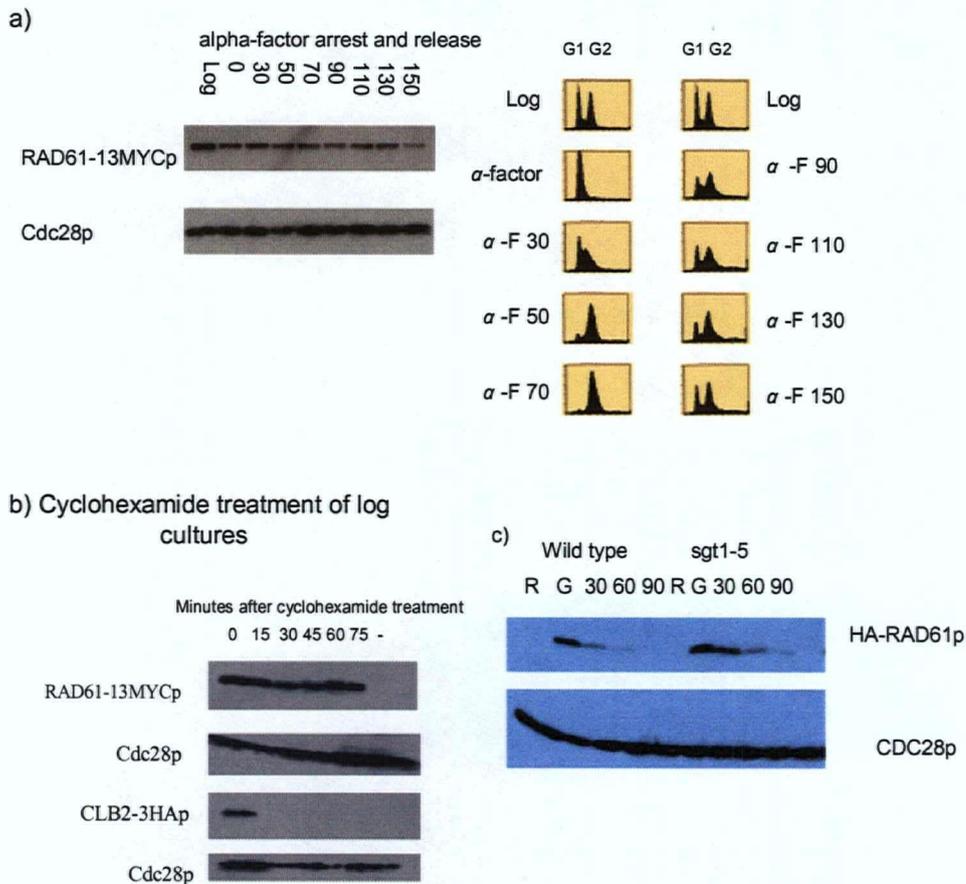


Figure 2-7 – Rad61-13MYCp is stable through the cell cycle with a long half-life.

A) Cell cultures from YBC198 were arrested in alpha-factor for 2 hours at 30°C, washed, and released into YPD at 30°C. FACS and protein samples were taken at the time points indicated. Protein sample concentrations were determined using OD280 readings, and Cdc28p was used as a loading control. B) 10 µg/mL of cyclohexamide was added (YBC198) to log phase cells (OD600 of 0.6) and protein samples were extracted at the time points indicated. Protein sample concentrations were assessed with OD280 readings and equal amounts loaded, with Cdc28p used as a loading control. C) A GAL-HA-RAD61 plasmid was transformed into wild type cells (YPH499) and *sgt1-5* (YKK57) cells. Liquid cultures were grown in selective media to maintain the plasmid overnight. Equal amounts of cells (by absorbance readings at OD600) were resuspended in rich media with glucose replaced by raffinose and grown for 2 hours. Galactose was then added to 2%, cells grew for another 2 hours and then glucose was added to repress transcription of HA-RAD61. Time points are after repression by addition of glucose. Cdc28p was used as a loading control.

kinetochore proteins tagged with GFP or VFP is well documented and is characterized by a punctuate lobe adjacent to the nuclear side of the spindle pole body. As the spindle pole body is duplicated and separated, the sister kinetochores appear as two lobes that localize on the nuclear faces of the spindle pole bodies (Measday et al., 2002; Pot et al., 2003). By these criteria, Rad61-VFP did not display characteristic kinetochore localization, but instead showed a diffuse nuclear staining at all stages of the cell cycle in ~150 cells examined (Figure 2-8b and 2-8c). We used Nic96-CFP to visualize the nuclear membrane and showed that Rad61-VFP was contained within the nucleus.

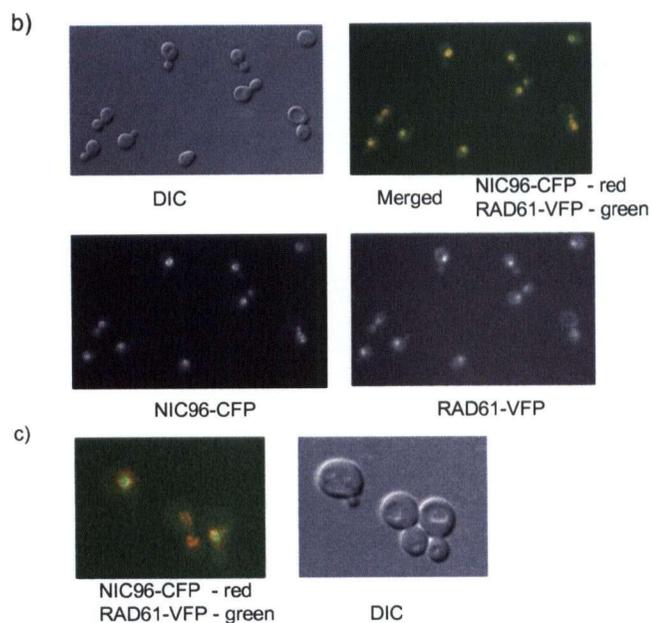
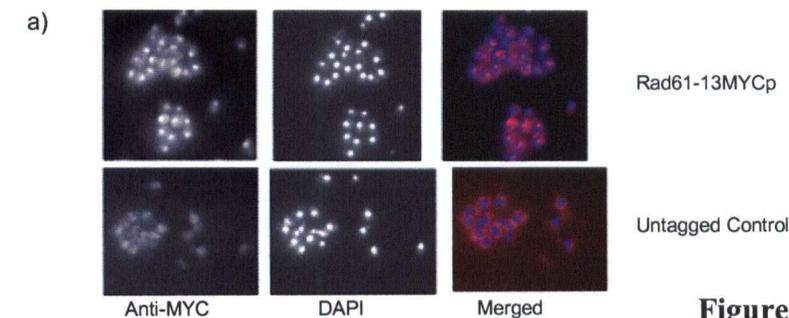


Figure 2-8 – Rad61p localizes to the nucleus by indirect immunofluorescence and live cell imaging.

A) Indirect Immunofluorescence of RAD61-13MYC cells (YBC198). DAPI appears blue and MYC appears red. ~150 cells were examined at different stages of the cell cycle, the figure is a representative image. **B) Fluorescence microscopy on live cells (YBC673).** These images are flattened Z-stacks (11 planes at 0.2 μm per plane). Nic96-CFP appears red and Rad61-VFP appears green. ~150 cells were examined, the figure is a representative image. **C) Increased magnification of live cell imaging of Rad61-VFP Nic96-CFP cells.**

A sub-fraction of Rad61p binds chromatin

Rad61p localized to the nucleus but not in the distinct pattern characteristic of kinetochore proteins. It was therefore of interest to determine whether the nuclear localization of Rad61p involved any binding of chromatin. To address this issue, two assays were employed. The first assay utilized the technique of chromosome spreading whereby cells and nuclei are lysed with a detergent and spread onto a specially prepared microscope slide (that has been washed and boiled in 0.1% HCl). Chromatin is bound onto the slide and soluble proteins are washed away. The protein of interest is visualized

using fluorescent secondary antibodies and DAPI is used to visualize chromatin. In chromosome spreads, Rad61-13MYCp signal was found to colocalize with DAPI in >100 nuclei examined and by this assay can bind to chromatin (Figure 2-9a, left panel). Ctf8-13MYCp is a chromatin bound protein and was used as a control (Figure 2-9a, right panel). The second assay used was chromatin purification, which involved centrifugation of spheroplasted and lysed cells to isolate chromatin away from soluble proteins. In this assay, chromatin bound protein is purified with the chromatin in the high speed centrifugation step. The amount of Rad61p in equal volumes of the total mixture, the supernatant mixture (containing the soluble fraction of the protein), and the pellet mixture (containing the chromatin bound fraction of the protein) can then be assessed by western blot analysis. We used an antibody to acetylated H4 as a control for a chromatin bound protein and an antibody to Carboxypeptidase Y (CPY) as a control for a cytoplasmic protein. By the chromatin binding assay a sub-fraction of Rad61-VFP was isolated in the chromatin fraction while a larger fraction of the protein was contained in the soluble fraction (Figure 2-9b). We obtained similar results using Rad61-13MYCp at different stages of the cell cycle (data not shown). By two different assays we found that a sub-fraction of Rad61p in the nucleus was binding to chromatin. It therefore appears that Rad61p is involved directly in an aspect of chromosome biology necessary for chromosome segregation. We addressed the question of the chromosomal locations of Rad61p binding in Chapter 3 using Chromatin Immunoprecipitation (ChIP experiments).

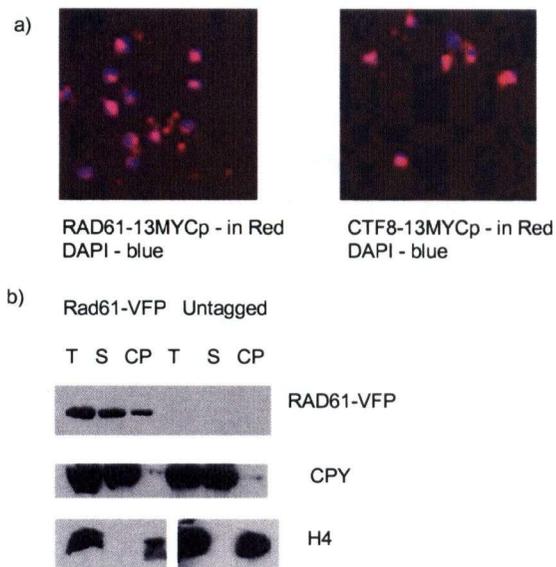


Figure 2-9 – A sub-fraction of Rad61p binds chromatin.

A) Chromosome spreads on Rad61-13MYC strain (YBC198) and Ctf8-13MYC strain (YPH1474). Anti-MYC and secondary fluorescent antibodies were incubated at 1/1000 at room temperature. DAPI was contained in the mounting media, DAPI appears blue, MYC appears red. ~100 nuclei were examined and this is a representative figure. Ctf8-13MYCp was used as a control for binding to chromatin. B) Chromatin Purification Experiment. Equal amounts of total, supernatant and chromatin pellet fractions were loaded and anti-GFP (detecting the VFP tag) Westerns performed on strains expressing Rad61-VFP (YBC671) or a control untagged strain (YPH499). Acetylated Histone H4 is used as a control for binding to chromatin and Carboxypeptidase Y (CPY) is used as a control cytoplasmic protein.

***RAD61* is not involved in Homologous Recombination to repair DNA damage**

The major mechanism for repair of DNA damage in budding yeast is by homologous recombination. In haploid cells, DNA repair by homologous recombination is conducted after S phase when there is an identical sister chromatid as a template for homologous recombination. We were interested to see if Rad61p was involved in

homologous recombination / repair because *rad61*Δ caused sensitivity to DNA damaging agents.

The HO endonuclease in budding yeast is required for mating type switching, a mechanism whereby the mating type locus is switched to the silent mating type locus in homothallic strains. The HO endonuclease induces a double strand break at a specific site in the mating type locus and then the homologous recombination machinery repairs this double strand break using the silent mating type locus as the template. Mutants with defective homologous recombination cannot survive when the HO endonuclease is expressed as the double strand break cannot be repaired. When we overexpressed the HO endonuclease (using a galactose inducible promoter) in wild type, *rad61*Δ, and *rad52*Δ (involved in homologous recombination) strains, the *rad61*Δ and wild type strains were both able to grow whereas the *rad52*Δ strain was unable to grow (Figure 2-10a, compare the right hand panels, galactose media). MMS will methylate DNA and cause DNA replication forks to stall and collapse and create double strand breaks. *rad61*Δ strains are sensitive to MMS (Figure 2-6a) and we were interested to see if Rad61p's localization was affected by MMS treatment and the generation of double strand breaks. We investigated the localization of Rad61-VFP fusion protein in MMS treated cells found that the localization remained similar to untreated cells (data not shown).

There was still the possibility that *RAD61* could be important, but not essential, for targeting the homologous recombination machinery to sites of double strand break for repair. *RAD52* is part of a large epistasis group that functions in homologous recombination. Upon induction of double strand breaks, it has been shown that Rad52p co-localizes with the double strand break sites (Lisby et al., 2003). Rad52p and the

homologous recombination machinery form “repair centres” that DNA double strand breaks will co-localize with to be repaired. We tested whether deleting *RAD61* could affect the formation and localization of RAD52-CFP foci in response to the generation of double strand breaks. We used strains developed in the Rothstein laboratory that contained *RAD52-CFP*, an HO target site marked with LAC operator sequences, and a constitutively expressed RFP-LAC repressor protein. We transformed in a vector that contained the HO endonuclease under the control of a galactose inducible promoter (pJH 132). We induced double strand breaks by inducing expression of the HO endonuclease by addition of galactose and we monitored sites of double strand breaks with RFP fluorescence (see Figure 2-10b, RFP and overlay panels, yellow and red arrows). We also monitored Rad52-CFP foci formation (see Figure 2-10b, upper right panel, yellow arrow shows a Rad52-CFP foci, red arrow does not) and we counted the number of cells that contained double strand break sites that had co-localizing Rad52-CFP foci. Figure 2-10b shows an example of a co-localizing Rad52-CFP foci to the double strand break site (yellow arrows) and a double strand break site with no Rad52-CFP foci (red arrows). Table 2-4 shows the results; wild type and *rad61Δ* strains displayed similar numbers of cells with double strand break sites visualized (28.3% to 31.1%) and had similar number of Rad52-CFP foci co-localizing with those double strand break sites (52% to 53.8%).

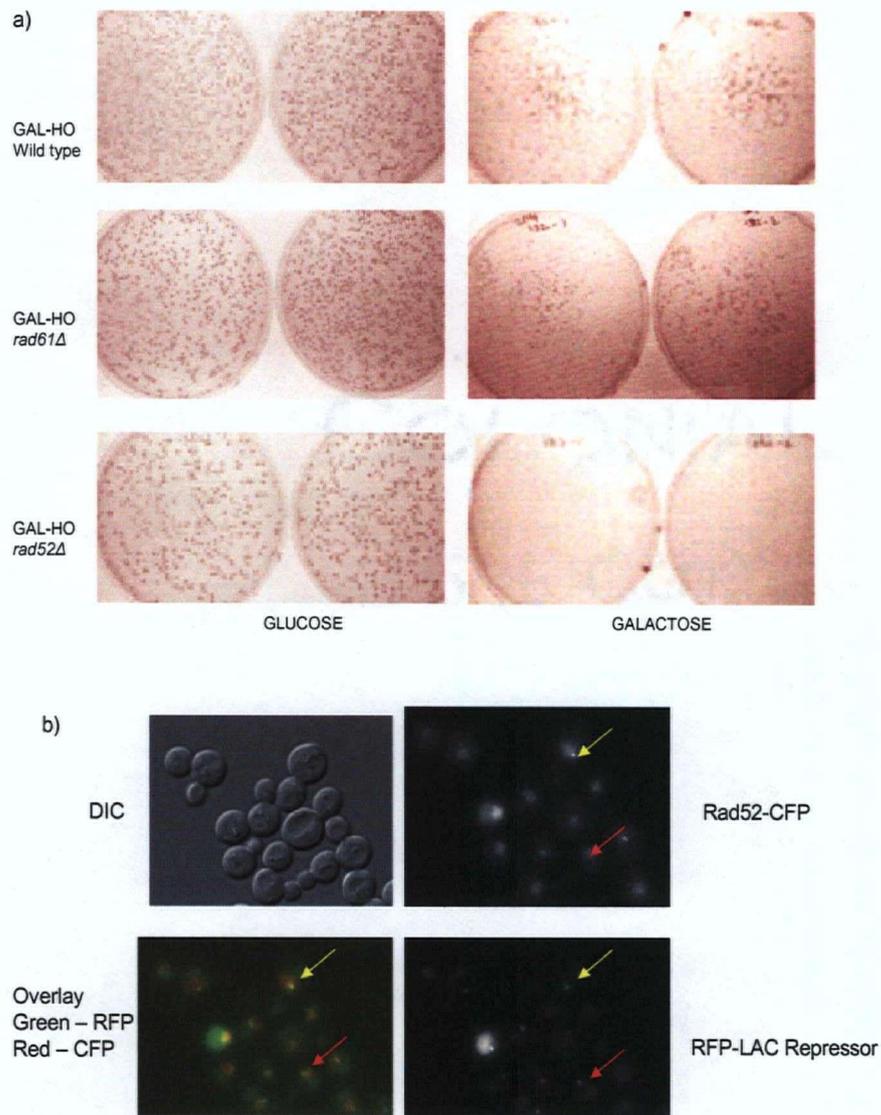


Figure 2-10 – *rad61Δ* strain is not sensitive to overexpression of HO endonuclease and does not affect Rad52p foci formation.

A) Wild type (YPH499), *rad61Δ* (YBC204), and *rad52Δ* (YBC675) cells were transformed with a GAL-HO plasmid. Cell cultures were grown overnight in selective media to maintain plasmid, and approximately equal amounts by absorbance were plated onto rich media either containing glucose as carbon source or galactose as the carbon source and allowed to grow at 25°C for 5 days. B) Strains containing RAD52-CFP, as well as RFP-LAC repressor fusion protein and an HO cut site marked by the LAC operator sequence (YBC658), were transformed with GAL-HO. Co-localization of RAD52-CFP and the RFP marked HO cut site is induced after induction of HO by addition of galactose. Representative images from a strain that was also *rad61Δ* is shown (YBC661). RFP-LAC repressor appears green and Rad52-CFP appears red. The yellow

Figure 2-10 legend continued

arrows mark an instance of co-localization, while the red arrows mark an RFP-LAC repressor imaged double strand break that does not have a Rad52-CFP foci co-localized to it.

Thus, we concluded that Rad52-CFP foci formation and co-localization with double strand break sites is unaffected in *rad61Δ* cells.

Table 2-4 Double Strand Break Induction – Rad52 foci formation is unaffected in *rad61Δ* cells

Strain	# of cells	# of DS break sites RFP foci	Rad52-CFP colocalizing with DS break sites
Wild type (YPH658), GAL- HO	346	98	51
Wild type (YPH658), GAL- HO as %		28.3%	52%
<i>rad61Δ</i> (YBC661), GAL- HO	209	65	35
<i>rad61Δ</i> (YBC661), GAL- HO as%		31.1%	53.8%

Discussion

The rationale for finding proteins that genetically or physically interact with Sgt1p at the budding yeast kinetochore was to identify novel proteins that played structural or regulatory roles important for kinetochore function. High copy suppression screens have been used successfully to find biologically relevant protein partners (Connelly and Hieter, 1996; Kitagawa et al., 1999). It was reasonable to expect that by identifying high copy suppressors of the temperature sensitivity phenotype of *sgt1-3* we could identify proteins important for Sgt1p function, but we were unable to find proteins that were clearly linked to chromosome segregation to study.

Neither of the deletion mutants of the uncharacterized ORFs that suppressed the *sgt1-3* mutant missegregated a chromosome fragment and neither of the deletion mutants in the two ORFs harboured phenotypes indicative of a potential role in chromosome segregation. The *SOG2* and *SOG3* ORF reports in the Yeast Proteome Database indicate that the functions of these proteins are unknown (YPD, www.incyte.com). We also tested if overexpressing Sog2p or Sog3p had an effect on *sgt1-5* strains (with an SCF defect) to test if Sog2p or Sog3p could be substrates of the SCF but found no effect upon overexpression of either protein (data not shown).

Using *SGT1* as a bait in a genome wide two hybrid screen yielded results that were more biologically relevant to the study of chromosome segregation. Finding many known interacting proteins of Sgt1p indicated that the screen was working but the fact that Rad61p could not be immunoprecipitated in a complex with Sgt1p indicates that the interaction may be transient *in vivo* or that the two hybrid result is an artifact that happened to isolate a protein involved in chromosome segregation. The epitope tagged

constructs of the two proteins may also interfere with their physical interaction. We addressed the question of potential Rad61p protein partners in Chapter 3 using mass spectrometry approaches.

Rad61p has all the characteristics of a protein that is involved in chromosome segregation. It is localized to the nucleus and a sub-fraction binds chromatin. The deletion mutant exhibits a chromosome missegregation phenotype, as do the truncation mutants found in the original *ctf* mutant collection. Furthermore, the diploid null mutant displays a spindle checkpoint dependent cell cycle accumulation in G2/M. We did not see this phenotype in the haploid null mutant. Diploid null mutants displaying a defect (e.g. cell cycle progression delay) that is not seen in a haploid null mutant has been observed before (Hyland et al., 1999). A diploid mutant may show more of an effect compared to a haploid mutant because diploid cells have twice as many chromosomes in the nucleus; phenotypes related to chromosome dynamics and segregation could be more accentuated as a result.

The homozygous diploid null mutants also displayed resistance to intermediate levels of the microtubule de-polymerizing drug, benomyl. Benomyl resistance has been demonstrated in deletion mutants of genes involved in microtubule dynamics such as *vik1Δ* (Manning et al., 1999) or *kip3Δ* (Cottingham and Hoyt, 1997). Kip3p is a kinesin-related microtubule motor protein and Vik1p is a protein partner of Kar3p, another kinesin-related microtubule motor protein. Microtubules in *kip3Δ* mutant cells are longer and more stable compared to microtubules in wild type cells (Cottingham and Hoyt, 1997). We examined microtubules in *rad61Δ / rad61Δ* diploid cells but found no difference compared to wild type diploid cells (data not shown). The resistance to

benomyl of the *rad61Δ / rad61Δ* strain could be due to the delay in progression through G2/M allowing cells time to stabilize microtubules and the mitotic spindle.

We tested for localization of the protein and phenotypes that were indicative of a role at the budding yeast kinetochore but consistently found that Rad61p did not appear to be a kinetochore component. The deletion mutant of *RAD61* is sensitive to DNA damaging agents (in particular to DNA double strand breaks) and we were able to confirm this phenotype in our strain background. We followed up on this data by looking at aspects of homologous recombination repair of DNA but also found that Rad61p had no detectable defect in Rad52p mediated homologous recombination repair. We also did not detect differences in Rad61p localization in response to DNA damaging agents (such as MMS and ionizing radiation) and we do not believe that Rad61p is involved directly in DNA repair.

An exact mechanism for Rad61p's role was not elucidated by direct tests for specific functions such as homologous recombination repair and kinetochore function. We decided to explore different genomic and proteomic approaches in order to understand the function of Rad61p in chromosome segregation. The reagents in our laboratory and the techniques that have been pioneered in budding yeast were used to gain insight into the role of Rad61p in chromosome segregation.

CHAPTER 3:

**Approaches to dissecting the role of Rad61p in
chromosome segregation**

Introduction

To further characterize Rad61p and elucidate its biochemical functions, we decided to employ systematic genetic interaction technologies that have recently been developed. Specifically, we employed diploid-Synthetic Lethal Analysis on Microarrays (SLAM) to identify gene deletion mutants that were synthetically lethal in combination with *rad61Δ*. Finding synthetic interactions on a genomic scale has helped to elucidate the function of genes by placing the query genes into clusters of genes involved in the same pathway (Tong et al., 2004; Pan et al., 2004). We utilized epitope tagged constructs of Rad61p which allowed us to immunoprecipitate the protein from budding yeast extracts and attempted to identify proteins or DNA that interacted with Rad61p. We also cloned *RAD61* into *E. coli* expression vectors that fused GST to the N-terminus of the protein. We expressed this construct in *E. coli* and used yeast lysate to identify proteins that could bind to the purified GST-Rad61 protein.

These experiments were used as approaches to dissect out the function of Rad61p in chromosome segregation and represented initial screens that required further characterization and validation that allowed us to gain insight into Rad61p function.

Materials and Methods

Yeast strains, growth conditions and media

Yeast strains unless otherwise indicated were in the YPH 499 strain background (S288C). In particular, the strains used in the cohesion assays were in the W303 background. Yeast strains used in Chapter 3 are listed in Table 3-1. *DED1* temperature sensitive alleles were obtained as gifts from Patrick Linder. The cloned mutant alleles were obtained on YCplac111 LEU CEN plasmids. The plasmids were transformed into heterozygous *DED1 / ded1Δ* strains, which were sporulated to produce haploid strains with *ded1Δ* complemented by the *DED1* allele containing plasmids.

Diploid-SLAM synthetic lethal screen

The diploid-SLAM (Synthetic Lethality Analysis on Microarrays) was performed as previously described in Pan et al., (2004). A *RAD61* URA3 deletion cassette was transformed into the heterozygous deletion set pool. The diploid-SLAM was performed by Xuewen Pan in Jef Boeke's laboratory at Johns Hopkins. Random spore analysis was carried out using the query strain containing *rad61Δ::NAT* mated to deletion mutant strains of interest. The diploids were sporulated and we selected for haploids using the Mat a specific HIS3 selectable marker (Tong et al., 2001). We tested for growth of the haploids on G418 plates (200 μg/mL in YPD) to select for query deletion strains, clonNAT plates (1 μg/mL in YPD) to select for *rad61Δ*, and G418 and clonNAT plates to select for the double mutants. The ratio of colonies that grew on the different selection plates indicated synthetic interactions. If no colonies grew on the G418 clonNAT plates, a synthetic lethal interaction was scored.

Clustering analysis on diploid-SLAM results

Two dimensional hierarchical agglomerative clustering was performed on the data from the Tong et al., (2004) yeast genetic interaction database with the diploid-SLAM data added as a query. The query genes were clustered based on Average linkage and analysis and presentation of the data was as described in Tong et al., (2004). Clustering was performed by Cluster 3.0 and visualized with Treeview 1.0.8.

Cohesion Assay

Cohesion assays were performed as described in Mayer et al., (2001). Cells were counted using a Zeiss Axioplan 2 microscope using the 100x objective lens.

ChIP-chip Assay

ChIP-chip analysis was performed as previously described in Horak et al., (2002). A strain containing RAD61-13MYC tagged at the endogenous locus and YPH 499 (wild type untagged control strain) were processed in parallel, labeled and hybridized onto the same DNA chip. ChIP-chip analysis was performed by Anthony Borneman in Michael Snyder's laboratory at Yale University. Confirmation PCRs were performed using primers to CEN3, CEN16 and HMR (Measday et al., 2002).

Immunoprecipitation and GST purification Mass Spectrometry

Co-immunoprecipitations were performed as described in Measday et al., (2002) with the following modifications. IP samples were eluted from the 9E10 MYC-conjugated beads (Covance) by incubation at 65°C for 15 minutes. Eluted samples were trypsin digested and purified using a cation exchange column. Samples were then dried using Speed Vac Plus and tandem Mass Spectrometry was performed. Tandem mass spectrometry and analysis was performed as described in Lee et al., (2004) by Mark Flory in Rudi Aebersold's laboratory at the University of Washington. For the GST-Rad61 purification we cloned RAD61 into the pGEX vectors creating a GST-Rad61 fusion protein product. pGEX-Rad61 was transformed into *E.coli* (BL21 strain) and expression of the fusion protein was induced with 1 mM isopropyl-beta-D-thiogalactopyranoside (IPTG) at 37°C for 3 hours. GST-Rad61 was contained in the soluble fraction in the bacterial cells and purified using glutathione agarose beads (Sigma). GST-Rad61 bound beads were then washed 4 times in 0.1% Triton in Phosphate Buffered Saline Solution (T-PBS) and subsequently incubated with yeast lysate overnight at 4°C. After washing 4 times with T-PBS, samples were eluted at 65°C for 15 minutes and subsequently treated the same as the IP samples used for tandem Mass Spectrometry analysis.

Co-Immunoprecipitations from Yeast Extract Cells

Co-immunoprecipitations were performed as previously described in Measday et al., (2002) using equal amounts of protein.

Methods from Chapter 2

We performed chromosome spreads, chromatin binding, fluorescence and indirect immunofluorescence microscopy, and FACS analysis of cells as described in Chapter 2

Materials and Methods.

Table 3-1 List of yeast strains used in this chapter

YPH499	Mata <i>ura3-52 lys2-801 ade2-101 his3Δ200 leu2Δ1 trp1Δ63</i>	Sikorski and Hieter., 1989
YPH500	Mata <i>ura3-52 lys2-801 ade2-101 his3Δ200 leu2Δ1 trp1Δ63</i>	Sikorski and Hieter., 1989
YBC285	Mata <i>ura3-52 lys2-801 ade2-101 his3Δ200 leu2Δ1 trp1Δ63 rad61Δ::HIS3 cep3-1</i>	This study
YBC289	Mata <i>ura3-52 lys2-801 ade2-101 his3Δ200 leu2Δ1 trp1Δ63 rad61Δ::HIS3 ctf13-30</i>	This study
YBC255	Mata <i>ura3-52 lys2-801 ade2-101 his3Δ200 leu2Δ1 trp1Δ63 rad61Δ::HIS3 slk19Δ::kanMX6</i>	This study
YBC329	Mata <i>ura3-52 lys2-801 ade2-101 his3Δ200 leu2Δ1 trp1Δ63 rad61Δ::HIS3 chl4Δ::kanMX6</i>	This study
YBC400	Mata <i>ura3-52 lys2-801 ade2-101 his3Δ200 leu2Δ1 trp1Δ63 rad61Δ::HIS3 sgt1-3::LEU2</i>	This study
YBC423	Mata <i>ura3-52 lys2-801 ade2-101 his3Δ200 leu2Δ1 trp1Δ63 rad61Δ::HIS3 ctf19Δ::TRP1</i>	This study
YBC439	Mata <i>ura3-52 lys2-801 ade2-101 his3Δ200 leu2Δ1 trp1Δ63 rad61Δ::HIS3 ctf3Δ::TRP1</i>	This study
YBC676	Mata <i>rad61Δ::HIS3 cin8Δ::kanMX6</i>	This study
YBC698	Mata <i>ura3-52 lys2-801 ade2-101 his3Δ200 leu2Δ1 trp1Δ63 rad61Δ::HIS3 sgt1-5::LEU2</i>	This study
YBC699	Mata <i>ura3-52 lys2-801 ade2-101 his3Δ200 leu2Δ1 trp1Δ63 rad61Δ::HIS3 skp1-3::LEU2</i>	This study
YBC700	Mata <i>ura3-52 lys2-801 ade2-101 his3Δ200 leu2Δ1 trp1Δ63 rad61Δ::HIS3 skp1-4::LEU2</i>	This study
YPH1477	Mata <i>ade2-1 trp1-1 can1-100 his3-11,15 leu2::LEU2tetR-GFP ura3::3xURA3tetO112 PDS1-13MYC-TRP1</i>	Mayer et al., 2001
YBC534	Mata <i>ade2-1 trp1-1 can1-100 his3-11,15 leu2::LEU2tetR-GFP ura3::3xURA3tetO112 PDS1-13MYC-TRP1 rad61Δ::HIS5</i>	This study
YBC535	Mata <i>ade2-1 trp1-1 can1-100 his3-11,15 leu2::LEU2tetR-GFP ura3::3xURA3tetO112 PDS1-13MYC-TRP1 rad61Δ::HIS5</i>	This study
Y819	Mata <i>trp1-1:lacO-TRP-LEU2 his3-11,15:lacR::GFP-HIS3 leu2-3,112 ura3-1 ade2-1 can1-100</i>	Mayer et al., 2001
YBC564	Mata <i>trp1-1:lacO-TRP-LEU2 his3-11,15:lacR::GFP-HIS3 leu2-3,112 ura3-1 ade2-1 can1-100 rad61Δ::kanMX6</i>	This study
YBC565	Mata <i>trp1-1:lacO-TRP-LEU2 his3-11,15:lacR::GFP-HIS3 leu2-3,112 ura3-1 ade2-1 can1-100 rad61Δ::kanMX6</i>	This study
YBC583	Mata <i>ade2 ura3 trp1 leu2 SCC3-3HA-HIS3</i>	K. Nasmyth
YBC584	Mata <i>ade2 ura3 trp1 leu2 SMC1-6HA-HIS3</i>	K. Nasmyth
YBC585	Mata <i>ade2 ura3 trp1 leu2 SMC3-6HA-HIS3</i>	K. Nasmyth
YBC619	Mata <i>ade2 ura3 trp1 leu2 SCC2-6HA-HIS3 pep4Δ::LEU2</i>	K. Nasmyth

Table 3-1 List of yeast strains used in this chapter,

YBC688	Mata <i>ura3-52 lys2-801 ade2-101 his3Δ200 leu2Δ1 trp1Δ63 ded1Δ::kanMX6</i> CFIII (<i>CEN3.L</i>) <i>URA3 SUP11</i> containing YCplac111- <i>ded1-56</i>	This study
YBC689	Mata <i>ura3-52 lys2-801 ade2-101 his3Δ200 leu2Δ1 trp1Δ63 ded1Δ::TRP1</i> CFIII (<i>CEN3.L</i>) <i>URA3 SUP11</i> containing YCplac111- <i>ded1-57</i>	This study
YBC690	Mata <i>ura3-52 lys2-801 ade2-101 his3Δ200 leu2Δ1 trp1Δ63 ded1Δ::kanMX6</i> CFIII (<i>CEN3.L</i>) <i>URA3 SUP11</i> containing YCplac111- <i>ded1-58</i>	This study
YBC691	Mata <i>ura3-52 lys2-801 ade2-101 his3Δ200 leu2Δ1 trp1Δ63 ded1Δ::kanMX6 rad61Δ::HIS3</i> containing YCplac111- <i>ded1-51</i>	This study
YBC692	Mata <i>ura3-52 lys2-801 ade2-101 his3Δ200 leu2Δ1 trp1Δ63 ded1Δ::kanMX6 rad61Δ::HIS3</i> containing YCplac111- <i>ded1-54</i>	This study
YBC693	Mata <i>ura3-52 lys2-801 ade2-101 his3Δ200 leu2Δ1 trp1Δ63 ded1Δ::kanMX6 rad61Δ::HIS3</i> containing YCplac111- <i>ded1-55</i>	This study
YBC694	Mata <i>ura3-52 lys2-801 ade2-101 his3Δ200 leu2Δ1 trp1Δ63 ded1Δ::kanMX6 rad61Δ::HIS3</i> containing YCplac111- <i>ded1-57</i>	This study
YBC695	Mata <i>ura3-52 lys2-801 ade2-101 his3Δ200 leu2Δ1 trp1Δ63 ded1Δ::kanMX6 rad61Δ::HIS3</i> containing YCplac111- <i>ded1-58</i>	This study
YH1444	Mata <i>ade2 trp1 leu2 can1 his3-11,15::GFP(pAFS144,thermostable)-LacI-HIS3 leu2-3,112 ura3-1 CEN15(1.8 kb)-LacO-URA3</i>	Goshima and Yanagida., 2000
YBC696	Mata <i>ade2 trp1 leu2 can1 his3-11,15::GFP(pAFS144,thermostable)-LacI-HIS3 leu2-3,112 ura3-1 CEN15(1.8 kb)-LacO-URA3 ded1Δ::kanMX6</i> containing YCplac111- <i>ded1-51</i>	This study
YBC697	Mata <i>ade2 trp1 leu2 can1 his3-11,15::GFP(pAFS144,thermostable)-LacI-HIS3 leu2-3,112 ura3-1 CEN15(1.8 kb)-LacO-URA3 ded1Δ::kanMX6</i> containing YCplac111- <i>ded1-51</i>	This study
YBC701	Mata <i>ade2 trp1 leu2 can1 his3-11,15::GFP(pAFS144,thermostable)-LacI-HIS3 leu2-3,112 ura3-1 CEN15(1.8 kb)-LacO-URA3 rad61Δ::HIS3</i>	This study

Table 1 List of yeast strains used in this chapter, continued

YBC603	<i>Mata trp1 ade2 ura3 leu2 RAD61-13MYC-TRP1 SCC3-3HA-HIS3</i>	This study
YBC604	<i>Mata trp1 ade2 ura3 leu2 RAD61-13MYC-TRP1 SCC3-3HA-HIS3</i>	This study
YBC607	<i>Mata trp1 ade2 ura3 leu2 RAD61-13MYC-TRP1 SMC1-6HA-HIS3</i>	This study
YBC608	<i>Mata trp1 ade2 ura3 leu2 RAD61-13MYC-TRP1 SMC1-6HA-HIS3</i>	This study
YBC611	<i>Mata trp1 ade2 ura3 leu2 RAD61-13MYC-TRP1 SMC3-6HA-HIS3</i>	This study
YBC612	<i>Mata trp1 ade2 ura3 leu2 RAD61-13MYC-TRP1 SMC3-6HA-HIS3</i>	This study
YBC649	<i>Mata trp1 ade2 ura3 RAD61-13MYC-TRP1 SCC2-6HA-HIS3 pep4Δ::LEU2</i>	This study
YBC650	<i>Mata trp1 ade2 ura3 RAD61-13MYC-TRP1 SCC2-6HA-HIS3 pep4Δ::LEU2</i>	This study
YBC589	<i>Mata ura3 trp1 ade2 leu2 rad61Δ::HIS3 SMC3-6HA-HIS3</i>	This study
YBC677	<i>Mata ura3-52 lys2-801 ade2-101 his3Δ200 leu2Δ1 trp1Δ63 ALT1-VFP-kanMX6</i>	This study
YBC678	<i>Mata ura3-52 lys2-801 ade2-101 his3Δ200 leu2Δ1 trp1Δ63 DED1-13MYC-kanMX6</i>	This study
YBC679	<i>Mata ura3-52 lys2-801 ade2-101 his3Δ200 leu2Δ1 trp1Δ63 DED1-13MYC-kanMX6</i>	This study
YBC680	<i>Mata ura3-52 lys2-801 ade2-101 his3Δ200 leu2Δ1 trp1Δ63 VPS3-13MYC-kanMX6</i>	This study
YBC681	<i>Mata ura3-52 lys2-801 ade2-101 his3Δ200 leu2Δ1 trp1Δ63 VPS3-13MYC-kanMX6</i>	This study
YBC682	<i>Mata ura3-52 lys2-801 ade2-101 his3Δ200 leu2Δ1 trp1Δ63 DED1-13MYC-kanMX6 RAD61-VFP-kanMX6</i>	This study
YBC683	<i>Mata ura3-52 lys2-801 ade2-101 his3Δ200 leu2Δ1 trp1Δ63 DED1-13MYC-kanMX6 RAD61-VFP-kanMX6</i>	This study
YBC684	<i>Mata ura3-52 lys2-801 ade2-101 his3Δ200 leu2Δ1 trp1Δ63 DED1-VFP-kanMX6</i>	This study
YBC685	<i>Mata ura3-52 lys2-801 ade2-101 his3Δ200 leu2Δ1 trp1Δ63 ded1Δ::kanMX6 CFIII (CEN3.L) URA3 SUP11 containing YCplac111-ded1-51</i>	This study
YBC686	<i>Mata ura3-52 lys2-801 ade2-101 his3Δ200 leu2Δ1 trp1Δ63 ded1Δ::kanMX6 CFIII (CEN3.L) URA3 SUP11 containing YCplac111-ded1-54</i>	This study
YBC687	<i>Mata ura3-52 lys2-801 ade2-101 his3Δ200 leu2Δ1 trp1Δ63 ded1Δ::TRP1 CFIII (CEN3.L) URA3 SUP11 containing YCplac111-ded1-55</i>	This study

Results

rad61Δ has genetic interactions with kinetochore and cohesion gene mutations

To potentially gain insight into Rad61p function, we tested if *rad61Δ* displayed genetic interactions with mutants known to be defective in different aspects of chromosome biology. We mated a *rad61Δ* strain with strains carrying mutations in genes involved in kinetochore or cohesion function (Table 3-2), sporulated the double heterozygous diploids, and assessed the phenotypes of double mutant haploids following tetrad dissection. *rad61Δ* displayed synthetic interactions with central kinetochore gene mutations (*ctf3Δ*, *chl4Δ*, *ctf19Δ*), a microtubule associated protein gene (*kar3Δ*) and also with cohesin and cohesin loading factor mutations (*scc2-4*, *smc3-42*).

Table 3-2 *rad61Δ* genetic interactions

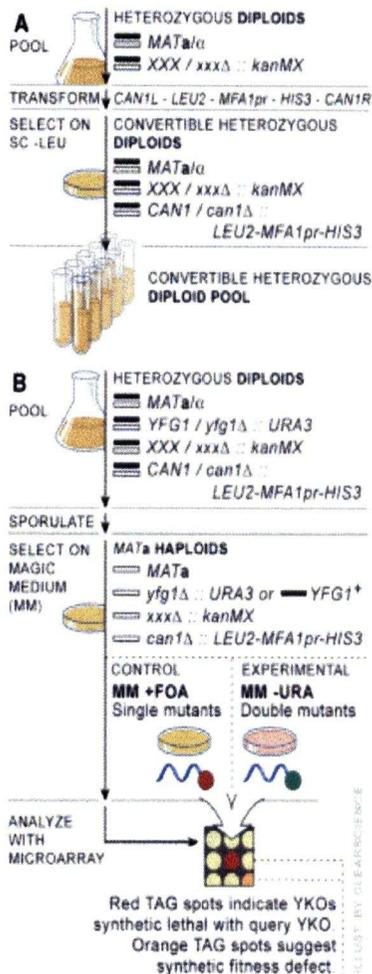
Strain	Genetic Interaction
<i>rad61Δ cep3-1</i> (YBC285)	Viable
<i>rad61 Δ ctf13-30</i> (YBC289)	Viable
<i>rad61 Δ skp1-3,4</i> (YBC699,700)	Viable
<i>rad61 Δ sgt1-3,5</i> (YBC400,698)	Viable
<i>rad61 Δ cin8 Δ</i> (YBC676)	Viable
<i>rad61 Δ slk19 Δ</i> (YBC255)	Viable
<i>rad61 Δ ctf3 Δ</i> (YBC439)	CSL
<i>rad61 Δ ndc10-42</i>	² SL
<i>rad61 Δ kar3 Δ</i>	SL
<i>rad61 Δ chl4 Δ</i> (YBC329)	CSL
<i>rad61 Δ ctf19 Δ</i> (YBC423)	CSL
<i>rad61 Δ scc2-4</i>	SL
<i>rad61 Δ smc3-42</i>	SL

1 CSL – conditional synthetic lethality - the double mutant can survive at 25°C but the restrictive temperature of a temperature sensitive allele is lowered or for two non-essential gene deletion mutants, there is lethality at higher temperatures for the double mutant.

2 Synthetic Lethality - Two non-essential gene deletion mutants are inviable in combination or a non-essential gene deletion and a temperature sensitive allele in an essential gene are inviable in combination.

CEP3, *CTF13*, *NDC10* – inner kinetochore proteins
SKP1, *SGT1* – inner kinetochore and SCF proteins
SLK19, *CTF3*, *CHL4*, *CTF19* – central kinetochore
CIN8, *KAR3* – microtubule motors
SCC2 – Cohesin loading protein
SMC3 – subunit of core cohesion complex

Testing specific mutants for genetic interactions with *rad61Δ* does not provide a comprehensive data set, and is therefore limited. To address this issue we performed a diploid-SLAM screen to attempt to identify all non-essential genes in the genome wide gene deletion set that had synthetic interactions with *rad61Δ*. The diploid-SLAM screen requires the generation of a double heterozygote diploid “pool” that is heterozygous for both the deletion mutant of interest (in this case *rad61Δ*) and each of the deletion mutants in the budding yeast deletion set (refer to Figure 3-1 for a schematic of the diploid-SLAM technique). Systematic diploid generation is accomplished by transformation of a pool of the entire heterozygous diploid deletion collection with a *rad61Δ* cassette with an auxotrophic marker (URA3). The heterozygous deletion mutant diploid product is selected for and then induced to sporulate. After sporulation, an auxotrophic marker, HIS3, that is only expressed in a specific cells, is used to select for haploid cells. Further selection using the markers for the deleted genes is also used to isolate double mutant haploids. Haploid double mutant combinations that exhibit synthetic lethality or slow growth in the pool of all possible double mutant combinations with the query strain will be selectively lost from the population upon outgrowth. Each of the deletion mutants in the deletion collection contain unique molecular tags, “barcodes”, which can be used to assess if the population contains that deletion mutant. Genomic DNA was prepared from populations that were *rad61Δ* or *RAD61*⁺ and the “barcodes” amplified by PCR, labeled with Cy3 (*rad61Δ*) or Cy5 (*RAD61*⁺) and both hybridized to a DNA microarray that contains the anti-sense sequences to the “barcodes”. The relative abundance of the “barcodes” of each of the deletion mutants in the two pools indicates the synthetic interaction.



From Pan et al., (2004)

Figure 3-1 – Schematic of the diploid-SLAM technique.

See the text for details and also Pan et al., (2004). A) Transformation of the heterozygous diploid pool with *HIS3* under the *MFA1* promoter to allow selection of haploids. B) Heterozygous diploid pools are transformed with *yfg1Δ::URA3* and sporulated. Haploids are selected and the relative abundance of strains is analyzed with a microarray.

See Table 3-3 for the results of the screen. We performed random spore analysis on a subset of the results and found in all instances that the synthetic interactions were re-confirmed (6/6 interactions tested by random spore analysis were re-confirmed, see Table 3 for the deletion mutants tested). We were also able to detect a synthetic interaction between *rad61Δ* and all four deletion mutants that had previously been directly tested (*ctf3Δ*, *kar3Δ*, *chl4Δ*, and *ctf19Δ*), which gave more confidence in the results of the screen. As expected from the directed double mutant analysis, *rad61Δ* displayed synthetic interactions with the central kinetochore gene mutations and also with cohesin and cohesin loading factor mutations, and with other factors that are involved in the establishment of sister chromatid cohesion. The results of the diploid-SLAM were clustered with the data from Tong et al. (2004) using a clustering program first developed for use with microarray transcription data. The program was used to cluster binary synthetic interaction data, with 0 representing no synthetic lethality and 1 representing synthetic lethality. *rad61Δ* clustered with *scc1-73* (a temperature sensitive mutant of the core cohesin complex member Scc1p) as well as *CHL1*, a DNA helicase that is involved in sister chromatid cohesion (Figure 3-2).

Table 3-3 Diploid SLAM Results

RANK	GENE NAME (or ORF)	
1	SGO1	
2	CTF19	*
3	CHL4	*
4	MCM21	*
5	RSC1	
6	CSM3	
7	KAR3	*
8	TOF1	
9	CIK1	
10	CTF4	*
11	BRE2	
12	MCM16	
13	CTF18	
14	MGA2	
15	BUB3	
16	YPL017C	
17	YLR111W	
18	CTF8	*
19	DUN1	
20	YDR018C	
21	RAD27	
22	CIN8	
23	CTF3	
24	MCM22	
25	LST4	
26	LSM1	
27	DCC1	

• * Tested and confirmed with Random Spore Analysis

Genes involved in chromosome segregation

- Kinetochore
- Cohesion
- Checkpoint
- MT/kinetochore

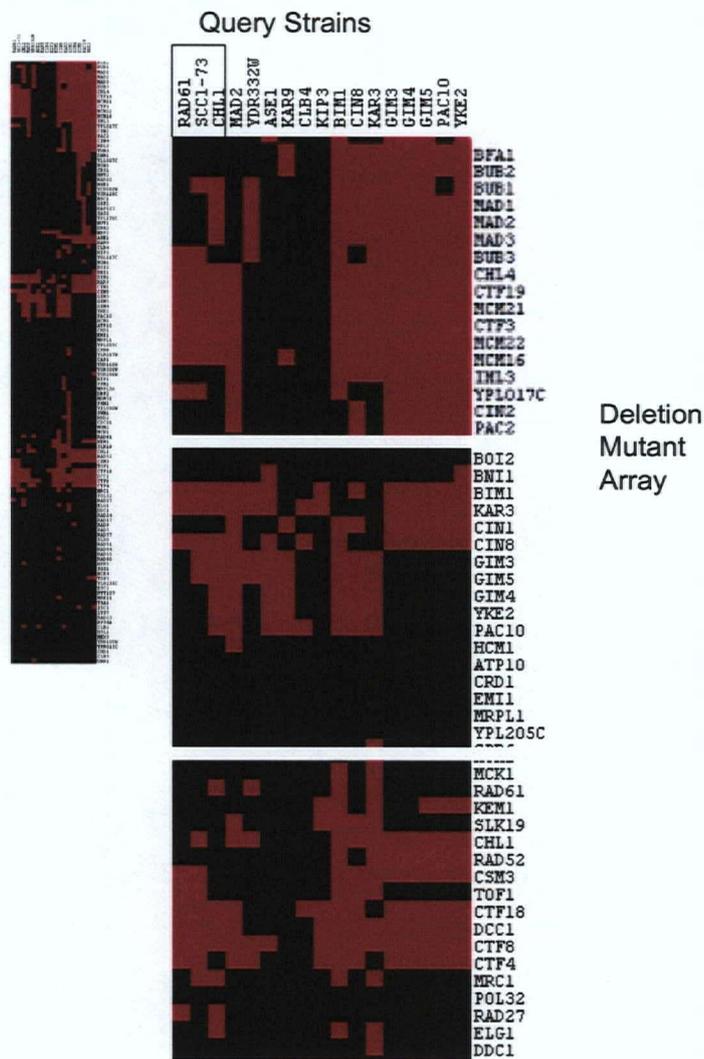


Figure 3-2 - Clustering of d-SLAM genetic interactions with genome wide SGA screens from Tong et al., 2004.

RAD61 clusters with *scc1-73* and *CHL1* (boxed genes). Query strains form columns with the deletion mutant array as rows. Red indicates a synthetic lethal interaction. Clustering was performed by Cluster 3.0 and visualized with Treeview 1.0.8.

Rad61p is involved in sister chromatid cohesion and potentially in establishment

RAD61's genetic interactions clustered with genes involved in cohesion so we were interested in testing the possibility that Rad61p was important for sister chromatid cohesion. To test this possibility we performed a sister chromatid cohesion assay. Cells that have chromosome locations marked with TET or LAC operator sequence arrays are arrested in either G1 phase (before DNA replication has occurred) using the mating pheromone alpha-factor, or in G2/M (after DNA replication) using a microtubule depolymerizing drug, nocodazole. Sister chromatids are visualized using GFP tagged LAC repressor or TET repressor fusion proteins that will bind to the operator arrays. Precociously separated sister chromatids will appear as two distinct GFP signals whereas sister chromatids that remain together will appear as one signal (two GFP "dots" versus one "dot" in nocodazole arrested cells). The alpha-factor arrested cells serve as a control for aneuploidy; cells should have only one GFP signal before DNA replication. When we performed this assay, we found that *rad61Δ* strains displayed a sister chromatid cohesion defect. In wild type strains, the percentage of cells with two distinguishable GFP signals in G2/M was consistently 9%. In *rad61Δ* strains, the percentage was approximately 18%. Presumably the 9% of cells that display two GFP signals in wild type cells arrested with nocodazole represent a basal rate of breathing of the paired chromatids. This assay was performed for two sites on chromosome arms as well as a site close to the centromere; the magnitude of the defect is comparable at all three sites (Figure 3-3a).

To determine if a chromatin bound fraction of Rad61p could be potentially binding at a chromosomal sequence known to be enriched for cohesin binding, we

performed a ChIP assay using PCR primers flanking that site. In the ChIP assay, protein-DNA interactions are fixed using formaldehyde treatment (to cross-link protein-DNA interactions). The cells are then lysed and the cell lysate is sonicated in order to shear DNA to 300 to 500 base pairs in length. The protein of interest that has been epitope tagged is immunoprecipitated using antibodies to the epitope tag and the immunoprecipitated mixture is incubated at 65°C in order to reverse the formaldehyde cross-linking; DNA is subsequently isolated by phenol extraction. Analysis of the DNA fragments that immunoprecipitated with the protein of interest can be assessed by PCR using primers flanking the region of interest. Although we could clearly see cohesion DNA in the Smc1p control ChIP, no enrichment of binding of Rad61p to the cohesin binding site was observed (Figure 3-3e). It was possible that Rad61p may be interacting with the cohesin complex that is not on chromatin and we tested this possibility by co-immunoprecipitation experiments performed on yeast extracts. We performed Anti-HA IPs using yeast extracts from strains that carried either endogenously tagged *SCC3-3HA*, or *SMC1-6HA*, or *SMC3-6HA* individually or in combination with endogenously tagged *RAD61-13MYC*. We could not detect any association of Rad61p with Scc3p, Smc1p or Smc3p (Figure 3-3b; see lanes 2, 6, and 10). Thus, Rad61p does not appear to interact directly with the core cohesin complex at cohesin binding sites.

To test whether or not Rad61p was involved in loading of cohesin onto chromatin, we performed chromosome spreads in both wild type and *rad61Δ* strains that expressed an epitope tagged cohesin component (Smc3-6HAp). Cohesin is loaded onto chromatin in G1 (after release from alpha-factor arrest) and dissociates in anaphase. *rad61Δ* strain background had no effect on the kinetics of loading or the dissociation of cohesin (Figure

3-3d). We examined ~50 nuclei at each of the time points in Figure 3-3d and representative images of Smc3-6HAp on or off chromatin for wild type and *rad61Δ* strains are shown. To test if there could be an interaction between Rad61p and the complex that loads the cohesin complex onto chromatin, we performed a co-immunoprecipitation assay on yeast extracts prepared from two strains that had endogenously tagged Rad61-13MYCp and Scc2-6HAp. We found that there was no detectable association between the two proteins in extracts prepared from both strains tested (Figure 3-3c, see lanes 6 and 8).

A new class of non-essential genes that seem to be involved in the establishment of sister chromatid cohesion has recently been described. Many of the genes have a defect in sister chromatid cohesion that is comparable to that observed in *rad61Δ* mutant strains (Mayer et al., 2001; Mayer et al., 2004; Warren et al., 2004). It is believed that the timing of cohesion establishment is during or right after DNA replication. Rad61p by virtue of having a sister chromatid defect and not being involved either as a core cohesin complex member or the loading of cohesin, may similarly be involved in the establishment of cohesion.

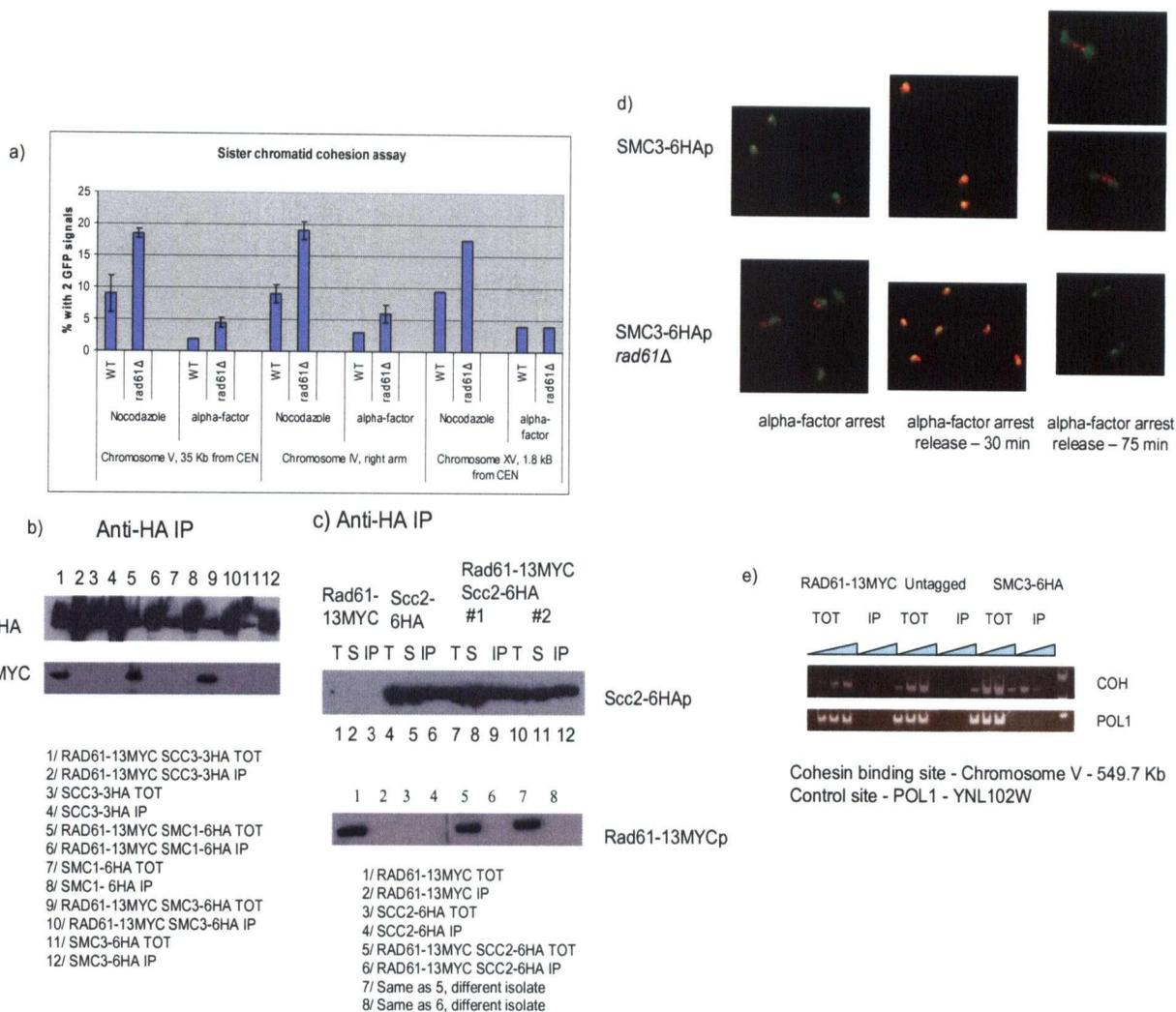


Figure 3-3 – *rad61Δ* strains display a cohesion defect that is likely a result of defective establishment.

A) Sister Chromatid Cohesion Assay. We counted the number of cells with 1 or 2 GFP signals. The error bars represent 200 cells counted in 2 different experiments. No error bars – represents 200 cells counted. We tested three different chromosomal loci, one on Chromosome V (35 kb from CEN) (YPH1477, YBC534,535), Chromosome IV arm (Y819, YBC564,565), and Chromosome XV (1.8 kb from CEN) (YPH1444, YBC701). B) Anti-HA Co-IP on yeast extract expressing Rad61-13MYCp and Scc3-3HAp (lanes 1 and 2) (YBC603), Scc3-3HAp (lanes 3 and 4) (YBC583), Rad61-13MYCp and Smc1-6HAp (lanes 5 and 6) (YBC607), Smc1-6HAp (lanes 7 and 8) (YBC584), Rad61-13MYCp and Smc3-6HAp (lanes 9 and 10) (YBC611), or Smc3-6HAp (lanes 11 and 12) (YBC585). Total and IP samples were run on gels for Western blot using Anti-HA or Anti-MYC with the strains only expressing a single tag as negative controls.

Figure 3-3 Figure legend continued

C) Anti-HA Co-IP performed on yeast extracts from strains expressing Rad61-13MYCp (upper panel lanes 1 to 3, bottom panel lanes 1 and 2) (YBC198), Scc2-6HAp (upper panel lanes 4 to 6, bottom panel lanes 3 and 4) (YBC619), and two strains expressing Rad61-13MYCp and Scc2-6HAp (upper panel lanes 7 to 12, bottom panel lanes 5 to 8) (YBC649, 650). The two single tagged strains were used as negative controls. Western blots were performed using Anti-MYC and Anti-HA. D) Chromosome Spreads (YBC585, Smc3-6HA) and (YBC589, Smc3-6HA *rad61*Δ). We performed chromosome spreads using Anti-HA primary and a fluorescent secondary (GAN-F) at 1/1000 dilutions. Smc3-6HA appears red and DAPI appears green. At each of the time points we examined ~50 nuclei, and representative images are shown. E) ChIP PCR assays on strains expressing Rad61-13MYCp (YBC198), Smc3-6HAp (YBC585), or untagged control strain (YPH499) using primers for a cohesin binding site and *POL1* as a non-cohesin binding site control.

By ChIP-chip analysis Rad61p associates with regions around centromere DNA

In an effort to understand Rad61p's role in binding chromatin we sought to determine if the chromatin binding occurred at specific loci on chromosomes. We performed a Chromatin Immunoprecipitation followed by microchip analysis (ChIP-chip experiment) in collaboration with Michael Snyder's laboratory at Yale University. The ChIP technique (formaldehyde fixation, lysis, sonication, immunoprecipitation, reversal of cross-link and DNA extraction) is similar (see Materials and Methods) to that used to assess the binding of Rad61p to the putative cohesin binding site. In ChIP-chip, the detection of binding sites involves hybridization of immunoprecipitated DNA to sequences on a DNA chip. Genomic DNA that has been sheared and isolated is labeled and used as a probe on a DNA microchip (containing intergenic sequences of the yeast genome). A parallel ChIP is performed on an untagged control strain to produce genomic DNA that is labeled and used as a control for non-specific DNA binding in the IP. The DNA samples from both ChIP experiments are labeled with different fluorescent markers and hybridized to the same DNA chip. Binding sites are determined based on the

hybridization ratio of the Rad61-13MYCp immunoprecipitated DNA sample over the untagged control immunoprecipitated DNA sample with a threshold that must be met in order to be scored as a binding site (Horak et al., 2002; Anthony Borneman, personal communication). The data is then presented on a map of the yeast chromosomes with diamonds depicting the binding sites with the diamonds stacked if the binding sites cluster. Four separate immunoprecipitation experiments were performed. We found that there was specific binding to a majority of the centromere regions (12 of the 16 chromosomes) in budding yeast with little other specific binding sites in the rest of the genome (Figure 3-4a). Confirmation PCRs were performed using template DNA from Rad61-13MYCp tagged strains or from untagged strains and there did appear to be enrichment at CEN DNA sequences compared to non-CEN sequences such as *HMR*, *TEC1* and *MGA1* (Figure 3-4b, upper panels are CEN DNA sequences, compare left and right lanes. Lower panels are the control negative sequences).

The ChIP-chip analysis and confirmation PCRs were performed in Michael Snyder's laboratory at Yale University; when we performed confirmation PCRs using CEN3 and *HMR* sequences we found that using our protocols, a known kinetochore protein, Ndc10-13MYCp, could immunoprecipitate CEN DNA while Rad61-13MYCp could not (Figure 3-4c, compare IP lanes of RAD61-13MYC and NDC10-13MYC). The contradictory results are likely due to differences in the protocol employed. The fixation time of the ChIP-chip analysis was longer and may have cross-linked proteins that are further from the core kinetochore. Differences in sonication treatment of the yeast lysate could shear DNA to different average sizes and this would affect the sequences that could be detected in the analysis. The nuclear localization of Rad61p does not correspond to

known kinetochore proteins (Figures 2-8b and 2-8c) and we conclude that Rad61p's preferential localization to centromeres is transient, sub-stoichiometric, or indirect through other proteins.

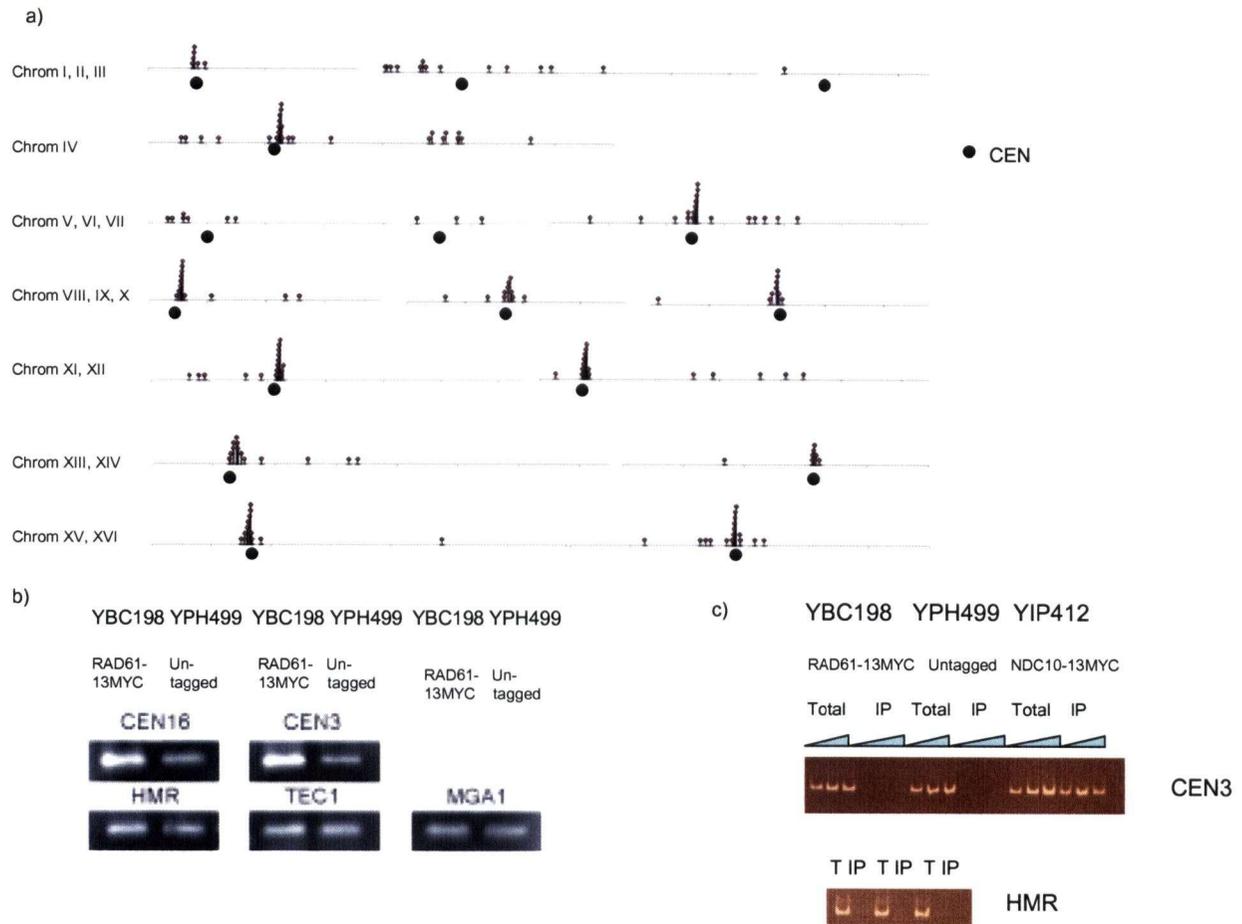


Figure 3-4 – Rad61p by ChIP-chip analysis is specifically enriched at Centromere DNA.

A) Chromosomes I to XVI are visualized with diamonds representing spots of Rad61p specific binding over the wild type (over the threshold SD of 4) Centromeres are indicated by the black dots. B) ChIP PCR assays using strains expressing Rad61-13MYCp or an untagged control strain were performed using Primers to CEN III, XVI, and control regions *HMR*, *TEC1* and *MGA1*. C) Chromatin Immunoprecipitation Assay using primers to CEN3 and HMR. Strains expressing Ndc10-13MYCp or Rad61-13MYCp, and an untagged control strain were used for the ChIP assay. Increasing concentrations of the template DNA sequence were used for the total and IP samples in the 3 strains for the CEN3 PCR. HMR was used as a control for non-CEN background binding.

Interaction between Rad61p and Ded1p

As an alternative approach to dissecting the function of Rad61p, we explored different methods of finding proteins that interacted with Rad61p. We performed immunoprecipitation experiments on budding yeast extracts from a RAD61-13MYC strain. Eluted IP samples were digested with trypsin, purified and tandem mass spectrometry was performed. Dr. Mark Flory in Rudi Aebersold's laboratory at the University of Washington performed the tandem mass spectrometry analysis. We were consistently unable to identify more than 2 peptides of Rad61p in the elution mixture. We decided to modify our approach and expressed a GST-Rad61 construct in *E. coli*, purified the construct with glutathione agarose beads and then incubated the beads with budding yeast lysate in order to find potential interactions. We were able to recover approximately 200 peptides of Rad61p as well as other peptides specifically in the GST-Rad61 experiments versus GST alone (Alt1p, Ded1p, Vps3p, Kar2p, Snu13p, Tfp1p, and Tub1p) (see Table 3-4). We tested the top three proteins (Alt1p, Ded1p and Vps3p) for interactions with Rad61p by co-immunoprecipitation experiments using epitope tagged constructs of Rad61p and each of the potential interacting proteins and we found a reproducible interaction between Rad61p and Ded1p (Figure 3-5a, see lane 3 and lane 15 of the panels). We did not detect interactions between Rad61p and Vps3p (Figure 3-5a, lane 9 in the upper panels, and lane 18 in the bottom panel) or with Rad61p and Alt1p (Figure 3-5b, see lane 6). A peptide of Ded1p had also been identified previously in the initial RAD61-13MYC IP experiments.

Table 3-4 GST-Rad61 purified protein incubated with

Protein	# of peptides
ALT1	9
VPS3	5
DED1	5
KAR2	2
SNU13	2
TFP1 *	2
TUB1	2

* Common Contaminant

Ded1p is an essential protein and is an RNA helicase of the DEAD-box family with a documented role in the initiation of translation (Iost et al., 1999; Chuang et al., 1997). Ded1p also has a nuclear role (discussed below) but its documented localization is cytoplasmic (as would be expected from a gene involved in protein synthesis). Ded1p has also been implicated as part of the spliceosome (Burckin et al., 2005) and as a potential interactor with Cdc28p (the CDK in budding yeast) and therefore it may have additional functions inside the nucleus (Honey et al., 2001). We were able to show that Ded1-13MYCp and Rad61-VFPp epitope tagged constructs could interact when expressed at endogenous levels from their endogenous loci in budding yeast cells (Figure 3-5c, see lane 6). In fission yeast there is intriguing evidence that Ded1p could be post-translationally modified and that translation of other proteins could be affected in response to different forms of cellular stress (Liu et al., 2002). We decided to check the

interaction between Rad61p and Ded1p when cells have undergone DNA damage induced by MMS. We found that the interaction between Rad61p and Ded1p did not detectably change in cells that had been treated with MMS (data not shown).

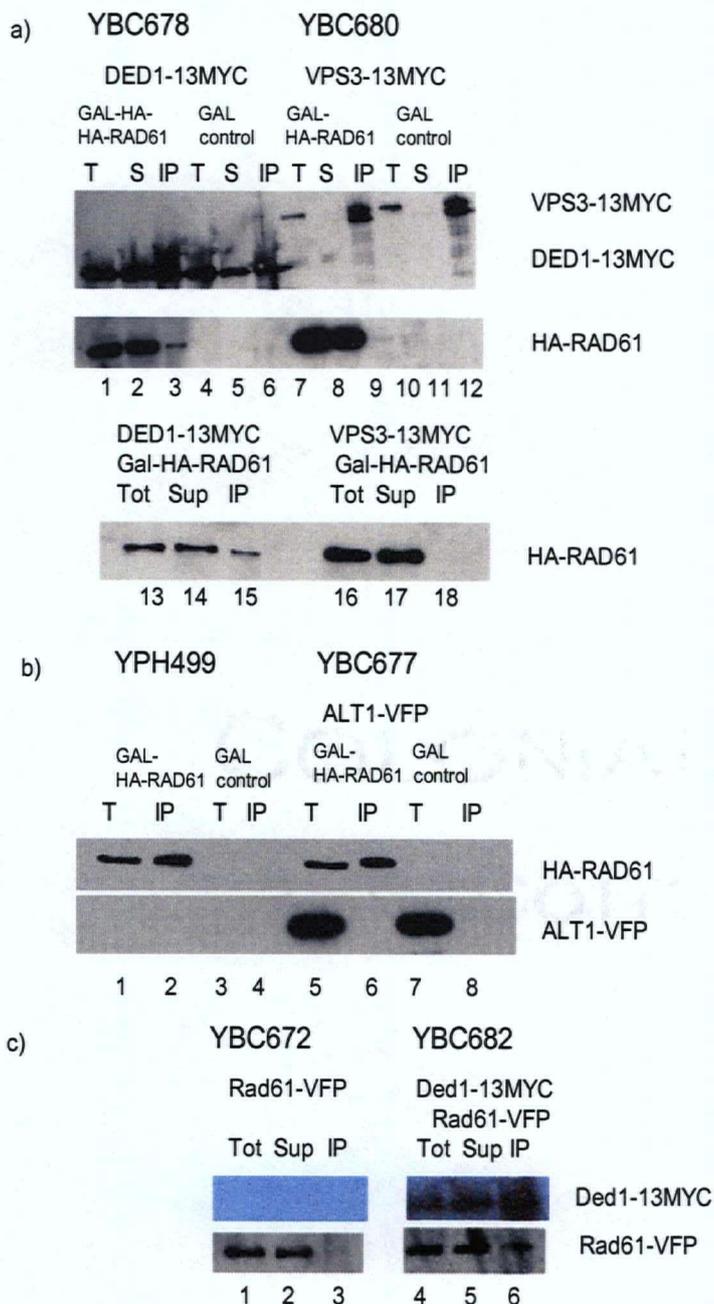


Figure 3-5 – Protein-protein interactions of Rad61p and Ded1p and unconfirmed interactions of Rad61p with Alt1p and Vps3p.

Figure legend on the next page.

Figure 3-5 – Protein-protein interactions of Rad61p and Ded1p and unconfirmed interactions of Rad61p with Alt1p and Vps3p.

A) Strains expressing DED1-13MYC with a GAL-HA-RAD61 plasmid (lanes 1 to 3 and 13 to 15), DED1-13MYC with a GAL control plasmid (lanes 4 to 6 in the upper panels), VPS3-13MYC with a GAL-HA-RAD61 plasmid (lanes 7 to 9 in upper panels, and lanes 16 to 18 in lower panel), or VPS3-13MYC with a GAL control plasmid (lanes 10 to 12 in upper panels), were grown in galactose (2%) to induce HA-Rad61p expression. Yeast extracts were then prepared and IPs carried out with Anti-MYC beads. Western blots were performed and Anti-MYC or Anti-HA used to visualize proteins. Lower panel is re-running of the samples from the upper panels. B) Untagged strains with GAL-HA-RAD61 plasmid (lanes 1 and 2) or with a GAL control plasmid (lanes 3 and 4), and strains expressing ALT1-VFP with a GAL-HA-RAD61 plasmid (lanes 5 and 6), or with a GAL control plasmid (lanes 7 and 8), were grown in 2% galactose to induce HA-Rad61p expression. Yeast extracts were then prepared and Anti-HA IPs were carried out. Anti-GFP and Anti-HA Westerns were performed on the samples. C) Yeast extracts from strains expressing Rad61-VFP (lanes 1 to 3), or Rad61-VFP and Ded1-13MYCp (lanes 4 to 6) at endogenous levels were prepared and Anti-MYC IPs performed. Anti-GFP and Anti-MYC Western blots were performed to detect proteins.

Ded1p binds chromatin and has a role in genome integrity

Ded1p has well documented roles in initiation of translation and at the spliceosome but because of its interaction with Rad61p we were interested to see if Ded1p could potentially have other roles in the nucleus. Because Rad61p binds chromatin, it was of interest to determine if there could be a fraction of Ded1p that bound chromatin. By chromatin purification experiments (performed in duplicate on two independent clones expressing Ded1-13MYCp), Ded1-13MYCp binds to chromatin (Figure 3-6a). Acetylated H4 was used as a control for chromatin binding and CPY was used as a control cytoplasmic protein; both of the controls behaved as expected and Ded1-13MYCp contained a chromatin bound fraction. Chromosome spreads also showed that Ded1-13MYCp could bind chromatin and that the staining looked to be spread across chromatin with no discernable foci (data not shown). When we looked at

the localization of all the Ded1p by live cell microscopy using Ded1-VFP, we found that the majority of the protein localized to the cytoplasm (Figure 3-6b).

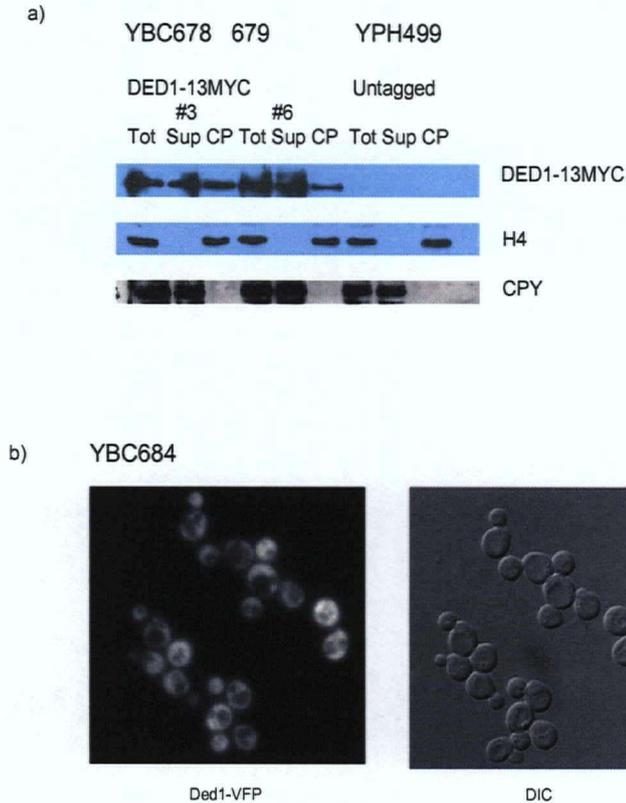


Figure 3-6 – Ded1p binds chromatin but the majority of the protein is in the cytoplasm.

A) Chromatin purification experiments were done as described. Equal amounts of total, supernatant and chromatin pellet fractions of two Ded1-13MYCp expressing strains, and an untagged control strain, were run and a Western blot using Anti-MYC was used to visualize the amounts of Ded1p in each fraction. Acetylated H4 and CPY were used as controls for chromatin and cytoplasmic proteins. B) Live cell fluorescence microscopy on Ded1-VFP cells. Cells were immobilized by low melting point agarose (see Materials and Methods). We examined ~100 cells and this a representative image using one focal plane.

DED1 is an essential gene in budding yeast and certain temperature sensitive alleles have been shown to cause both translation and splicing defects at the non-permissive temperature. We were interested to see if temperature sensitive alleles of *DED1* may have defects in chromosome segregation at permissive temperatures and also whether or not they had cohesion defects when shifted to non-permissive temperatures. Six temperature sensitive alleles of *DED1* on yeast centromere plasmids were obtained from Patrick Linder's laboratory (*ded1-51*, *ded1-54*, *ded1-55*, *ded1-56*, *ded1-57*, and *ded1-58*). We transformed the plasmids into diploid strains that had one copy of the *DED1* gene deleted and also contained a non-essential chromosome fragment that contained the SUP11 marker (in order to assess the amount of chromosome missegregation of the strain containing the *ded1* allele). We also transformed the plasmids into another diploid strain that had one copy of *DED1* gene deleted and that was homozygous null for *RAD61*. After sporulation of the diploids, we isolated haploids that contained the *ded1Δ* complemented by the *ded1* temperature sensitive alleles on the plasmids. The restrictive temperatures of the strains containing *ded1Δ* complemented by the different *ded1* mutant alleles are shown in Table 3-5.

All strains containing a combination of mutant alleles of *ded1* and *rad61Δ* were able to grow. However, the restrictive temperatures of strains containing two temperature sensitive alleles, *ded1-51* and *ded1-57*, in combination with *rad61Δ*, were lowered, indicating a genetic interaction between the mutations of the genes (Table 3-5). To assess if the *ded1* mutant alleles caused strains to lose chromosomes, we compared the red/white sectoring (indicating the loss of the non-essential chromosome fragment) between the strains containing the *ded1* mutant alleles and a wild type strain. The strains

containing the *ded1* alleles did not have strong sectoring phenotypes at 25°C although *ded1-55* containing strains at higher temperatures showed an increased amount of red sectors compared to the wild type strain (Table 3-5 and Figure 3-7). *ded1-55* mutant strains contained 238 half-red sectors in a population of 6018 colonies counted (3.95%) versus 18 half-red sectors in a population of 4113 wild type colonies counted (0.44%) representing an increase of approximately 9x that of wild type strains.

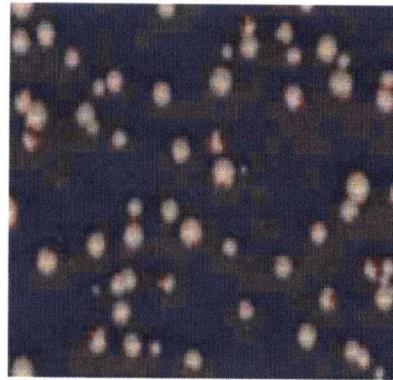
Table 3-5 *DED1* mutants and their sectoring phenotypes

DED1 Mutant	Mutation	Restrictive Temperature	Sectoring at 35°C (or 33°C if lethal at 35°C)	Genetic Interaction with <i>RAD61</i>
<i>ded1-51</i> YBC685	L 403 S M to V in C-terminal	35°C	Wild type	CSL
<i>ded1-54</i> (686)	L 403 S	37°C	Wild type	NI
<i>ded1-55</i> (687)	M 183 R	37°C	~9x wild type	NI
<i>ded1-56</i> (688)	P 526 L	37°C	Wild type	---
<i>ded1-57</i> (689)	H 430 R	35°C	Wild type	CSL
<i>ded1-58</i> (690)	I 404 T	37°C	Wild type	NI

NI No Interaction
CSL Conditional Synthetic Lethality



Wild Type
YPH1020



ded1-55
YBC687

Figure 3-7 – *ded1-55* mutants sector at 35°C.

There is an increase number of red sectors in the *ded1-55* strain compared to wild type. By half-sector analysis, *ded1-55* strains lose the chromosome fragment at a rate ~ 3 to 4x that of wild type.

In order to assess if the chromosome missegregation phenotype of the *ded1* mutant alleles could be caused by sister chromatid cohesion defects, strains were constructed that had the *ded1-51* allele covering the *ded1Δ* with a chromosomal locus marked in order to assess precocious sister chromatid separation. Cells were arrested with α -factor and released into media containing nocodazole either at 30°C (permissive temperature) or 37°C (restrictive temperature). At 15 minute time points cell cultures were shifted from 30°C to 37°C, in order to inactivate Ded1p function at different points. Wild type cells and the *ded1-51* strain arrested as expected in α -factor and nocodazole at 30°C (Figure 3-8a) but the *ded1-51* strain at 37°C had a delay in progression through S phase. We found that increasing the time at 30°C allowed a larger fraction of cells to duplicate their DNA and be subsequently arrested in G2/M by nocodazole (Figure 3-8a, compare rows 3 to 8). The binding of Ded1p to chromatin (Figure 3-6a) suggests that

this S phase progression delay could be a direct consequence of inactivating Ded1p. Alternatively, the delay could be caused by aberrant transcription or translation of G1 or S cyclins similar to a temperature sensitive allele found in hamster BHK21 cells, ts ET24 cells, with a mutation in DDX3X (a DEAD-box gene), which caused a G1/S arrest with a decrease in the mRNA of cyclin A (Fukumura et al., 2003). A mutant defective in the translation initiator eIF4E in budding yeast, which caused a G1 to S phase progression defect, was also rescued by expression of Cln3p (Danaie et al., 1999). There were very few large budded cells with duplicated DNA in the *ded1-51* strains arrested with nocodazole at 37°C. Assessing precocious sister chromatid separation was therefore difficult. Preliminary results indicate that there may be a defect in sister chromatid cohesion in *ded1-51* strains at 37°C as approximately half of the large budded cells contained 2 GFP signals (46 / 90; 51%) with the 2 GFP signals in the same “mother” compartment (Figure 3-8b).

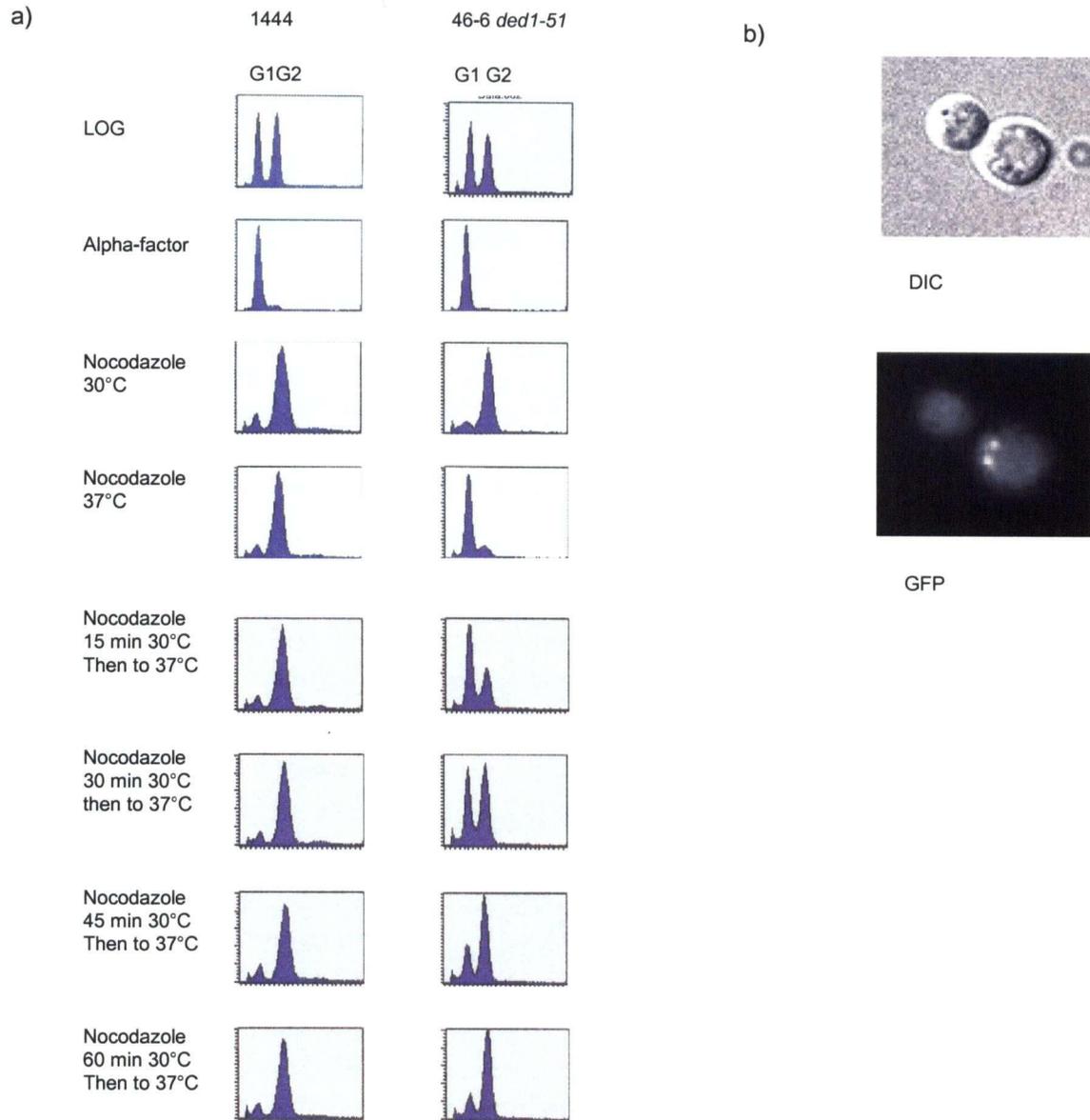


Figure 3-8 – *ded1-51* strains have a cell cycle defect and potential sister chromatid cohesion defect.

A) FACS profiles of wild type (YPH1444) and a *ded1-51* (YBC696) ts mutant arrested in alpha-factor and released either into YPD-nocodazole at 30°C or 37°C. At 15 minute time points cell cultures were also shifted from 30°C to 37°C. G1 and G2 peaks are labeled. B) An example cell from the sister chromatid cohesion assay showing a large budded cell containing 2 GFP signals both in the same “mother” compartment of the cell.

Discussion

In an effort to understand the functions of Rad61p *in vivo* we used both genetic and biochemical (proteomic) approaches. The diploid-SLAM technique allowed the identification of all the deletion mutants in the genome wide deletion set that were synthetically lethal with *rad61Δ*. Direct tests for synthetic interactions had been performed previously with *rad61Δ* and deletion mutants of genes important for kinetochore and sister chromatid cohesion function and all four interactions that had been identified previously (*ctfΔ*, *chl4Δ*, *kar3Δ*, *ctf19Δ*) were also identified in our diploid-SLAM screen. There were no false negatives from the diploid-SLAM results compared to the direct tests. Clustering the results of the synthetic lethality profile of *rad61Δ* led us to test for cohesion defects in *rad61Δ* strains because *rad61Δ* clustered next to mutants in cohesion genes. We indeed observed a cohesion defect but were unable to show any physical interaction of Rad61p with either the core cohesin complex or with the Scc2/4 cohesin loading complex. By chromosome spreads, cohesin loading, association and dissociation from chromatin appear unaffected in *rad61Δ* strains. Rad61p may be involved in establishing sister chromatid cohesion.

To investigate the possibility that Rad61p was binding to specific chromosomal loci, we collaborated with Michael Snyder's laboratory and performed ChIP-chip analysis on strains containing Rad61-13MYCp expressed at endogenous levels. Using the ChIP-chip assay, Rad61p bound specifically at regions clustered around centromeres. Extensive work purifying the components of the yeast kinetochore in recent years has not identified Rad61p as a component of the budding yeast kinetochore (De Wulf et al., 2003; Measday and Hieter, 2004). We also did not see the typical localization pattern

seen for kinetochore associated proteins when we examined localization of Rad61p through the cell cycle (Figure 2-8b, 2-8c). It is possible that Rad61p is binding CEN DNA transiently and/or this transient binding is difficult to detect because of Rad61p binding to general chromatin (Figure 2-9a). The fixation time used in the ChIP-chip assay is longer than that used in our follow-up experiments (Figures 3-4b and 3-4c) and this may have an effect in detecting interactions that are less direct and occurring through other proteins at the kinetochore. Rad61p may be associated at the periphery of the budding yeast kinetochore. The binding of Rad61p to regions clustered around CEN DNA is also reminiscent of the cohesin complex association with centromere regions (Lengronne et al., 2004; Glynn et al., 2004).

The biochemical approach that we initially undertook to find proteins that physically interact with Rad61p was to perform immunoprecipitations on extracts from yeast expressing endogenous levels of Rad61-13MYCp. The eluted samples were purified and the proteins digested with trypsin, with the tryptic peptides subjected to tandem mass spectrometry analysis. We were consistently unable to purify more than 2 peptide fragments of Rad61p even when we scaled the culture to five litres. Rad61p may be labile and the immunoprecipitation and washing combined with the trypsin digests and purification may have caused much of the protein to be degraded. We did however isolate one peptide fragment of Ded1p in one of our experiments, but because of the low number of Rad61p peptides it was not immediately followed up on. While we were able to see intact Rad61-13MYCp by western blotting of our IP samples (data not shown), there also appeared to be degradation bands suggesting loss of sample due to degradation.

The alternative approach that we decided to pursue was that of producing a large amount of GST-Rad61 protein in *E. coli*, purifying the protein using glutathione agarose beads and then incubating the beads with yeast lysate. We performed this experiment with a GST vector construct alone as a negative control for yeast proteins that would bind GST and the glutathione agarose beads. This approach helped us to isolate hundreds of Rad61p peptide fragments and also specific peptides that were present in the GST-Rad61 and not in the GST alone control experiment. Testing the top three results confirmed that Rad61p did indeed bind to one of these proteins, Ded1p, and that this interaction occurred during a normal cell cycle (the IP was performed using log phase cells) with both proteins expressed at their endogenous levels. We had performed the initial characterization of the binding of Ded1p and Rad61p using an over-expressed HA-Rad61 fusion protein and it was important to show that the proteins could interact when they were both expressed at their endogenous levels in yeast extracts.

Ded1p belongs to a large family of putative RNA helicases that have nine conserved amino acid motifs including the characteristic Asp-Glu-Ala-Asp (DEAD) box (Rocak and Linder, 2004). Along with the DEAH and DExH-box families they form a large super-family of helicases that are highly conserved and are present in bacteria, yeast, and humans (Abdelhaleem et al., 2003). The DEAD-box helicases use the energy released from ATP hydrolysis to cause re-arrangements of RNA including unwinding RNA duplexes and displacing proteins from RNA molecules (Linder, 2003; Rocak and Linder, 2004). There are 27 DEAD-box proteins in budding yeast and they have been implicated in many aspects of RNA metabolism including RNA synthesis, translation, rRNA processing, ribosomal bio-synthesis and RNA degradation (Rocak and Linder,

2004). Purified Ded1p has been shown to have RNA helicase activity *in vitro* and also to be able to displace proteins from RNA molecules without unwinding the RNA duplex (that is without acting as a helicase); both activities occur in an ATP dependent manner (Fairman et al., 2004). Ded1p's role in initiating translation is well established and a *ded1* cold sensitive allele shows defects in global protein synthesis when shifted to non-permissive temperature. A decline in polyribosome levels occurs almost immediately upon shift to lower temperatures consistent with a direct role of Ded1p in translation initiation (Chuang et al., 1997). The putative role for RNA helicases at translation initiation is unwinding RNA duplex structures at the 5' Un-Translated Region (5'-UTR) allowing the ribosome to scan the mRNA to the AUG (Iost et al., 1999). Localization of Ded1p has also been shown to be cytoplasmic, which was re-confirmed in our strain background, and consistent with its role in mRNA translation.

Ded1p also has a nuclear role as a member of the spliceosome and also as a Cdc28 interacting protein isolated by mass spectrometry analysis of purified Cdc28p (Honey et al., 2001). *DED1* was originally isolated as a suppressor of the *prp8-1* mutation, a gene involved in splicing (Jamieson et al., 1991). Using splicing-sensitive microarrays (DNA chips that contain sequences that correspond to intronic sequences and to sequences at 5' and 3' UTRs), *ded1* temperature sensitive alleles were found that clustered with genes involved in mRNA translation and also with genes involved in splicing (Burckin et al., 2005). Ded1p could also bind to small nuclear RNAs (snRNAs) indicative of a role in splicing (Burckin et al., 2005). It was concluded that Ded1p had functions both in translation and in splicing and that any link between the two functions would need further characterization.

What could be the potential role of Rad61p and Ded1p together in chromosome segregation? It seems likely that any role would be direct, as both proteins bind chromatin and can physically interact. The genetic interactions between mutant alleles of *ded1* and *rad61Δ* and also of *rad61Δ* and mutants in kinetochore and sister chromatid cohesion function, are consistent with a direct role in chromosome segregation. The biochemical function of the two proteins has not been elucidated and there remains the possibility that the role of Ded1p in chromosome segregation is in affecting the expression level of a protein important for chromosome segregation.

Ded1p has been implicated in both translation and splicing and it is possible that its role in those two processes could contribute to chromosome loss through indirect action on genes involved in chromosome segregation. For example, *Prp17*, a gene coding for a protein of the spliceosome was initially characterized as *Cdc40* and mutant strains displayed cell cycle defects. The cell cycle defects of a *prp17* mutant could be suppressed by deleting the intron of *ANCI*, a gene that is implicated in cell cycle control (although the exact function of Anc1p is unclear) (Dahan and Kupiec, 2004).

Approximately 3.8% of yeast genes contain introns (the majority having only one intron) but these yeast genes are estimated to account for 27% of the total mRNA transcripts in typical cells (many ribosomal proteins contain an intron as well as genes coding for actin and tubulin) (Ares et al., 1999). Using microarray chips that contain intronic sequences (the splicing-sensitive chips mentioned above), researchers have recently been able to determine effects of deleting specific genes involved in splicing and which genes containing introns are affected (Burckin et al., 2005). Specific effects on different intronic genes by deletion of different members of the splicing machinery (and also of

genes involved in mRNA metabolism) can be assessed. Some of the genes that have introns that are interesting in terms of potential chromosome segregation defects and/or cell cycle control defects include *GLC7*, a phosphatase that de-phosphorylates Ip1p substrates, *MOB1*, part of the mitotic exit network, *TUB1* and *TUB3*, coding for alpha-tubulin, *ACT1* coding for actin, and *PHO85* involved in cell cycle control (Saccharomyces Genome Database, SGD).

Ded1p's role in translation initiation could be another potential way that Ded1p has an indirect role on chromosome segregation. Proteins that are involved in cell cycle regulation, kinetochore function and cohesion could be potentially reduced in a *ded1* hypomorph mutant. In the fission yeast *S. pombe*, the homologue of Ded1p has been found to associate with Cdc2 (the CDK in fission yeast) and with Chk1 (a protein kinase that is involved in response to DNA damage) (Liu et al., 2002). It was shown that Ded1p could be post-translationally modified in response to heat shock and to depletion of carbon (but not to DNA damage) and it was concluded that Ded1p may be playing a role in affecting protein translation and synthesis in response to cellular stress (Liu et al., 2002). An attractive idea would be that Ded1p is affecting translation of proteins that are needed to respond to the cellular stress of damage. In budding yeast, a mutant allele of *DED1*, *ded1-18*, has been found to impair brome mosaic virus (BMV) RNA2 translation while still maintaining general translation of other mRNA and allowing growth (Noueiry et al., 2000). Ded1p could either have a direct effect in selecting mRNA for translation or the helicase activity of the mutant allele could be decreased below a threshold required to unwind RNA secondary structure in the BMV RNA2 mRNA to allow translation initiation (Noueiry et al., 2000).

Could Rad61p have a role with Ded1p in translation initiation or mRNA splicing?

The *rad61Δ* deletion mutant does not have growth defects at any of the temperatures tested and it seems unlikely that Rad61p would have a function in initiating translation, although it may have a role in translating a subset of proteins that are important for chromosome segregation. Testing the expression levels of a subset of proteins that are known to be involved in kinetochore or cohesion function could be attempted. In this regard we have checked protein levels of cohesin complex members and some members of the kinetochore complexes and have found no detectable difference in expression levels between wild type and *rad61Δ* cells (data not shown). A role for Rad61p in splicing could be addressed by using splicing-sensitive chips to check if there were splicing defects in *rad61Δ* cells versus wild type. Different components of the splicing machinery appear to have potentially different effects on specific subsets of yeast genes that have introns. A global approach to assessing all the yeast genes with introns would be the best way to assess any splicing defect that may exist in *RAD61* deletion mutants. The other possibility is to check protein expression of genes that have documented roles in cell cycle regulation and chromosome dynamics.

From the experiments that were performed on Ded1p it seems likely that Ded1p has roles other than splicing in the nucleus. A fraction of Ded1p binds chromatin, but it is unclear what function this chromatin binding Ded1p fraction is performing. It would be very informative to perform ChIP-chip experiments on Ded1p and characterize any particular chromosomal locations to which Ded1p is binding. It would be interesting to see if Ded1p is binding the same locations as Rad61p, which would suggest that the chromatin binding fractions of the proteins are interacting. Ded1p's chromatin binding

can also be assessed in *rad61Δ* strains to investigate if Ded1p chromatin binding is dependent on Rad61p. At this point it is unclear whether Rad61p is interacting with Ded1p on chromatin or whether there is some small fraction of Rad61p in the cytoplasm that cannot be detected by microscopy. The conditional synthetic lethality between *rad61Δ* and temperature sensitive alleles of *ded1* points to the importance of the interaction between the two proteins. Preliminary data showing sister chromatid cohesion defect in *ded1-51* may point to a mechanism for the chromosome missegregation phenotype.

CHAPTER 4:

Conclusions and Discussion

Approaches to Identifying novel components involved in chromosome segregation

In an effort to identify novel components that are involved in chromosome segregation, and in particular, kinetochore function, we used *SGT1* as a genetic entry point. Sgt1p is a protein partner of Skp1p and plays a role with Skp1p at the budding yeast kinetochore and the SCF complex. We were interested in finding proteins that could play a role in regulation of the kinetochore and since Sgt1p plays a role in activating the CBF3 complex, we reasoned that it would be a suitable entry point. We attempted two large scale screens, a high copy suppression screen and a genome wide two-hybrid screen, and we identified a novel factor in chromosome segregation, Rad61p, that is apparently not directly involved in Sgt1p function, but is important in sister chromatid cohesion.

Genome Wide Two-hybrid screen of *SGT1* identifies *RAD61*

The approach that led to the identification of Rad61p as a novel factor in chromosome segregation was a genome wide two-hybrid screen using *SGT1* as the “bait”. We were able to identify genes encoding known protein partners of Sgt1p, including Skp1p and F-box proteins such as Cdc4p, Grr1p and Met30p. These results gave us confidence that we were identifying proteins that were biologically relevant to Sgt1p function. We were especially interested in Rad61p, because the deletion mutant of the gene displayed a chromosome missegregation phenotype. We were unable to detect an interaction between Sgt1p and Rad61p by co-immunoprecipitation experiments using logarithmically growing yeast extracts and we concluded that the interaction was transient, sub-stoichiometric, or a potential artifact of the two-hybrid screen. We found

when we performed a ChIP-chip analysis with long fixation times using formaldehyde to cross-link DNA-protein interactions that Rad61p could bind preferentially to regions close to centromeres. The two-hybrid interaction may represent an indirect interaction between Rad61p and Sgt1p through the kinetochore and proteins indirectly associated with the kinetochore.

In addition to a chromosome missegregation phenotype, *rad61Δ* diploid strains also displayed a G2/M progression delay that was dependent on Mad2p. When we investigated the morphology of the cells, we found that *rad61Δ* strains contained more cells with short spindles and the nucleus at the neck, compared to wild type cells. This was indicative of a delay in progressing through the metaphase to anaphase transition consistent with the delay depending on Mad2p. Mutants displaying a similar phenotype have been found by mutation of genes encoding proteins involved in processes such as kinetochore, microtubule, and sister chromatid cohesion function (Hyland et al., 1999; Mayer et al., 2001). As we continued to characterize Rad61p, we found that it was localized to the nucleus and also bound chromatin. These characteristics were consistent with a direct role in chromosome segregation. *rad61Δ* mutants are also sensitive to DNA damaging agents, especially to DNA damaging agents that cause a double strand break (Game et al., 2003; this study). We explored the possibility that Rad61p could be involved in homologous recombination mediated DNA repair but found that it was not necessary for repair of a specific double strand break and that it was not necessary for DNA “repair centres” as determined by Rad52p foci formation.

Rad61p and its role in sister chromatid cohesion

Using the diploid-SLAM technique to find synthetic interactions of *rad61Δ*, and combining that data with the large database of synthetic interactions uncovered by Tong et al., (2004), we were able to cluster *rad61Δ* with *scc1-73* and *chl1Δ*, two known players in sister chromatid cohesion. When we examined *rad61Δ* strains, we indeed found that they had a sister chromatid cohesion defect. Rad61p belongs to a group of non-essential proteins that when deleted have a moderate sister chromatid cohesion defect. Many of these proteins have been provisionally categorized as proteins involved in establishing sister chromatid cohesion during or immediately after DNA replication because they are not part of the core cohesin complex, are not needed to maintain cohesion once established, and are not involved in loading cohesin onto chromatin.

The DNA damage sensitivity of *rad61Δ* mutant strains may be the result of the moderate defect in sister chromatid cohesion. Several other non-essential gene deletion mutants exhibiting moderate sister chromatid cohesion defects are also sensitive to DNA damaging agents, including *chl1Δ*, *ctf8Δ*, *dcc1Δ*, and *ctf4Δ* (Bennett et al., 2001). We also found that *scc1-73* and *smc3-42* containing strains, with defects in the core cohesin components, were sensitive to bleomycin and MMS (data not shown). Recent studies have shown that sister chromatid cohesion is required for post-replicative DNA double strand break repair (Sjogren and Nasmyth, 2001; Strom et al., 2004; Unal et al., 2004). Cohesin is recruited to the sites of double strand break and this recruited cohesin is functional for sister chromatid cohesion (Strom et al., 2004). How sister chromatid cohesion is generated outside of S phase remains unclear. Establishment of cohesion may simply depend on proximity of the sister chromatids provided in S phase

immediately behind the replication fork and in G2 and M (at double strand break sites that have recruited cohesin) by sister chromatid cohesion on the arms established during S phase (Unal et al., 2004). There may be other factors that are important and it would be interesting to see if deleting *RAD61* or any of the non-essential genes involved in sister chromatid cohesion could affect cohesin recruitment or the establishment of sister chromatid cohesion at sites of double strand breaks. Once established, sister chromatid cohesion likely facilitates homologous recombination repair between sister chromatids by tethering the broken ends together and ensuring the right template sequence is available.

Rad61p does not interact with the cohesin complex (or with chromosomal loci known to be cohesin binding sites) and does not interact with Scc2p, part of the cohesin loading complex. It has also not been identified as a member of any of the non-essential complexes that are important for sister chromatid cohesion (Hanna et al., 2001; Mayer et al., 2001; Mayer et al., 2004; Warren et al., 2004). Identifying proteins that interact with Rad61p could shed light onto its role in sister chromatid cohesion.

Rad61p and Ded1p interaction

We incubated a large amount of purified GST-Rad61 recombinant protein with yeast lysate and found specific proteins that bound to GST-Rad61 and not to the GST control. We tested the top three “hits” as determined by the number of peptides that were identified by mass spectrometry, and found that we could reproduce the interaction between Rad61p and Ded1p in cells expressing endogenous levels of the two proteins. Ded1p is an RNA helicase of the DEAD-box family, a large family of ATP dependent RNA helicases that function in RNA metabolic processes such as splicing, ribosome bio-

synthesis, and translation initiation. We showed that a portion of Ded1p binds to chromatin in addition to the documented localization in the cytoplasm. The functional significance of the interaction between Ded1p and Rad61p is supported by the observed genetic interactions between *rad61Δ* and two temperature sensitive alleles of *DED1*.

Ded1p and its role in chromosome segregation

One of the temperature sensitive alleles of *DED1* (*ded1-55*) exhibits an increase in chromosome missegregation at a semi-permissive temperature, as evidenced by a nine fold increase in the rate of loss of a chromosome fragment at 35°C. The *ded1-55* mutation (M183R) is not in a conserved motif but is in a predicted β -sheet (Tanner et al., 2003) and the mutation may disrupt the secondary structure of the protein. The data demonstrating that *ded1-51* has a sister chromatid cohesion defect may account for the chromosome missegregation phenotype. It is possible that Ded1p could be having an indirect effect on sister chromatid cohesion through mRNA translation or splicing affecting the expression level of one or more proteins that are necessary for sister chromatid cohesion, but we believe this is unlikely because of its localization onto chromatin and the genetic interactions of *ded1* temperature sensitive alleles with *rad61Δ*. The clustering of *rad61Δ* with *scc1-73* and *chl1Δ* (*Scc1p* is a core component of cohesin and *Chl1p* is a DNA helicase important for sister chromatid cohesion) based on synthetic lethal interactions with deletion mutants of the yeast genome wide deletion set suggests a direct function for Rad61p in sister chromatid cohesion. The genetic interaction between *ded1* ts alleles and *rad61Δ* suggests that Rad61p and Ded1p binding in yeast cells is functionally significant. Clustering analysis placing uncharacterized genes into well

known pathways has identified novel components of those pathways (Tong et al., 2004). Ded1p could be directly involved with Rad61p in sister chromatid cohesion in addition to its roles in translation and splicing. There are examples of other proteins that play multifunctional roles in cells; for example, Noc3p was initially found to be required for rRNA processing and pre-ribosome maturation (in the nucleolus) but also subsequently demonstrated to be a chromatin binding protein important for the initiation of DNA replication (Zhang et al., 2002). Ded1p could be playing multiple roles in the budding yeast cell in translation, splicing, and sister chromatid cohesion.

RNA Helicases in Human Cancers

A recent survey using the PSI-protein BLAST program identified 36 members of the DEAD-box family and 14 members of the closely related DEAH-box family in the *Homo Sapiens* non-redundant peptide sequence database (Abdelhaleem et al., 2003). The functions of the putative DEAD-box RNA helicases that have been investigated are the same as the DEAD-box RNA helicases in budding yeast; namely RNA metabolic processes such as mRNA splicing, ribosome biogenesis, translation, mRNA export and mRNA stability (Abdelhaleem et al., 2003). Many of the DEAD-box family members remain un-characterized but two important characteristics of the DEAD-box family members that have been investigated have been described; there is a dysregulation of expression in cancer and an involvement in differentiation (Abdelhaleem, 2005).

Human RNA helicases have been found to be overexpressed in colorectal cancer (*DDX5* (p68) (Causevic et al., 2001), *DDX6* (Nakagawa et al., 1999)), as well as other cancers and down-regulated in acute lymphoblastic leukemia (*DHX32* (DEAH-box

family member), (Abdelhaleem, 2002)). There is also evidence that human RNA helicases can interact with proteins implicated in cancer. For example, DDX2 interacts with Pcd4, a protein with transformation-suppressing activity (and Pcd4's overexpression can inhibit transformation), and this activity is linked to its binding to DDX2 (Yang et al., 2003a; Yang et al., 2003b). *DDX10* was found to be involved in a leukemia-associated chromosomal translocation Inv11(p15q22) generating an in-frame fusion of the *DDX10* to *NUP98*. There are many more examples of RNA helicase links with cancer (see the recent review from Abdelhaleem, (2004) for a list of the human RNA helicases and the cancer-related evidence). While the closest human homologues to Ded1p in humans (DDX3X, DDX3Y, and DDX41) do not have documented roles in cancer and tumorigenesis, Ded1p is also highly homologous to the other DEAD-box family members (especially in the nine conserved domains).

The accepted role of human RNA helicases in RNA processing can contribute to tumorigenesis in at least three ways, through altering splicing, transcription and translation of mRNA (Abdelhaleem, 2004). Altering expression of oncogenes and/or tumour suppressor genes could lead to tumorigenesis and altering RNA helicase activity (either by mutations, deletions of the gene, or by overexpression) could be one of the "hits" needed for tumour progression. We have shown evidence that a temperature sensitive mutation in budding yeast *DED1* can cause an increase in the rate of loss of a non-essential chromosome fragment. There is no clear evidence to show that this phenotype is a direct consequence of Ded1p function on chromatin. The chromosome missegregation phenotype of the *ded1* ts allele may be the result of altering the expression of a gene that is involved in chromosome segregation. Finding the gene that

is responsible for the loss of the chromosome fragment based on its altered expression in the *DED1* mutant would provide a rationale for how mutating RNA helicases could affect processes such as chromosome segregation. A connection between RNA helicases and genes important for chromosome segregation fidelity would be an interesting avenue of research to explore in colorectal cancer cell lines that are both aneuploid and overexpressing RNA helicases.

Could RNA helicases be playing a more direct role in chromosome segregation and tumorigenesis? Could there be effects other than on RNA metabolism? A recent report showed that some of the RNA “helicases” (including Ded1p) could displace proteins from RNA duplexes without “unwinding” the RNA duplex; that is without its RNA helicase activity (Fairman et al., 2004) indicating that novel activities of these proteins can still be uncovered. Recombinant Dpb9p (a budding yeast DEAD-box protein involved in ribosome biogenesis) was also found to have DNA helicase activity *in vitro* although the physiological significance of this is unclear (Kikuma et al., 2004). However, Ded1p does not show DNA helicase activity (Patrick Linder, personal communication).

There is evidence that Ded1p can physically interact with a checkpoint kinase, Chk1p, in fission yeast extracts (Liu et al., 2002) and that *DED1* has genetic interactions with *CDC2* (CDK in fission yeast). In budding yeast Ded1p has been identified as a protein interactor with Cdc28p. We also identified Ded1p as a protein interacting with a nuclear localized protein, Rad61p. A fraction of both Rad61p and Ded1p binds chromatin. These results suggest a potential role for Ded1p on chromatin but definitive proof of a biochemical function has not been obtained.

***RAD61* and potential human homologues**

A BLAST search using the Rad61p protein sequence reveals two human sequences with weak homology to Rad61p. LOC57821 is a putative human protein and has had little characterization performed and is the most significant human protein found from a Rad61p protein query (over a region of 230 amino acids, there is 18.4% identity and 29.4% similarity). LOC57821 has been identified as one of the putative homologues of ctf genes (specifically of *RAD61*) that are candidates for sequencing in colorectal cancer cell lines with the CIN phenotype (Karen Yuen, personal communication). APE (AKT-Phosphorylation Enhance), KIAA1212, is 19.3% identical and 29.4% similar to Rad61p over a region of 190 amino acids. APE has been recently shown to be a novel interactor of Protein Kinase B (PKB), a kinase involved as a key mediator of signal transduction with a wide variety of substrates, all of which have the consensus motif RXXRX(S/T) (Anai et al., 2005). APE may function in DNA synthesis and apoptosis in cooperation with PKB.

Conclusion

Rad61p was initially identified as a potential protein partner of Sgt1p based on a genome wide two-hybrid screen using *SGT1* as the "bait". Rad61p and Sgt1p did not detectably interact in co-immunoprecipitation experiments performed on yeast extracts and Rad61p did not appear to be an SCF substrate. *RAD61* is not essential, but deleting the gene has consequences for the fidelity of chromosome segregation, increasing the rate of loss of endogenous chromosomes to ~100x that of wild type strains. The chromosome

Figure 1 - Putative Human homologues of Rad61p in Human Cells

a)

LOC57821	45	QENLNQDIKNEIVVEHKKRHSDPKSLQDEVSPRRNDIISVPGIPLDPISSDSSENSF
RAD61	31	DDVNSVTPVVSSTISSSIAD-HPIEGLLDEPRKAQSSSSFDGAN-EKPSQLDSKRND
LOC57821	105	QESKRESOKLEEEEDVEVRRYIMBKIYQANKLQNOQEPVNDKREKCLKFKDQLMDL
RAD61	89	QNVKTISSDTS-----MAFMKDEKLSAFNFLDGSKASKRKRRTYQKHDANITS
LOC57821	162	EYPP---LEDITISKNYFENERNMGKISQLCHSNDFQEDVLLSITNGSCEENK-
RAD61	139	SIEPDVQDEDSITMINEFESIRKIKYNDINEFIEKLPRADDDLNKKLEENEMKMDDS
LOC57821	214	--DRGILVERDCKEELNLQDTASOGILPPIINANSTENDPQQLPRSSNSVSGT
RAD61	195	IENNSIRTSKDKKYGKFRITILNKN----KENEIMGEEVDQKANTLSLNNADNSNA
LOC57821	268	KKEDSTAKIHAVTHSSSTCEPZAY 290
RAD61	247	EKEGLTSTNHYNELKNMCEETIKY 269

b)

RAD61	60	EPRKAQSSSSFDGAN EKPSQ--LDSKRNDQNVKHIITSSDHS-MAFMKDEKLSAFNFLD
KIAA1212	671	ENRRLKKAIDSFKNLTFQLESLEKENSQLDENEFQRRNVESLKCASMKMAQLQLENKEL
RAD61	117	GSKASKRKRRTYQKHDANITSSIEPDVQ--DEDSITMINEFESIRKIKYNDINEFIEKLP
KIAA1212	731	ESEKEQLKKGLELLKASFKTERLEVSVOGLDIENQRLEKTLNENKKIQLSESEIQ
RAD61	172	KLPRADDDLNKKLEENEMKMDDSIENNSIRTSKDKKYGKFRITILNKNKENEIMGEE
KIAA1212	788	DLEMNQTEQKNLEELKISSKRLEQLEKENKSLQETSQLEKDKKQLEKENKRERQ
RAD61	228	EVDQKANTLSLNNADNSNAEKEGLT 252
KIAA1212	844	QAEIKDTLEENNVKIGNLEKENKT 868

Figure 1 - Putative Human homologues of Rad61p in Human Cells. A) Rad61p and LOC57821 are 18.4% identical and 29.4% similar near the N-terminal of each protein. B) Rad61p and KIAA1212 (APE) are 19.3% identical and 29.4% similar in a limited region, near the N-terminal of Rad61p and in the middle of APE.

missegregation phenotype of *rad61Δ* strains is the result of a defect in sister chromatid cohesion and Rad61p may play a role in sister chromatid cohesion establishment based on its chromatin localization. Rad61p physically interacts with a DEAD-box RNA helicase, Ded1p, and this interaction is functionally significant as evidenced by genetic interactions between temperature sensitive alleles of *DED1* and *rad61Δ*. Ded1p is a multifunctional protein with characterized roles in initiating translation and also as a member of the spliceosome. We found that a sub-fraction of Ded1p binds to chromatin and a mutant allele of *DED1* loses a non-essential chromosome fragment at a rate ~9x that of wild type strains at a semi-permissive temperature. Preliminary data also points to a sister chromatid cohesion defect for *ded1-51* strains pointing to a possible mechanism for the chromosome missegregation phenotype. Taken together, these results suggest that Rad61p and Ded1p cooperate together on chromatin in a process that is necessary for sister chromatid cohesion. Human homologues of Rad61p and Ded1p would therefore be candidates to screen for mutations that may be causing the CIN phenotype in human cancer cell lines.

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