Systematic Exploration of Essential Yeast Gene Functions

with Temperature-sensitive Mutants

By

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A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy

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The budding yeast *Saccharomyces cerevisiae* is the most well characterized model organism for systematic analysis of fundamental eukaryotic processes. Approximately 19% of *S. cerevisiae* genes are considered essential. Essential genes tend to be more highly conserved from yeast to humans when compared to nonessential genes. The set of essential yeast genes spans diverse biological processes and while the primary role of most essential yeast genes has been characterized, the full breadth of function associated with essential genes has not been examined, due, at least in part, to the lack of adequate genetic reagents for their conditional and systematic perturbations.

To systematically study yeast essential gene functions using synthetic genetic array analysis and to complement the current yeast deletion collection, I constructed a collection of temperature-sensitive yeast mutants consisting of 795 ts strains, covering 501 (~45%) of the 1,101 essential yeast genes, with ~30% of the genes represented by multiple alleles. This is the largest collection of isogenic ts yeast mutants constructed to date.

I confirmed the correct integration of over 99% of the ts alleles using PCR-based strategy and the identity of the ts allele by complementation of the ts phenotype with its
cognate plasmid. The ts mutant collection was characterized by high-resolution profiling of the temperature sensitivity of each ts strain, distribution analysis of gene ontology molecular function and biological process, and comparison of ts allele strains to the strains carrying Tet-repressible alleles of essential genes. The results demonstrated that the ts collection is a powerful reagent for the systematic study of yeast essential gene functions and provides a valuable resource to complement the current yeast deletion collection.

I validated and demonstrated the usefulness of the ts collection in a number of different ways. First, I carried out detailed temperature profiling of each mutant strain using liquid growth assays and found that ts mutants that define particular biological pathways often show highly similar profiles. Second, I showed that the ts mutant array can be used to screen compounds for suppression of growth defects and thus is useful for exploration of chemical-genetic interactions. Third, I demonstrated that the ts collection represents a key reagent set for genetic interaction analysis because essential genes tend to be highly connected hubs on the global genetic network. Fourth, I further validated the ts array as a key resource for quantitative phenotypic analysis by using a high-content screening protocol to score six different fluorescent markers, diagnostic for different subcellular compartments or structures, in hundreds of different mutants. Quantification of the marker behaviour at the single-cell level enabled integration of this data set to generate a morphological profile for each ts mutant to reveal both known and previously unappreciated functions for essential genes, including roles for cohesion and condensin genes in spindle disassembly.
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LIST OF ABBREVIATIONS

Individual amino acids are abbreviated in the text as three letters (e.g. alanine is abbreviated Ala). Lengths and volumes are annotated using the prefix symbols approved by the International Systems of Units (e.g. micron is abbreviated μm, microliter is abbreviated μl).

Δ deletion (of a gene)
°C degree Celsius
AP/MS affinity purification/mass spectrometry
ATP adenosine triphosphate
bp base pair(s)
canavanine L-canavanine sulfate salt
CAP chromosome associated protein
*C. elegans* *Caenorhabditis elegans*
CEN centromere
clonNAT nourseothricin
CPC chromosomal passenger complex
cs cold-sensitive
DAmP decreased abundance by mRNA perturbation
*D. melanogaster* *Drosophila melanogaster*
DMSO dimethyl sulfoxide
DNA deoxyribonucleic acid
FEAR Cdc fourteen early anaphase release
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>FPP</td>
<td>farnesyl diphosphate</td>
</tr>
<tr>
<td>G418</td>
<td>geneticin</td>
</tr>
<tr>
<td>GFP</td>
<td>green fluorescent protein</td>
</tr>
<tr>
<td>GGPP</td>
<td>geranylgeranyl diphosphate</td>
</tr>
<tr>
<td>GO</td>
<td>gene ontology</td>
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<tr>
<td>GST</td>
<td>glutathione-S-transferase</td>
</tr>
<tr>
<td>HCS</td>
<td>high content screening</td>
</tr>
<tr>
<td>HMG-CoA</td>
<td>3-hydroxy-3-methylglutaryl-coenzyme A</td>
</tr>
<tr>
<td>HMGR</td>
<td>HMG-CoA reductase</td>
</tr>
<tr>
<td>kanMX</td>
<td>kanamycin resistant cassette</td>
</tr>
<tr>
<td>kb</td>
<td>kilobase pair(s)</td>
</tr>
<tr>
<td>MAT&lt;sup&gt;a&lt;/sup&gt;</td>
<td>yeast mating type a</td>
</tr>
<tr>
<td>MAT&lt;sup&gt;α&lt;/sup&gt;</td>
<td>yeast mating type alpha</td>
</tr>
<tr>
<td>MEN</td>
<td>mitotic exit network</td>
</tr>
<tr>
<td>MoBY-ORF</td>
<td>molecular barcoded yeast ORF</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger RNA</td>
</tr>
<tr>
<td>MSG</td>
<td>monosodium glutamic acid</td>
</tr>
<tr>
<td>MT</td>
<td>microtubule</td>
</tr>
<tr>
<td>natMX</td>
<td>nourseothricin resistant cassette</td>
</tr>
<tr>
<td>OD</td>
<td>optical density</td>
</tr>
<tr>
<td>ORF</td>
<td>open reading frame</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
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<tr>
<td>RFC</td>
<td>replication factor C</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<td>--------------</td>
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<tr>
<td>RFP</td>
<td>red fluorescent protein</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>RNAi</td>
<td>RNA (mediated) interference</td>
</tr>
<tr>
<td><em>S. cerevisiae</em></td>
<td><em>Saccharomyces cerevisiae</em></td>
</tr>
<tr>
<td><em>S. pombe</em></td>
<td><em>Schizosaccharomyces pombe</em></td>
</tr>
<tr>
<td>SAC</td>
<td>spindle assembly checkpoint</td>
</tr>
<tr>
<td>SC</td>
<td>synthetic complete media</td>
</tr>
<tr>
<td>SCC</td>
<td>sister chromatid cohesion</td>
</tr>
<tr>
<td>SD</td>
<td>minimal synthetic defined/synthetic minimal glucose</td>
</tr>
<tr>
<td>SGA</td>
<td>synthetic genetic array</td>
</tr>
<tr>
<td>SGD</td>
<td>Saccharomyces Genome Database</td>
</tr>
<tr>
<td>SL</td>
<td>synthetic lethal</td>
</tr>
<tr>
<td>SMC</td>
<td>structural maintenance of chromosomes</td>
</tr>
<tr>
<td>SMF</td>
<td>single mutant fitness</td>
</tr>
<tr>
<td>SS</td>
<td>synthetic sick</td>
</tr>
<tr>
<td>TEF</td>
<td>translation elongation factor</td>
</tr>
<tr>
<td>Tet</td>
<td>tetracycline-regulatable promoter-replacement</td>
</tr>
<tr>
<td>ts</td>
<td>temperature-sensitive</td>
</tr>
<tr>
<td>WT</td>
<td>wild-type</td>
</tr>
<tr>
<td>Y2H</td>
<td>yeast two hybrid</td>
</tr>
<tr>
<td>YEPD</td>
<td>yeast extract peptone dextrose (rich medium)</td>
</tr>
<tr>
<td>YKO</td>
<td>yeast knockout</td>
</tr>
<tr>
<td>ZA</td>
<td>zaragozic acid A</td>
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CHAPTER ONE

General Introduction

1.1 Budding yeast as a model organism

The budding yeast *Saccharomyces cerevisiae* is one of the best characterized model organisms for systematic analysis of fundamental eukaryotic processes. *S. cerevisiae* was the first eukaryotic genome to be sequenced, largely because it has a relatively small genome of 12,052 kb (Goffeau et al., 1996). Yeast has short non-coding regions and less than 7% of its ~6,000 genes contain introns (Grate and Ares, 2002). These factors have facilitated the identification of genes in yeast and enabled the rapid construction of genomic resources. The life cycle of budding yeast is well suited to classical genetic studies. Importantly, *S. cerevisiae* can be propagated in either a haploid or diploid form, which lends itself well to genetic manipulation and the analysis of recessive and dominant mutations. In addition, yeast is highly amenable to plasmid transformation and has very efficient homologous recombination, which allows the efficient integration of genetically engineered DNA sequences into the genome.

The extensive conservation of many cellular processes between yeast and higher organisms, especially with regard to basic cellular metabolism and cell division, makes yeast an excellent model eukaryote (Botstein et al., 1997). The sequencing of yeast (Goffeau et al., 1996) and human genomes (Lander et al., 2001; Venter et al., 2001) has revealed thousands of yeast proteins that share amino-acid sequence similarity with at least one human protein (Hughes, 2002). Several hundreds of these conserved proteins are implicated in human disease (Botstein et al., 1997). Furthermore, 30-40% of human disease-related genes have counterparts in the yeast genome (Foury and Kucej, 2002).
Many fundamental questions in molecular biology have been answered in yeast, and yeast continues to play a central role in eukaryotic cell biology.

In this introductory chapter, I provide an overview of tools and approaches that have been used for systematic analysis of yeast genes with a focus on the technologies I have adapted to understand the genetics of essential genes. In the following sections I describe: (1) essential genes and conditional mutants; (2) the yeast deletion collection and the application of pooled screens using microarrays; (3) the development of synthetic genetic array (SGA) analysis and its power in identifying genetic interactions; (4) the coupling of SGA with high content screening to understand cell biological phenotypes.

1.2 Essential genes and conditional mutants

1.2.1 Essential genes

Essential genes and nonessential genes are genetic descriptions referring to the functional consequence of deleting a gene with respect to its effect on an organism’s viability (Jordan et al., 2002). A gene is considered to be essential if it is required for survival (Gerdes et al., 2006). In contrast, a nonessential gene is dispensable for viability, and its inactivation yields viable individuals.

Several interesting observations have been made regarding the discernible characteristics of essential genes in model organisms. Essential genes are more evolutionarily conserved than nonessential genes, which are more functionally dispensable and/or redundant (Jordan et al., 2002; Yang et al., 2003). Essential genes are likely to be hubs in protein-protein interaction networks (Fraser et al., 2003; Goh et al., 2007; He and Zhang, 2006; Hirsh and Fraser, 2001; Jeong et al., 2001; Wall et al., 2005;
Yu et al., 2004) and in genetic interaction networks (Davierwala et al., 2005; Tong et al., 2004). Also essential genes are more likely to be abundantly and ubiquitously expressed in cells and tissues (Butte et al., 2001; Warrington et al., 2000) and have smaller-sized introns (Bortoluzzi et al., 2003; Lopez-Bigas and Ouzounis, 2004; Rocha, 2006). Essential genes in yeast tend to be more highly conserved in humans: 38% of essential yeast proteins have clear counterparts in human, versus 20% for nonessential genes (Hughes, 2002).

The yeast deletion collection, created by an international consortium of laboratories, consists of a comprehensive set of over 20,000 *S. cerevisiae* gene deletion strains. The collection contains heterozygous diploid strains corresponding to deletions in each of the ~6,000 yeast genes, including the ~1,000 essential genes, and a homozygous diploid, a *MATα*, and a *MATα* strain for each of the ~5,000 nonessential genes. Indeed, the *S. cerevisiae* gene deletion project revealed that ~19% of yeast genes are essential genes (required for viability in standard laboratory growth medium). Recently the analysis of a genome-wide gene deletion set for the fission yeast *Schizosaccharomyces pombe* revealed that ~26% of fission yeast genes (1,260/4,836) were essential and ~74% (3,576/4,836) were nonessential for viability of haploid cells (Kim et al., 2010).

The percentage of essential genes in prokaryotic and eukaryotic genomes varies. Genome-wide screens for gene essentiality found that the percentage of essential genes in different bacteria ranged from 6% to 40% (Gerdes et al., 2006; Lamichhane et al., 2003). *Mycoplasma genitalium* has the smallest genome of any organism that can be grown in pure culture and has a minimal metabolism and little genomic redundancy and over 70%
About 7% (1,170/16,757) of Caenorhabditis elegans genes were essential as defined by embryonic or larval lethality or sterility (with or without associated post-embryonic defects) using RNA interference (RNAi) to inhibit gene function. In this study ~86% of the 19,427 predicted genes of C. elegans were tested (Kamath et al., 2003). Based on about 5,000 knockout mutants obtained from the 20,000 genes of C. elegans, ~20% of genes are essential (personal communication from Dr. Donald G. Moerman). Furthermore, ~51% of C. elegans orthologues of S. cerevisiae essential genes (Mewes et al., 2000) have a nonviable RNAi phenotype (Kamath et al., 2003). Genes involved in basic metabolism and maintenance of the cell are significantly more likely to have a nonviable RNAi phenotype and genes involved in more complex processes that are expanded in metazoa, such as signal transduction and transcriptional regulation, are enriched in the viable post-embryonic phenotype class, consisting of defects in post-embryonic development (for example, in movement or body shape) without associated lethality or slowed growth (Kamath et al., 2003). Yeast and worm genes essential for viability have a similar distribution within the different functional classes (Fraser et al., 2000), suggesting that similar types of genes are required for viability of yeast and animal cells.

Analysis of the published data for 27 different chromosomal regions that were subjected to extensive mutagenesis revealed that approximately 30% (3,600/12,000) of genes are essential for viability in Drosophila (Miklos and Rubin, 1996). Large insertional mutagenesis screens in zebrafish, using mouse retroviral vectors as the
mutagen, revealed that around 20% of the 2,400 genes are required for the development of a zebrafish embryo (Golling et al., 2002). More than 70% of embryonic-essential fish genes have clear orthologues in yeast (Amsterdam et al., 2004). The apparent high conservation of essential gene function suggests that studying yeast essential genes can help us to better interpret essential gene function in animals.

1.2.2 Conditional mutants

Genetic analysis of essential proteins in yeast has traditionally relied on conditional mutants. Conditional alleles enable a functional version of the gene under a permissive condition and a compromised version under the non-permissive condition. Conditional alleles in yeast include temperature-sensitive (ts) (Hartwell, 1967), cold-sensitive (cs) (Moir et al., 1982), temperature-inducible degron (td) (Dohmen et al., 1994; Kanemaki et al., 2003), tetracycline-regulatable promoter-replacement (Tet) (Mnaimneh et al., 2004), and temperature-sensitive inteins (Tan et al., 2009).

One systematic approach for constructing ts alleles involves the fusion of the coding sequence of a given gene to a heat-inducible-degron domain, which modulates the stability of the protein (Dohmen et al., 1994; Kanemaki et al., 2003). The heat-activated degron is long-lived at 23°C but becomes short-lived at 37°C and linking this portable ts-degron to a protein of interest results in destruction of the protein at 37°C (Dohmen and Varshavsky, 2005). The degron system appears highly variable in its effectiveness. Nearly 40% of fusion proteins did not result in inviability when 104 essential genes were fused to the N-degron domain (Kanemaki et al., 2003). Another limitation of the ts-
The degron technique is that it cannot be applied to proteins whose function is incompatible with N-terminal insertions (Dohmen and Varshavsky, 2005).

Another systematic approach for creating mutant alleles of essential genes involves replacement of the native promoter with a tetracycline-regulatable promoter. A set of tetracycline-regulatable promoter-replacement (Tet) alleles was constructed by replacing the native promoter (~100 bp upstream of the start codon) with a \( \text{kanMX-TetO}_7-\text{CYC1}_{TATA} \) cassette (Mnaimneh et al., 2004). The parent yeast strain was also engineered to express tetracycline-controlled transactivator (tTA), which is composed of the activator domain of the herpes simplex virus VP16 fused to the tetracycline-inducible repressor from the Tn10-encoded tetracycline-resistance operon. The tTA protein binds the \( \text{TetO}_7-\text{CYC1}_{TATA} \) promoter and activates gene expression but can be repressed by addition of doxycycline to the growth medium (Gari et al., 1997). In other words, tetO-driven expression occurs in the absence of the effector (tetracycline or other molecules of the same antibiotic family) while addition of antibiotic prevents the tTA protein from binding to tetO promoter sequence and switches off gene expression. Much like the degron system, Tet alleles are useful for regulating expression of a subset of the essential genes, presumably due to contextual effects of the \( \text{TetO}_7-\text{CYC1}_{TATA} \). Specifically, ~600 Tet alleles were created and 74% showed a small colony phenotype in the presence of doxycycline (promoter in the “off” state). Of these, ~20% were also growth-impaired in the absence of doxycycline (promoter in the “on” state) (Mnaimneh et al., 2004).

Hypomorphic alleles of essential genes can often be created by inserting an antibiotic-resistance marker into the 3’UTR to reduce mRNA abundance thus reducing gene expression (Schuldiner et al., 2005). The so-called “decreased abundance by mRNA
perturbation” or DAmP alleles are not conditional alleles because the reduced gene expression is constitutive. The DAmP method appears to work very well for ~30% of essential genes but variably for most essential genes. DAmP alleles may lower the abundance of an essential gene product, but if the reduced protein level does not lead to a fitness defect, then the DAmP allele will tend not to display genetic interactions, and thus may be less informative for functional characterization as discussed in Chapter 3 of this thesis.

Recently a temperature-sensitive intein approach for generating conditional alleles was reported (Tan et al., 2009; Zeidler et al., 2004). An intein is an intervening protein sequence that is embedded within a precursor protein sequence and spliced out during protein maturation (Perler et al., 1994; Perler et al., 1997). More than 200 inteins have been identified from unicellular organisms, including bacteria, archaea, and eukaryotes (http://www.neb.com/neb/inteins.html). Splicing of the inteins (protein splicing) is essential for the function of the host proteins and protein-splicing failure leads to a loss of function of the host proteins. A temperature-sensitive intein (ts intein) is an intein that self-splices only at the permissive temperature to generate a wild-type host protein (Tan et al., 2009; Zeidler et al., 2004). The ts intein approach has been used in bacteria (Liang et al., 2007), yeast (Tan et al., 2009; Zeidler et al., 2004), and Drosophila (Zeidler et al., 2004). A collection containing 41 ts inteins, which function at different permissive temperatures ranging from 18°C to 30°C, was recently created (Tan et al., 2009) and can be selected for generating ts mutants in a variety of organisms. The advantage of the ts intein strategy is that it can be used for any gene and it is readily available.
The most common conditional allele in yeast is the temperature-sensitive allele. A temperature-sensitive (ts) mutant retains the function of a gene at a low (permissive) temperature but not at high (restrictive) temperature. Temperature-sensitive mutants can be used effectively to gain information about essential gene function and have been generated for many different functional classes of genes (Edgar and Lielausis, 1964). Collections of temperature-sensitive mutants have been established in various model microbes, including *S. cerevisiae*, *Salmonella typhimurium*, and *Escherichia coli* (Ben-Aroya et al., 2008; Harris et al., 1992; Kaback et al., 1984; Schmid et al., 1989; Sevastopoulos et al., 1977). A major goal of my thesis work was to construct a collection of strains carrying ts alleles for yeast essential genes, which is compatible with large-scale genetic analyses.

1.3 Yeast deletion collection and barcode microarray assay

The yeast genome-deletion project, a worldwide collaborative effort, systematically created deletion/substitution mutants for almost all of the annotated yeast ORFs, each of which was replaced by a kanamycin resistance cassette (*kanMX4*) that confers resistance to the drug G418 (Winzeler et al., 1999). These yeast knockout (YKO) mutants were created by chromosomal integration of a PCR-generated disruption cassette via homologous recombination (http://www-sequence.stanford.edu/group/yeast_deletion_project/PCR_strategy.html). There are four different YKO collections in the commonly used S288c laboratory strain: (1) *MATα* haploid (referred to as BY4741); (2) *MATα* haploid (referred to as BY4742); (3) *MATα/α* homozygous diploid (referred to as BY4743); (4) *MATα/α* heterozygous diploid.
(Winzeler et al., 1999). The first three YKO collections contain only strains carrying disruption alleles of nonessential genes and the heterozygous diploid YKO collection contains disruptions in both essential and nonessential ORFs. The YKO mutant collections have opened the door to systematic, genome-wide functional analyses.

Each deletion mutant is marked by two unique 20 mer nucleotide sequences flanked by sequences common to all mutants. These ‘molecular barcodes’ enable the identification of each strain within a mixed population of mutants through PCR-based amplification of the barcodes using the flanking common sequences followed by microarray-based quantitative detection (Giaever et al., 2002). This highly parallel technique is particularly useful for fitness testing of large numbers of mutants in small volumes of media. In this strategy, strains are pooled and grown in parallel in liquid culture under selective conditions (for example, in the presence of a drug). Genomic DNA is isolated from the mixed culture and a pool of barcode PCR amplimers is prepared and hybridized to high-density DNA microarrays containing oligonucleotides corresponding to the barcodes. The relative abundance of each strain in the pool is then assessed by measuring the abundance of the relevant gene signal from the microarray readout compared to a control. Using this approach, the entire yeast deletion set can be pooled, grown competitively and assessed quantitatively in only a few milliliters of growth medium (Giaever et al., 2002). The approach has recently been adapted for use with a sequencing-based readout (Bar-seq) (Smith et al., 2009; Smith et al., 2010).

1.4 Genetic interactions and synthetic genetic array (SGA) analysis

1.4.1 Genetic interactions
The yeast haploid deletion mutant collection sparked the development of a high-throughput genome-wide method to identify and study genetic interactions between nonessential genes in *S. cerevisiae* (Tong et al., 2001; Tong et al., 2004). Genetic interactions have long been studied in model organisms as a means of identifying functional relationships among genes or their corresponding gene products (Boone et al., 2007; Hartman et al., 2001). Genetic interactions can be classified into three different groups: negative interactions, positive interactions and gene dosage interactions (Figure 1-1) (Dixon et al., 2009).
A. Negative Interactions

**Nonessential Pathways (Between Pathway GIs)**

- A → X → Y → Z
- B → Y → Z
- C → Z

- Wild type
- Viable
- Lethal (sick)

**Essential Pathways (Within Pathway GIs)**

- A → a
- B → b
- C

- Wild type
- Viable
- Lethal (sick)


B. Positive Interactions

**Loss-of-function Suppression**

- A → a → b → c
- B → c

- Wild type
- Lethal (sick)
- Viable

**Gain-of-function Suppression**

- A → a → b
- B → b → c

- Wild type
- Lethal (sick)
- Viable


C. Gene Dosage

**Dosage Lethality**

- A → a → b
- B → B → B

- Wild type
- Viable
- Lethal (sick)

**Dosage Suppression**

- A → a
- B → B

- Wild type
- Lethal (sick)
- Viable

Adapted from Dixon et al. (2009)

Figure 1-1 Three Different Types of Genetic Interactions
A. Negative interactions. Negative interactions can occur between two non-essential genes that belong to parallel pathways (between-pathway genetic interactions, left panel) or between two hypomorphic alleles of essential genes encoding components within the same pathway (within-pathway genetic interactions, right panel). Uppercase and lowercase represent wild type and mutant (either deletion or hypomorphic allele), respectively. Dashed arrows show decreased activity of the mutant. Black and blue letters indicate wild type and red letters indicate mutants.

B. Positive interactions. Inactivation of a negative regulator (a) may result in overexpression of downstream gene (B) and lead to buildup of a toxic gene product C. A loss-of-function mutation in a downstream component (b) could reduce flux through the same pathway and suppress the toxic effects caused by mutation of the upstream component (a). Gain-of-function suppression (shown as an allele of c*) may occur when a downstream pathway component is mutated (c*) such that it now has upregulated or novel activity and therefore suppresses the lethal/sick phenotype of an upstream mutation (b).

C. Gene dosage. Accumulation of gene product B can cause lethality/sickness (dosage lethality) when an upstream negative regulator A is mutated (left panel). Overexpression of a downstream component B can suppress the lethality/sickness caused by mutation of an upstream component (a) (dosage suppression, right panel).
Negative interactions (also called aggravating or synergistic interactions) occur when double mutants exhibit a more severe phenotype than expected based on the fitness of each single mutant alone. This is referred to as synthetic lethality or sickness. Synthetic lethality or sickness in yeast results when two mutations in different genes are each viable as single mutations but lead to lethality or sickness when combined in the same haploid genome (Hartman et al., 2001; Hartwell, 2004). Synthetic lethality or sickness is of particular interest because it can identify genes whose products buffer one another and are involved in the same essential biological process (Hartman et al., 2001; Hartwell, 2004). Thus synthetic lethality or sickness indicates a functional relationship between genes.

Positive interactions describe a situation in which double mutants exhibit a less severe phenotype than would be expected from a multiplicative model, in which the joint effect of two mutants is the product of their effects if they were acting alone. Positive interactions have also been referred to as alleviating or epistatic interactions (Dixon et al., 2009; Schuldiner et al., 2005).

Gene dosage interactions include dosage lethality and dosage suppression. Synthetic dosage lethality is detected when overexpression of a gene is lethal only if another, normally nonlethal, mutation is present (Boone et al., 2007; Kroll et al., 1996; Liu et al., 2009; Measday and Hieter, 2002). Dosage suppression can be achieved if a mutation in one gene rescues the lethality caused by mutation of another gene. For example, Bts1p (GGPP synthase) is a upstream of Bet2p in yeast isoprenoid pathway (Figure 3-1). Overexpression of $BTS1$ can suppress the lethality of the $bet2-1$ ts mutant at 37°C (Jiang et al., 1995).
Synthetic lethal interactions are interpreted in several ways: between-pathway models, within-pathway models and indirect effects (Dixon et al., 2009). Between-pathway models posit that the genetic interactions bridge genes operating in two pathways with redundant or complementary functions, and deletion of either gene is expected to abrogate the function of one but not both pathways. Within-pathway models predict that the genetic interaction occurs between protein subunits within a single pathway. A single gene is dispensable for the function of the overall pathway, but the additive effect of several gene deletions is lethal. The indirect effort models can occur because a deletion/mutation phenotype represents not just the absence or function reduction of a particular gene, but also the response of the cell to the absence or function reduction of that gene, which may include upregulating or downregulating diverse pathways. For non-essential genes, genetic interactions between deletion mutants usually occur between pathways that are redundant for an essential biological process. In contrast, genetic interactions between essential genes can occur between two hypomorphic alleles within the same biochemical pathway (within-pathway model).

1.4.2 Synthetic genetic array (SGA) analysis

Synthetic genetic array (SGA) analysis is an approach that automates the isolation of yeast double mutants, enabling large-scale mapping of genetic interactions (Tong et al., 2001). In the SGA analysis system, a series of robotic pinning steps is used to cross a $MAT^\alpha$ query mutation strain into an ordered $MAT^a$ mutant (deletion or conditional allele) array. Through mating, sporulation, haploid selection, and double mutant selection, inviable or slow growing double mutants are identified as synthetic lethal or sick (Figure
1-2) (Tong et al., 2001). The key to this system was the development of SGA reporters that allow the germination of only $MAT_a$ meiotic progeny from a population containing both $MAT_a$ and $MAT_\alpha$ spores.

Figure 1-2 Synthetic Genetic Array (SGA) Analysis

In SGA analysis, a $MAT_\alpha$ natMX-marked query strain is crossed to the $MAT_a$ kanMX-marked mutant array. After mating, sporulation, haploid selection and double mutant selection, inviable/slow growing double mutants are identified as synthetic lethal/sick. Synthetic lethality/sickness may indicate a functional relationship between genes.
To first look at genetic interactions on a global scale, our lab performed 132 SGA screens, focused on certain well characterized genes - termed query genes involved in actin-based cell polarity, cell wall biosynthesis, microtubule-based chromosome segregation, and DNA synthesis and repair (Tong et al., 2004). The resulting confirmed data set allowed construction of a genetic interaction network containing ~1,000 genes and ~4,000 interactions. Genes with related biological functions are connected by synthetic genetic interactions more often than expected by chance (Tong et al., 2004), revealing that synthetic genetic interactions tend to occur among functionally related genes. The number of interactions per query gene ranged from 1 to 146, with an average of 34 interactions per screen. Genetic interactions are generally non-overlapping with protein-protein interactions but two-dimensional hierarchical clustering of the interactions reveals that genes within the same pathway or complex often show similar patterns of genetic interactions. Clustering of previously uncharacterized genes with well-studied pathways enables the prediction of precise molecular roles. Presumably, a complete map of synthetic genetic interactions for yeast will identify sets of protein complexes and pathways that buffer each other. Based on the network connectivity, genetic clusters can be used to predict functions for uncharacterized genes. For example, PAR32, ECM30, and UBP15 had similar genetic interaction profiles to those of members of Gap1-sorting module and deletions of all three genes resulted in Gap1-sorting and transport defects (Costanzo et al., 2010).

As noted above, genes that show similar genetic interaction profiles often encode proteins within the same pathway or complex. For example, the uncharacterized gene YMR299C showed a genetic interaction profile that is highly similar with the genes
encoding the dynein-dynactin spindle orientation pathway (ARP1, JUM1, NUM1, DYN1, PAC1, NIP100, PAC11, DYN2) (Tong et al., 2004). Consistent with a role in the spindle orientation pathway, a ymr299cΔ mutant exhibits abnormal cytoplasmic microtubules and defects in mitotic spindle positioning similar to dyn1Δ and arp1Δ (Figure 1-3). Clustering genetic interactions of uncharacterized genes with genetic interactions from components of defined functions and/or pathways should enable the prediction of specific biological functions for uncharacterized genes. Thus SGA stands to be a very powerful approach to uncover gene functions for uncharacterized ORFs and to discover novel functions for previously characterized genes.
Figure 1-3 Predicting Gene Function from Genetic Interaction Patterns

Two-dimensional hierarchical clustering of synthetic genetic interactions determined by SGA analysis suggests that the novel ORF \textit{YMR299C} may be a component of the dynein-dynactin complex. Rows, 76 query genes; columns, 108 array genes. Synthetic genetic interactions are represented as red squares.

Adapted from Tong et al., 2004
Large-scale mapping of synthetic lethal interactions has shown that ~20% of the query genes have no detectable genetic interactions with the nonessential gene deletion array under the conditions tested (growth on standard media). Only nonessential genes are represented in the yeast deletion collection because essential genes cannot be deleted in a yeast haploid strain. As such, ~1,100 essential genes are missed in the yeast deletion array, accounting for 18.7% of the yeast genome. It is possible that many of the query genes that fail to show genetic interactions with the nonessential array would do so with an essential strain array. This possibility was a major motivation for my thesis project which aimed to construct an SGA-compatible array of ts mutants in essential genes. In fact, I performed an SGA screen with a temperature-sensitive allele of an essential gene RFC5 (rfc5-1), which is involved in sister chromatid cohesion, DNA replication, and DNA damage response (Tong and Boone, 2006) and generated a profile of functionally informative genetic interactions (discussed more in Chapter Two).

1.5 High content screening

High content screening (HCS) is an automated imaging technology that facilitates the capture and analysis of thousands of cellular images following different genetic/chemical/environmental perturbations. HCS screening was first used in the fields of toxicology and drug discovery. In recent years, image-based assays for early-stage drug discovery have demanded an increase in the throughput and several developments have enabled high-throughput image acquisition and analysis. These technological advances have led to significant applications in both academic research and drug discovery. For example, HCS has been effectively used to identify targets of small
molecules using assays in mammalian cells (Eggert et al., 2004; Loo et al., 2007; Paran et al., 2007; Perlman et al., 2004; Rickardson et al., 2007). More recently, HCS has been coupled with RNAi-based gene knockdown to characterize gene function in culture cells (Bakal et al., 2007; Boutros et al., 2004; Moffat et al., 2006; Mukherji et al., 2006; Narayanaswamy et al., 2006; Pelkmans et al., 2005; Tanaka et al., 2005). Coupling genetic perturbation with HCS will enhance our understanding of functional connections between genes.

Our group developed an experimental system that combines SGA-based high-throughput strain manipulation and HCS to enable the visualization and quantitative measurement of specific morphological features for numerous single cells within a field (Vizeacoumar et al., 2009; Vizeacoumar et al., 2010). We took advantage of the deletion collection for yeast non-essential genes and SGA technology to introduce fluorescent reporters into every mutant in a collection, and samples were imaged in a systematic format. A major bottleneck in our approach is the automated image analysis. Several software solutions such as the open-source software “CellProfiler” are now available for complicated image segmentation. Image analysis converts the images to numbers that can then be subjected to several statistical tests to define mutants that deviate from the wild-type. Indeed machine-learning approaches are increasingly being used to establish a morphometric signature for a known set of mutants and identify novel components (Chen et al., 2007). For example, Ohya et al. have employed support vector machines (SVMs), which are capable of transforming the originally measured data to a high-dimensional space through kernel mapping, to predict gene functions based on morphological profiles, the rationale being genes within different functional groups are more separable in the
induced feature space (Ohya et al., 2005). Similarly, Bakal et al. trained a neural network where a non-linear mapping of the original data set was done by minimizing a certain objective function to identify local signaling networks that regulate cell protrusion, adhesion and tension (Bakal et al., 2007).

1.6 Mitotic spindle and spindle assembly checkpoint pathways

1.6.1 Mitotic spindle

The combination of SGA analysis with HCS (SGA-HCS) provides a powerful method for identifying and deciphering specific cellular functions, and this approach has been applied previously by our group to study the morphogenesis of the mitotic spindle (Vizeacoumar et al., 2010). I have extended this approach to identify mutants within the ts collection that affect specific pathways by crossing different subcellular reporters and querying for abnormal cellular phenotypes. I crossed several subcellular reporters to the essential gene collection including a GFP-TUB1 reporter to monitor spindle dynamics in the ts array. Since our assay of spindle phenotypes led to the identification of a novel role for cohesin and condensin in spindle disassembly, I outline some of the background required for spindle dynamics below.

Completion of the cell cycle requires the temporal and spatial coordination of chromosome segregation with mitotic spindle disassembly and cytokinesis. Chromosome segregation depends on a microtubule-based machine, the mitotic spindle, which is composed of three sets of microtubules (MT). Kinetochore MTs connect kinetochores on the sister chromatids to the spindle pole bodies, while astral (cytoplasmic) MTs are involved in interactions between the spindle pole body and the cell cortex to help position
the nucleus in the bud neck. Interpolar MTs link the two spindle pole bodies by interdigitating with each other to form an antiparallel MT array which is known as the spindle midzone (Figure 1-4) (Khmelinskii and Schiebel, 2008).

![Figure 1-4 Organization of the Mitotic Spindle](image_url)

**Figure 1-4 Organization of the Mitotic Spindle**

The spindle pole bodies (red), chromosomes (blue) and microtubules (MTs, black lines) are shown. (1) kinetochore MTs; (2) astral (cytoplasmic) MTs; (3) interpolar MTs. Overlapping interpolar MTs form the spindle midzone (4) with the onset of anaphase.
The spindle midzone is required to maintain the integrity of the anaphase spindle in organisms from yeast to humans. The kinetics of pole separation and chromosome segregation needs a stable spindle midzone. For example, interfering with the spindle midzone, either genetically or by laser ablation, accelerates the rate of spindle elongation in *C. elegans* embryos (Grill et al., 2001; Verbrugghe and White, 2004).

In budding yeast, Ase1p is a member of a conserved family of midzone-specific proteins with MT bundling activity. The midzone-specific proteins are required for proper midzone formation, and for promoting spindle extension and stability in anaphase, and later they play a role in cytokinesis (Balasubramanian et al., 2004; Juang et al., 1997; Mollinari et al., 2002; Norden et al., 2006; Schuyler et al., 2003).

The formation of the spindle midzone is regulated by the conserved protein phosphatase Cdc14p. Cdc14p is essential for mitotic exit and meiotic progression (Schild and Byers, 1980; Stegmeier and Amon, 2004; Taylor et al., 1997). Cdc14p dephosphorylates key mitotic targets leading to inactivation of mitotic cyclins (Visintin et al., 1998), proper spindle disassembly (Khmelinskii et al., 2007; Khmelinskii and Schiebel, 2008), and completion of cytokinesis (Hall et al., 2008). The activity of Cdc14p is regulated by two signaling pathways, the Cdc fourteen early anaphase release (FEAR) network (Pereira et al., 2002; Pereira and Schiebel, 2003; Stegmeier et al., 2002; Visintin et al., 2003; Yoshida et al., 2002) and the mitotic exit network (MEN) (Shou et al., 1999; Visintin et al., 1998; Visintin et al., 1999; Visintin et al., 2003).
1.6.2 Spindle assembly checkpoint pathways

After duplication of its chromosomes, the cell must segregate its chromosomes equally. Two important processes, sister chromatid cohesion and kinetochore spindle attachment, ensure the proper segregation of sister chromatids. Two kinetochores of each sister chromatid pair capture microtubules emanating from opposite spindle pole bodies to produce the metaphase plate at which all the chromosomes are bilaterally attached and aligned at the equator of the spindle (Bloom, 2005; Lew and Burke, 2003; Skibbens et al., 1993). Tension is produced when sister kinetochores are properly attached to opposite spindle pole bodies, so that the pole-ward force exerted on the chromosomes by the microtubules is counteracted by cohesion between sister chromatids.

The state of kinetochore spindle attachment is monitored by the spindle assembly checkpoint (SAC) pathway. Unattached kinetochores emit negative signals that activate the spindle assembly checkpoint pathway and prevent the onset of anaphase. In addition to detecting unattached kinetochores, the checkpoint pathway also senses the tension resulting from successful bilateral attachment of microtubules to the kinetochores (Nasmyth, 2005; Tanaka, 2005).

Once sister chromatids are separated, mitotic spindle disassembly is triggered by a complex of proteins referred to as the chromosomal passenger complex (CPC) and the kinetochore CTF19 complex (Buvelot et al., 2003; Vizeacoumar et al., 2010). Below I highlight the significance of these complexes and their roles in spindle disassembly.
1.6.3 Chromosome passenger complex (CPC)

The chromosomal passenger complex (CPC) is an important regulator of chromosome segregation during mitosis and is conserved from yeast to humans. The CPC regulates chromosome condensation, chromosome biorientation, signaling to the spindle checkpoint machinery, spindle assembly, and cytokinesis (Ruchaud et al., 2007a; Ruchaud et al., 2007b). Yeast CPC members include Ipl1p, Sli15p, Bir1p and Nbl1p (Chan and Botstein, 1993; Kang et al., 2001; Kim et al., 1999; Nakajima et al., 2009; Yoon and Carbon, 1999).

The functions of CPC are associated with dynamic changes in localization throughout the cell cycle. In the budding yeast *S. cerevisiae*, the CPC (e.g., Ipl1p) is diffusely associated with kinetochores in G1, S phase, and metaphase cells, localizes along the whole spindle. It becomes concentrated at the spindle midzone in anaphase cells, and focuses at the spindle pole bodies in telophase cells (Buvelot et al., 2003). This localization pattern is similar to that seen in mammalian cells (Ruchaud et al., 2007b).

Ipl1p is an Aurora kinase subunit of the conserved CPC that is involved in regulating kinetochore-microtubule attachments and maintaining condensed chromosomes during anaphase and early telophase (Cheeseman et al., 2002a; Kang et al., 2001; Nakajima et al., 2009; Vas et al., 2007). Sli15p is a subunit of the chromosomal passenger complex and involved in regulating kinetochore-microtubule attachments, activation of the spindle assembly checkpoint, and mitotic spindle disassembly (Biggins and Murray, 2001; Buvelot et al., 2003; Kim et al., 1999; Nakajima et al., 2009; Tanaka et al., 2002). Bir1p regulates chromosome segregation, and is required for chromosome bi-orientation and for spindle assembly checkpoint activation (Li et al., 2000; Makrantonii
and Stark, 2009; Nakajima et al., 2009; Yoon and Carbon, 1999). Recently identified NBL1 is an essential gene and nbl1 mutations cause chromosome missegregation and lagging chromosomes. Nbl1p colocalizes and copurifies with the CPC, and is essential for CPC localization, stability, integrity, and function (Nakajima et al., 2009).

1.6.4 The kinetochore CTF19 complex

Ctf19p (chromosome transmission fidelity protein 19) was identified as part of the kinetochore complex that functions as a link between the kinetochore and the mitotic spindle (Hyland et al., 1999). The central kinetochore complex is composed of at least 13 different proteins and the kinetochore CTF19 complex consists of Ctf19p, Mcm21p, and Okp1p (Hyland et al., 1999; Measday et al., 2002; Measday and Hieter, 2004; Ortiz et al., 1999; Pot et al., 2003). The CTF19 complex mediates centromere attachment to the mitotic spindle by forming interactions between the microtubule-associated outer kinetochore proteins and the centromere-associated inner kinetochore proteins (Cheeseman et al., 2002b). CTF19 regulates kinetochore architecture (Cheeseman et al., 2002b; McAinsh et al., 2003; Measday and Hieter, 2004; Westermann et al., 2007) and ensures loading of cohesin at centromeres prior to passage of the replication fork (Fernius and Marston, 2009). Defective pericentromeric cohesion was found in CTF19 complex mutants (Fernius and Marston, 2009). Recently, our group discovered a new role for CTF19 complex components in mitotic spindle disassembly (Vizeacoumar et al., 2010).
1.7 Sister chromatid cohesion

1.7.1 Sister chromatid cohesion is mediated by the cohesin complex

Sister chromatid cohesion is the physical link that associates two sister chromatids and is mediated by an essential core complex called cohesin (Guacci et al., 1997; Michaelis et al., 1997; Toth et al., 1999). Chromatid cohesion is a highly conserved process and I below outline some of the components identified in other eukaryotes. Unless otherwise stated, however, I focus on key components of the cohesion complex identified in *S. cerevisiae*.

The core cohesin complex consists of Scc1p, Scc3p, Smc1p, and Smc3p. These core components are found in *C. elegans* (Mito et al., 2003), *D. melanogaster* (Warren et al., 2000), and mammals (Ball Jr and Yokomori, 2001). Immunoprecipitation of any one cohesin subunit coprecipitates the other three subunits in stoichiometric quantities. All four subunits are nuclear proteins and associate with chromatin in an interdependent manner. Colocalization of the cohesin complex and chromatin can be detected in samples from S, G2, and metaphase cells, but not in early G1 cells (Michaelis et al., 1997). In *S. cerevisiae*, sister chromatid separation is induced by the cleavage of cohesins by the protease Esp1p (Guacci et al., 1997; Uhlmann, 2004). In *C. elegans*, RNA interference (RNAi) experiments revealed that depletion of any conserved cohesin homolog caused a defect in mitotic chromosome segregation but not in chromosome condensation and cytokinesis (Mito et al., 2003). In human cells, separase is recruited to mitotic chromosomes and cohesin cleavage by separase requires DNA in a sequence-nonspecific manner (Sun et al., 2009).
In addition to the core cohesin complex, the following components are also required for proper chromatid cohesion: (1) Pds5p which binds loosely to the cohesin complex; (2) a separate complex, containing Scc2p and Scc4p, which is essential for association of cohesin with chromosomes; (3) Eco1p, an acetyl transferase, which is required for establishing cohesion during S phase but not for cohesion maintenance (Nasmyth and Haering, 2009). Together these components function with the cohesin complex to ensure accurate chromosome segregation.

1.7.2 Structure and roles of cohesin

The Smc1p and Smc3p subunits of the cohesin complex form a heterodimer, which sits at the heart of the cohesin complex. Each subunit consists of an intramolecular antiparallel coiled coil domain and forms a rod-shaped protein with a globular “hinge” domain at one end and an ATP nucleotide-binding domain at the other end (Haering et al., 2002; Hirano and Hirano, 2002; Melby et al., 1998).

In every cell cycle, each chromosome is replicated in S phase to form two identical sister chromatids that are held together by sister chromatid cohesion (SCC), which is mediated by the cohesin complex. The cohesin complex is a major component of interphase and mitotic chromosomes, and this complex associates with chromosomes before their replication and is converted into a cohesive state as replication forks pass. Sister chromatids are held together by SCC continuously from the time of their formation until their separation during mitosis when cohesion is removed to allow chromosome segregation (Nasmyth and Haering, 2009).
In addition to its role in mediating sister chromatid cohesion, cohesin is also important for the repair of DNA double-strand breaks in mitotic (Cortes-Ledesma and Aguilera, 2006; Sjogren and Nasmyth, 2001) and meiotic cells (Ellermeier and Smith, 2005; Klein et al., 1999; van Heemst et al., 1999). Two recent studies reported the detection of cohesin subunits at spindle pole bodies (Wong and Blobel, 2008) or centrosomes (Kong et al., 2009). Cohesin depletion causes spindle pole defects, but it is difficult to exclude the possibility that these defects are an indirect consequence of prior mitotic defects caused by a lack of sister chromatid cohesion.

1.8 Condensin

Condensin is important during mitosis for the compaction and resolution of chromosomes and plays a key role in the condensation of chromosomes. Analysis of the in vivo localization of an Smc4p-GFP fusion protein revealed that condensin binds to in the repetitive regions of yeast chromosomes during the G2/M phase of the cell cycle (Freeman et al., 2000). Through interactions with RNA polymerase III transcription factor TFIIIC, the yeast condensin complex also binds to tRNA genes (D'Ambrosio et al., 2008; Haeusler et al., 2008). Condensin-binding sites on chromosomes were analyzed by chromatin immunoprecipitation and hybridization to whole-genome microarrays and validated by quantitative PCR. The results showed that the condensin-occupied sites span the length of every chromosome in yeast and also occur in specialized chromatin regions (near centromeres and telomeres) and in heterochromatic regions (Wang et al., 2005).
1.8.1 Components and structure of condensin

The budding yeast has a single condensin complex that consists of Ycg1p, Brn1p, Smc2p, Smc4p, and Ycs4p, and five genes encoding condensin components are essential (Freeman et al., 2000; Ouspenski et al., 2000; Strunnikov et al., 1995). All five condensin subunits are present in an equimolar ratio in the condensin complex (Freeman et al., 2000). Two SMC subunits (Smc2p and Smc4p) form a stable heterodimer, the “Smc2/4 complex”, and self-association of Smc2/4 heterodimers interacting reversibly appears to form heterotetramers (Stray and Lindsley, 2003).

1.8.2 Functions of condensin

Condensins associate with condensing chromosomes during mitosis and are involved in mitotic chromosome dynamics. Condensins are required for proper chromosome condensation and segregation in budding yeast (Bhalla et al., 2002; Freeman et al., 2000; Lavoie et al., 2002; Lavoie et al., 2000; Ouspenski et al., 2000; Strunnikov et al., 1995) and fission yeast (Saka et al., 1994; Sutani et al., 1999). Consistent with conservation of condensin function in eukaryotes, severe defects in chromosome segregation are seen when individual condensin subunits are inactivated in C. elegans (Hagstrom et al., 2002) and Drosophila (Steffensen et al., 2001).

Consistent with a broad role in chromosome regulating chromosome architecture, condensin also appears to play a role in regulating repression of gene expression at the silent mating type loci in yeast (Bhalla et al., 2002). Mutation of YCS4 alters the response of MATa haploid cells so that they continue to grow in the presence of α-factor, and the defect in α-factor-induced arrest is overcome when the silent mating locus HML is
deleted (Bhalla et al., 2002). This result is consistent with inappropriate expression of the HML locus in MATa haploids.

### 1.9 Summary of thesis

In this thesis, I described the construction of a novel collection of temperature-sensitive yeast mutants consisting of 795 ts strains, covering 501 (~45%) of the 1,101 essential yeast genes, with ~30% of the genes represented by multiple alleles. I have validated and demonstrated the use of the ts collection in a number of different ways. First, I collaborated with colleagues at the University of Gothenburg to carry out detailed temperature profiling of each mutant in liquid growth assays and found that mutants in particular pathways often showed highly similar profiles. Second, collaborating with members of Chris Bulawa’s lab in FoldRx Pharmaceuticals Inc., I showed that the ts array is useful for exploration of chemical-genetic interactions since it enables screens for compounds that suppress defects in vital pathways. Third, I demonstrated that the ts collection represents a key reagent set for genetic interaction analysis because essential genes tend to be highly connected hubs on the global genetic network in collaboration with the Boone and Andrews labs’ members. Fourth, I collaborated with members of the Boone, Andrews, Bloom and Zhang labs to further validate the ts array as a key resource for quantitative phenotypic analysis by using a high-content screening protocol to score six different fluorescent markers, diagnostic for different subcellular compartments or structures, in hundreds of different mutants. Quantification of the marker behaviour at the single-cell level enabled integration of this data set to generate a morphological profile.
for each ts mutant to reveal both known and previously unappreciated functions for essential genes, including roles for cohesin and condensin genes in spindle disassembly.
CHAPTER TWO

Construction, Characterization and Evaluation of
Temperature-sensitive Mutant Library

Work in Chapter 2 was published in the following papers:


**Contributions:**

I co-coordinated and led the project, and performed the majority of (1) searching and collecting ts alleles from more than 300 labs; (2) designing primers; (3) construction and confirmation of ts strains; (4) SGA analysis and data analysis.

S. Bahr, H. Lu, Y.Q Chen, and R.L. Brost assisted with ts strain collection, construction, and confirmation, and SGA analysis.

H. Ding assisted with the SGA data analysis.

A. P. Davierwala assisted with screening the Tet alleles.

J. Warringer (Department of Cell and Molecular biology, Medicinaregatan 9c, Göteborg University, 41390 Göteborg, Sweden) performed the high-resolution profiling of the temperature sensitivity of ts alleles.
2.1 Abstract

Essential genes are required for the viability of yeast haploid cells. To systematically study yeast essential gene functions using synthetic genetic array (SGA) analysis (Tong et al., 2001), I constructed a temperature-sensitive (ts) mutant array which contains 795 ts alleles representing ~45% of yeast essential genes. The integration of the ts allele was confirmed by PCR analysis and the identity of the ts allele was confirmed by complementation of the ts phenotype with the cognate plasmid. The ts mutant collection was characterized by high-resolution profiling of the temperature sensitivity of each ts strain, distribution analysis of gene ontology (GO) molecular function and biological process, and comparison of ts alleles to Tet alleles of essential genes. The usefulness of the ts allele collection was also evaluated by SGA analysis. These results demonstrated that the ts collection is a powerful reagent for systematic study of yeast essential gene functions and provides a valuable resource to complement the current yeast deletion collection.

2.2 Introduction

Gene disruption studies demonstrated that 1,105 (18.7%) of the ~5,800 protein-coding yeast genes (Cliften et al., 2003; Kellis et al., 2003) are essential for haploid viability when tested for spore germination at 30°C in rich medium with glucose as the carbon source (Giaever et al., 2002). Transcription, splicing, ribosome biosynthesis, translation, cell wall and membrane biogenesis, DNA replication, nuclear transport, and basic cytoskeletal functions are all required for cell proliferation, and so genes involved in these processes tend to be essential (Mnaimneh et al., 2004). Essential genes also tend
to be highly conserved in humans: ~38% of essential yeast proteins have counterparts in humans, versus ~20% for nonessential genes (Hughes, 2002). Nevertheless, the precise molecular and genetic functions of many essential yeast proteins have not been studied in detail, at least in part because essential genes cannot be easily studied using deletion mutants.

Because essential genes cannot be deleted in haploid strains, one way to systematically study yeast essential genes is to use a conditional system to modulate essential gene function. As described in Chapter 1, systems for modulating essential gene function include temperature-sensitive (ts) (Hartwell, 1967), cold-sensitive (cs) (Moir et al., 1982), temperature-inducible degron (td) (Kanemaki et al., 2003), tetracycline-regulatable promoter-replacement (Tet) (Mnaimneh et al., 2004), temperature-sensitive inteins (Tan et al., 2009), or decreased abundance by mRNA perturbation (DAmP) alleles (Schuldiner et al., 2005). The type of genetic perturbation most commonly used for studying essential genes, ts alleles, provides a simple and fine-tuned control of gene function, enabling permissive, semi-permissive, and restrictive conditions to be easily established. For example, at the permissive temperature, the phenotype of a ts mutant resembles that of the wild-type strain, whereas at the restrictive temperature, the activity of the essential gene is substantially reduced or abolished, resulting in a slow-growth or lethal phenotype.

I constructed a collection of 795 ts alleles for 501 yeast essential genes, which is useful to the yeast research community as a resource that complements the yeast non-essential deletion array.
2.3 Results

2.3.1 Construction of temperature sensitive strains

Over 1,300 yeast strains and DNA constructs carrying ts alleles were collected from more than 300 laboratories. Once the ts phenotypes were confirmed, I designed primers for moving the ts alleles into the S288c reference background (BY4741, MATa his3Δ1 leu2Δ0 ura3Δ0 met15Δ0) (Brachmann et al., 1998). Each ts allele was integrated into its native locus such that it was linked to a kanamycin-resistant cassette, kanMX, which was targeted to the 3’UTR region of the gene and placed 0-300 bp downstream of the stop codon (Table 1). I designed the integration such that the intervening sequences were not altered to avoid perturbing the expression of neighboring genes (Figure 2-1). In total, I constructed a set of 795 ts strains representing more than 500 essential genes (Table 2), accounting for ~45% of the yeast essential genes, which covers a representative proportion of the GO molecular functions and biological processes for yeast essential genes. Over ~30% of the essential genes in the ts mutant collection have multiple alleles (Table 2).
Figure 2-1 Construction of Temperature Sensitive Strains

A. Strategy for constructing strains harboring ts alleles of essential genes is depicted (not drawn to scale). A ts allele including 200-350 bp downstream of its stop codon was amplified by PCR using primers F1 and R2 (Table 1). The kanMX cassette was amplified by PCR using primers F3 and R4 (Table 1). The F1 primer sequence is unique to a region 50-400 bp upstream of the ts allele ATG while the R2 primer sequence is complementary to a region 200-350 bp downstream of the stop codon and includes an additional 32 to 36 bp sequence complementary to the 5’ end of the TEF promoter. The F3 primer sequence is unique to the TEF promoter and primer R4 sequence is complementary to the TEF terminator of the kanMX cassette and a 45 bp sequence immediately downstream of the ts allele stop codon. The F3 primer sequence is AGATCTGTAGCTGCGTCC and sequences for F1, R2, and R4 primers are listed in Table 1.

B-C. The wild-type ORF was replaced by ts allele::kanMX through homologous recombination.
2.3.2 Confirmation of the constructed ts strains

To confirm proper integration of the ts allele and *kanMX* cassette, I carried out two PCR reactions. One PCR reaction was used to test whether the *kanMX* cassette was linked to the expected target gene. A second PCR reaction was used to check whether the *ts allele::kanMX* was integrated at the expected genomic location (Figure 2-2). Correct integration was confirmed for ~99% of ts alleles (787/795) using this PCR-based strategy (Table 2).

To confirm that the ts phenotype was due to the expected allele, I transformed 794 of 795 ts strains with plasmids carrying a corresponding wild-type gene and then tested the resultant transformants for growth at both the permissive and restrictive temperatures (Figure 2-3). The plasmids were derived from either the low-copy molecular-barcoded yeast ORF (MoBY-ORF) library (Ho et al., 2009) or the high-copy yeast genomic tiling collection (Jones et al., 2008) (neither library contained a plasmid encoding *CDC39*). The high quality of the ts mutant library was confirmed because the ts phenotype of over 99% of (787/794) ts strains was rescued in a plasmid-dependent manner (Table 2).
Figure 2-2 PCR Confirmation of ts Strains

Two PCR reactions were used to confirm proper integration of the ts allele and \textit{kanMX} cassettes. Primers A and B were used to check the linkage of \textit{kanMX} cassette to the target gene. Primers C and D were used to verify the integration of the \textit{ts allele::kanMX} at the target gene locus. The primer sequences for confirmation PCR are listed in Table 4.
Figure 2-3 Functional Complementation by Plasmid

The ts mutant strains were transformed with plasmids carrying a corresponding wild-type gene (i.e., \( pACT1 \) or \( pRSC8 \)), and with a vector control. After growth for 3-5 days at 22°C, transformants were replica-plated (A) and/or streaked-out (B) and incubated at 22°C and at the restrictive temperature for 1-3 days.
2.3.3 High-resolution profiling of the temperature sensitivity of ts alleles

Next, I collaborated with Jonas Warringer and Anders Blomberg to apply high resolution growth profiling to characterize the temperature sensitivity of each mutant in the ts collection. Lag time, growth rate and growth efficiency (Warringer and Blomberg, 2003) were quantified across a range of temperatures to generate a growth profile for each ts mutant (Figure 2-4A). The reference strain BY4741 reached its maximal growth rate between 32°C and 36°C, whereas growth efficiency and growth lag more or less continuously decreased over the temperature range (Figure 2-4B). Individual ts strain growth profiles were normalized against a reference wild-type profile to provide a measure of relative growth defects at each temperature (Warringer and Blomberg, 2003). As expected, the frequency distribution of relative growth rates reflected the tendency towards exaggerated growth defects at higher temperatures (Figure 2-4C). In addition, after normalizing for defects at 22°C, the fraction of ts strains with significant temperature-dependent growth defects expanded with increasing temperature, from 24°C to 34°C (Figure 2-4D). Hierarchical clustering of liquid growth profiles revealed that ts mutants in functionally related genes showed similar growth behavior in response to temperature suggesting that temperature-dependent growth profiles may represent distinct phenotypic fingerprints reflecting an underlying physiological defect (Figure 2-4E). For example, subsets of ts mutants in components of DNA repair, cell division cycle, spindle pole body, RNA splicing, RNA polymerase II-dependent transcription, and ER to Golgi transport cluster together in specific cohorts (Figure 2-4E). These findings suggest that defects in different essential cellular functions may translate into diagnostic growth curves. For example, our data suggests that Cdc48p, which is a component of the Cdc48p-Npl4p-Ufd1p
AAA ATPase complex and participates in retrotranslocation of ubiquitinated proteins from the ER into the cytosol for degradation by the proteasome (Metzger et al., 2008; Nakatsukasa et al., 2008), may have roles in DNA repair (Figure 2-4F). Also, proteins involved in nuclear mRNA splicing (Msl5p and Yhc1p) may function in ER to Golgi transport (Figure 2-4F).
Figure 2-4 Profiling the Temperature Sensitivity of ts Strains

A. General interpretation of high-resolution liquid growth profiles of strains harboring ts alleles. Growth rate was determined by measuring the slope of the exponential phase of the growth curve and converted into population doubling time. Lag (hrs) is given by the intercept of the initial density and the slope, and growth efficiency (OD units) is calculated as the total change in density for cells having reached stationary phase.

B. Liquid growth variables, doubling time (rate), lag and efficiency of mitotic growth, for the reference WT strain (n=72) measured at various temperatures. Growth rate (red) is given as population doubling time (h), lag (blue) is given as time to initiate growth (h), and efficiency (black) is given as the total change in population density (OD units).

C. Frequency distribution of the relative growth rate defects (log2[WT/ts-strain]) for ts alleles at 22°C (blue), 30°C (black) and 40°C (red). Dotted line=0 (WT growth rate).

D. Fraction of ts strains that exhibit significant temperature sensitivity (p<0.001) compared to WT at the indicated temperatures. Growth rate (red), lag (blue), and efficiency (black) are shown.

E-F. Uncentered hierarchical clustering of ts allele temperature sensitivity profiles. Profile similarity was measured using a Pearson similarity metric and average linkage.
mapping. Functional clusters are indicated and alleles in each cluster are shown in colors.

The ts-strain sensitivity ratio is expressed as \( \log_2(WT/ts\ strain) \) at temperature \( X \) – \( \log_2(WT/ts\ strain) \) at 22°C.

2.3.4 GO molecular function and biological process distribution of ts mutant library

Using SGD Gene Ontology Slim Mapper (http://db.yeastgenome.org/cgi-bin/GO/goTermMapper), the GO molecular function distribution and biological process distribution of the entire yeast genome, all yeast essential genes and essential genes on the ts allele array were analyzed. In each molecular function (Figure 2-5A) or biological process (Figure 2-5B) group, the percentage of the essential genes on the ts mutant array is very similar to that of the essential genes in yeast, indicating that the ts mutant collection can represent the spectrum of the essential gene functions and biological processes in the yeast genome.
Figure 2-5 GO Molecular Function and Biological Process Distribution for Yeast Genome, Yeast Essential Genes and Genes in ts Collection

Genes for the whole yeast genome (5,795, black), essential genes in yeast genome (1,062, blue) and essential genes in the ts mutant collection (501, red) were entered into SGD Gene Ontology Slim Mapper (http://db.yeastgenome.org/cgi-bin/GO/goTermMapper). The percentage was calculated by gene number in a specific molecular function group (A) or biological process group (B) out of three categories (yeast genome, essential genes in yeast genome, or essential genes in the ts mutant collection).
2.3.5 Essential genes are hubs of a genetic interaction network

To illustrate the utility of the ts mutants for exploring essential gene functions, I conducted SGA analysis by crossing the nonessential gene deletion mutant \((bni1Δ::natMX)\) and the essential gene ts mutant \((rfc5-1::natMX)\) into three different collections: (1) an early version of the ts allele array containing 191 ts alleles (133 essential genes); (2) the Tet-array containing tetracycline-regulatable promoter-replacement alleles for 602 essential genes (Mnaimneh et al., 2004); and (3) the deletion array containing 4,700 nonessential genes (Tong et al., 2001). In these experiments the genetic interaction (GI) percentage for the ts collection and Tet collection (3.99% - 12.78%, GIs/gene) was much higher than the GI percentage for the deletion collection (0.45% - 1.38%, GIs/gene) (Table 3), suggesting that the ts array and the Tet array may provide an opportunity to detect more genetic interactions. These results indicate that the frequency of genetic interactions for essential genes may be higher than the frequency of genetic interactions for nonessential genes, consistent with previous work (Davierwala et al., 2005). This could suggest that essential genes are hubs of the genetic interaction network (Davierwala et al., 2005; Tong et al., 2004), which is similar to what was seen in the physical interaction network: protein-protein interactions were higher amongst essential proteins (44%) than nonessential proteins (17%) (Gavin et al., 2002).
2.3.6 Comparison of ts alleles to Tet alleles

To compare two conditional alleles (ts allele versus Tet allele), I crossed *sec1-1*, *sec15-1*, *arp2-14*, *bni1Δ*, and *rfc5-1* strains against a combined array containing only alleles of essential genes that were represented both in the ts mutant collection and Tet allele collection (Mnaimneh et al., 2004) using the SGA method. The genetic interaction profiles for *SEC1*, *SEC15*, and *RFC5* between the two different conditional alleles were similar (Figure 2-6). Specifically, 4/5, 8/9, and 7/11 genetic interactions were observed for both the ts allele and Tet allele when *SEC1*, *SEC15*, and *RFC5* respectively were used as a query. These results indicated that the ts allele is comparable to the Tet allele in these cases; however, I observed different genetic interaction profiles for *ARP2* (only one of 9 interactions common to both alleles) and *BNI1* (2 of 7 in both alleles), suggesting that these different conditional alleles can generate complementary data for essential genes (Figure 2-6).

<table>
<thead>
<tr>
<th>Query</th>
<th>Collection</th>
<th>Genes</th>
<th>Genetic Interactions</th>
<th>% (Gls/Gene)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>bni1Δ</em></td>
<td>Deletion</td>
<td>4700</td>
<td>65</td>
<td>1.38</td>
</tr>
<tr>
<td></td>
<td>ts Mutant</td>
<td>133</td>
<td>6</td>
<td>4.51</td>
</tr>
<tr>
<td></td>
<td>Tet Allele</td>
<td>602</td>
<td>34</td>
<td>5.65</td>
</tr>
<tr>
<td><em>rfc5-1</em></td>
<td>Deletion</td>
<td>4700</td>
<td>21</td>
<td>0.45</td>
</tr>
<tr>
<td></td>
<td>ts Mutant</td>
<td>133</td>
<td>17</td>
<td>12.78</td>
</tr>
<tr>
<td></td>
<td>Tet Allele</td>
<td>602</td>
<td>24</td>
<td>3.99</td>
</tr>
</tbody>
</table>
Figure 2-6 Comparison of ts Alleles and Tet Alleles

Two-dimensional clustering of the synthetic genetic interactions determined by SGA analysis when crossing sec1-1, sec15-1, arp2-14, bni1Δ, and rfc5-1 into a combined array containing both ts alleles and Tet alleles of a subset of essential genes. Rows, query genes; columns, conditional alleles. Blue, genetic interactions observed in both ts allele and Tet allele; red, genetic interaction observed in ts allele only; green, genetic interaction observed in Tet allele only.
2.3.7 Evaluating the usefulness of the ts mutant collection

To further evaluate the usefulness of the ts allele collection, I crossed a deletion allele of a nonessential gene \((bni1\Delta::natMX)\) against the ts mutant array. Bni1p is required for a proper bipolar budding pattern (Evangelista et al., 1997; Evangelista et al., 2002; Pruyne et al., 2002) and is involved in the regulation of actomyosin ring dynamics (Vallen et al., 2000), concentrating polarized growth to the bud tip during apical growth (Sheu et al., 2000), septation (Sheu et al., 2000), and localization of Bud8p to the bud tip (Ni and Snyder, 2001).

Septins are proteins associated with the neck filaments. The septin ring is seen at the bud neck using electron microscopy (Field and Kellogg, 1999). There are seven septin genes (\(CDC3, CDC10, CDC11, CDC12, SHS1, SPR3,\) and \(SPR28\)) in \(S.\) \(cerevisiae.\) \(CDC3, CDC10, CDC11,\) and \(CDC12\) are essential genes. Previous studies showed that \(bni1\Delta\) is synthetic lethal with septin alleles, \(shs1\Delta\) (Tong et al., 2001) and \(cdc12-ts\) (Akada et al., 1997; Longtine et al., 1996).

The NH2-terminal portion of Bni1p interacts with Cdc42p, whereas the COOH-terminal portion interacts with Pfy1p, Bud6p, and Act1p (Evangelista et al., 1997). Bni1p occurs as part of a complex that directs the assembly of actin filaments in response to Cdc42p signaling during polarized morphogenesis. It was previously shown that \(bni1\Delta\) is synthetic lethal with \(act1-120\) (Evangelista et al., 1997) and \(cdc42-118\) (Kozminski et al., 2003).

When I crossed the \(bni1\Delta::natMX\) query strain into an early version of the ts allele array containing 191 ts alleles (133 essential genes), I identified synthetic genetic interactions with \(ACT1, CDC3, CDC10, CDC11, CDC12,\) and \(CDC42.\) These results
were consistent with the known functions of \textit{BNII}, as all six genes are involved in budding, bud growth, establishment of cell polarity and cytokinesis.

I also crossed one essential gene as a ts allele (\textit{rfc5-1::natMX}) against the ts array. Rfc5p is a subunit of the Replication Factor C (RFC) complex. There are four different RFC complexes: (1) Ctf18p, Ctf8p, Dcc1p, and Rfc2p-Rfc5p form a RFC complex involved in sister chromatid cohesion, (2) Rfc1p-Rfc5p complex is involved in DNA replication, (3) Rad24p, Rfc2p-Rfc5p complex is involved in the DNA damage response, and (4) Elg1p, Rfc2p-Rfc5p complex is involved in DNA replication and DNA damage response (Bellaoui et al., 2003; Jones and Sgouros, 2001; Kenna and Skibbens, 2003).

Rfc5p is a component of all four RFC complexes. The cohesin complex links sister chromatids together at metaphase during mitosis. Sister chromatid cohesion is established during DNA replication and dissolved during the metaphase-to-anaphase transition (Campbell and Cohen-Fix, 2002). The core cohesin complex consists of Smc1p, Smc3p, Scc1p, and Scc3p (Nasmyth, 1999; Nasmyth, 2001). Loading complex requires Scc2p and Scc4p, which load the cohesin complex onto chromosomes (Ciosk et al., 2000).

When I crossed the \textit{rfc5-1} ts mutant into the ts allele array, I identified genetic interactions with \textit{SMC1}, \textit{SMC3}, \textit{SCC1}, and \textit{SCC2}, all of which are involved in sister chromatid cohesion (Figure 2-7).

Thirty-three genes, including 18 essential genes, are involved in mitotic sister chromatid segregation (\url{http://www.geneontology.org}). Five of 18 essential genes (\textit{CDC16}, \textit{CDC20}, \textit{CDC23}, \textit{CDC27}, and \textit{SMC1}) are represented on an early version of the ts array containing 191 ts alleles (133 essential genes). I identified four (\textit{CDC16}, \textit{CDC23}, \textit{CDC27}, and \textit{SMC1}) of five in the RFC5 screen (Figure 2-7).
Both Rfc5p and Pri2p are the members of the Pol12p associated complex (Gavin et al., 2002) and are involved in DNA replication. RFC5 also showed synthetic interactions with several genes involved in DNA replication when I crossed rfc5-1 to the ts allele array (Figure 2-7).

Thus, genetic interactions amongst functionally related genes for both BNI1 and RFC5 were identified from SGA analysis with the ts allele array, illustrating the potential of this collection as a valuable resource for exploring essential gene function.
Figure 2-7 rfc5-1::natMX Crossed into the ts Array

The ts query strain rfc5-1::natMX was crossed into the ts mutant array, which contained 191 ts alleles (133 essential genes), by SGA analysis. The double mutant selection plates were incubated at 22°C, 26°C, and 30°C. The synthetic genetic interactions shown here were confirmed by random spore analysis and/or tetrad analysis. Genes are represented as nodes, and interactions are represented as edges that connect the nodes. Functionally related genes are grouped by ovals.
2.4 Discussion

Approximately 19% (1,105) of all \textit{S. cerevisiae} genes are considered essential in the S288c reference genetic background because the haploid spores carrying a deletion allele of these genes fail to germinate and form a colony under standard laboratory conditions (Giaever et al., 2002). The essential gene set is largely the same within other \textit{S. cerevisiae} genetic backgrounds; however, it may vary in gene content by as much as ~5%, possibly due to complex genetic interactions associated with strain-specific genetic variation (Dowell et al., 2010). Essential gene function appears to be highly conserved because 83% of the \textit{S. cerevisiae} orthologs that are conserved in \textit{S. pombe} retain their essential gene functions (Kim et al., 2010).

Our ts mutant collection contains ts alleles of 501 (~45%) different \textit{S. cerevisiae} essential genes. Because each gene is linked to a \textit{kanMX} marker the alleles can be readily used in SGA-based automated genetic analysis, which enables their barcoding for competitive growth assays in pools (Yan et al., 2008), genetic network mapping (Costanzo et al., 2010), high-content screening (Vizeacoumar et al., 2010), and a host of other applications. Over ~30% of the essential genes in the ts mutant collection have multiple alleles (Table 2), which can be useful for dissecting the different roles of multifunctional proteins, such as actin. The ultimate aim is to expand the collection to include a range of ts alleles that cover all domains of essential genes. Indeed, previous efforts to dissect allele-specific interactions involving essential genes have provided significant insight into protein structure and function (Amberg et al., 1995; Dreze et al., 2009).
High resolution growth profiling of the temperature sensitivity of mutants in the strain collection revealed that ts mutations in functionally related genes showed similar growth behavior in response to temperature (Figure 2-4E-F). Thus, temperature-dependent growth profiles may represent distinct phenotypic fingerprints that reveal physiological defects associated with different essential cellular functions.

2.5 Materials and Methods

2.5.1 Yeast strains and medium

Strains used in this study were all *S. cerevisiae*. BY4741 (MATa, his3Δ1 leu2Δ0 met15Δ0 ura3Δ0) was used as a wild-type control and for constructing ts strains. Standard yeast media and growth conditions were used as previously described (Sherman, 1991; Sherman, 2002). Nonessential haploid deletion strains were made by the *Saccharomyces* Gene Deletion Project (Winzeler et al., 1999) and were obtained from Open Biosystems and EUROpean *Saccharomyces cerevisiae* ARchive for Functional analysis (EUROSCARF).

2.5.2 Checking ts phenotypes for the collected yeast strains

I refreshed the yeast strains on YEPD (or SC) plates for 2-4 days at 23°C. Single colonies of the yeast strains received from other labs and the control wild-type strain (BY4741) were re-streaked on YEPD (or SC) plates and incubated at different temperatures (23°C, 26°C, 30°C, 35°C, 37°C, and 38.5°C) for 3-5 days (Figure 2-8). The strains which grew well at 23°C and died or were sick at higher temperatures were used for constructing new ts strains in the deletion background BY4741.
Figure 2-8 ts Phenotype Confirmation on YEPD Plates

The ts strain candidates and the wild-type strains were streaked out on YEPD plates and incubated at different temperatures for 3-5 days. In these experiments the restrictive temperature for \textit{sec18-1} and \textit{sec12-1} was 30\degree C, and for \textit{sec7-1} was 37\degree C. This \textit{ypt1-1} strain was still able to grow at 38.5\degree C and rejected as not a ts strain.
2.5.3 Designing primers for constructing new ts alleles

I designed primers for constructing new ts alleles in the deletion background strain once the ts phenotypes of the received yeast strains were confirmed. The F1 primer and the R2 primer were used to amplify the ts allele. The F3 primer and R4 primer were used to amplify the $kanMX$ cassette (Figure 2-1). The F3 primer sequence is AGATCTGTTTAGCTTGCCCTCGTCC and sequences for F1, R2, and R4 primers are listed in Table 1.

2.5.4 Synthetic genetic array (SGA) analysis

Synthetic genetic array analysis (SGA) was carried out as described previously (Tong and Boone, 2006; Tong et al., 2001; Tong et al., 2004). First, a $MAT\alpha$ SGA query strain ($xxx\Delta::natMX$ or $ts\ allele::natMX$) was crossed into an ordered array of $MAT\alpha$ $xxx\Delta::kanMX$ deletion strains or $MAT\alpha$ ts mutants ($ts\ allele::kanMX$); second, the resultant diploids were selected on YEPD+G418+clonNAT plates; third, sporulation was induced by replicating diploids onto sporulation plates; fourth, $MAT\alpha$ meiotic progenies were germinated on SD-His-Arg-Lys medium supplemented with L-canavanine and thialysine; fifth, $MAT\alpha$ $xxx\Delta::kanMX$ meiotic progenies were selected on SD/MSG-His-Arg-Lys plates supplemented with L-canavanine, thialysine, G418; sixth, $MAT\alpha$ double mutants were selected on SD/MSG-His-Arg-Lys supplemented with L-canavanine, thialysine, G418 and clonNAT. The double mutant selection plates were put at 22°C, 26°C, and/or 30°C for ts mutant strains. Compared to colony size at 22°C the double mutants at 22°C and/or 30°C were scored as synthetic sick (SS), synthetic lethal (SL) or
no interaction (No). The putative synthetic genetic interactions were confirmed by random spore analysis and/or tetrad analysis.

### 2.5.5 Confirmation of temperature-sensitive strains by PCR

Two PCR reactions were carried out to confirm the correct integration of the ts allele and \textit{kanMX} cassette for the candidate ts strains. One PCR reaction was used to test whether the \textit{kanMX} cassette was linked to the target gene. Another PCR reaction was used to verify that the ts allele and \textit{kanMX} cassette were integrated at the target gene locus (Figure 2-2). The primer sequences for confirmation PCR are listed in Table 4.

### 2.5.6 Functional complementation by plasmids

Each ts strain was transformed with the cognate CEN plasmid from the MoBY-ORF library (Ho et al., 2009) and/or a high-copy plasmid from the yeast genomic tiling collection (Jones et al., 2008) (Open Biosystem, Cat# YSC4613, AL, USA) carrying the wild-type gene, or with a vector control. After growth for 3-5 days at 22°C, transformants were replica-plated and incubated at 22°C and at the restrictive temperature for 1-2 days.

### 2.5.7 Random spore analysis

A small amount of spores was resuspended in 1 mL of sterile water, mixed well, and plated out onto different media.

1. 20 µL on SD-His-Arg-Lys + canavanine/thialysine.
2. 40 µL on SD/MSG-His/Arg/Lys + canavanine/thialysine/G418.
3. 40 µL on SD/MSG-His/Arg/Lys + canavanine/thialysine/clonNAT.
(4) 80 µL on SD/MSG-His/Arg/Lys + canavanine/thialysine/G418/clinNAT.

The plates were incubated at 26°C and/or 30°C for 2-4 days (Figure 2-9). Colony growth under the four conditions was compared and double mutants were scored as synthetic sick (SS), synthetic lethal (SL) or no interaction (No).
Figure 2-9 Random Spore Analysis (bni1Δ::natMX cdc42-1::kanMX)

Resuspended spores were plated out on different media (see Material and Methods). The plates were incubated at 26°C or 30°C for 2-4 days. Most genetic interactions can be seen at 26°C. I repeated the experiment at higher temperatures if no genetic interaction was observed. For example, genetic interaction between cdc42-1::kanMX and bni1Δ::natMX was not clearly apparent at 26°C (left panel) but the double mutant was synthetic lethal at 30°C (right panel).
CHAPTER THREE

Application of the Temperature-sensitive Mutant Library

Work in Chapter 3 was published in the following papers:


Contributions:
I co-coordinated and led the project, and performed following experiments: (1) chemical-genetic analysis; (2) construction of reporter strains for HCS screening and run HCS screening by SGA method; (3) follow-up experiments for cohesin and condensin mutants with the following members.
F. J. Vizeacoumar acquired the HCS screening images, and involved in follow-up experiments.
J. Li, F. S. Vizeacoumar, R. Min, and K. Jin did HCS data analysis.
M. Costanzo, A. Baryshnikova, B. VanderSluis, J. Bellay and C. L. Myers generated and analyzed the SGA data (Figure 3-3, Figure 3-4, and Figure 3-5).
F. J. Vizeacoumar, A. Stephens, J. Haase and K. Bloom performed cohesin, condensin and CPC localization (Figure 3-13, Figure 3-14).
Z. Lin and A. C. Gingras performed the mass spectrometry analysis on physical interaction for cohesin, condensin and CPC complexes (Figure 3-12).
3.1 Abstract

Temperature-sensitive alleles represent an important reagent set for genetic interaction analysis because essential genes tend to be highly connected hubs on the global genetic network and are more evolutionarily conserved than nonessential genes. By evaluating their power to predict known annotations to the Gene Ontology, I found that the ts alleles of essential genes have greater predictive power than either deletions of nonessential genes or DAmP alleles of essential genes. Also, the ts collection provides a powerful resource for exploring chemical-genetic interactions because it enables screens for compounds that suppress defects in essential gene function. I validated the ts collection as an important new resource for quantitative phenotypic analysis using a high-content screening protocol. I crossed six different fluorescent markers, diagnostic for different subcellular compartments or structures, into hundreds of mutants and quantified the marker behavior at the single-cell level. Integrating this cell biological data generated a morphological profile for each ts mutant, revealing previously unappreciated functions for essential genes, including roles for cohesin and condensin genes in spindle disassembly.

3.2 Introduction

As discussed in Chapter 1, the budding yeast *S. cerevisiae* is the most well characterized model organism for systematic analysis of fundamental eukaryotic processes. Approximately 19% of *S. cerevisiae* genes are considered essential, because haploid spores carrying a deletion allele of these genes fail to germinate and form colonies under standard laboratory conditions (Giaever et al., 2002). The majority of *S.*
cerevisiae essential gene orthologs (~83%) are also essential for viability in the distantly related yeast, Schizosaccharomyces pombe, suggesting that there has been strong selective pressure to retain essential gene activity across large evolutionary distances (Kim et al., 2010). I wanted to evaluate the potential use of the ts mutant collection described in Chapter 2 in several large scale applications such as SGA, HCS and chemical-genetic analysis.

Our lab recently applied SGA analysis to map an extensive network of more than ~170,000 genetic interactions, including some interactions involving temperature-sensitive alleles of essential genes (Costanzo et al., 2010). This SGA genetic interaction dataset is composed of 1712 queries crossed to 3885 deletion strains. Of 1712 queries, 1378 are deletion mutants of nonessential genes and 334 are essential gene alleles (214 temperature-sensitive and 120 DAmP alleles) (Costanzo et al., 2010). This analysis provided a quantitative estimate of fitness for both the single and the double mutants and identified double mutants whose fitness defect is worse or better than expected for the combined effect of the single mutants to score either negative or positive genetic interactions, respectively.

Another sensitive approach to define genetic interactions is high content screening (HCS). In contrast to the terminal fitness assayed in SGA, this system integrates automated instrumentation, application software, and informatics/bioinformatics (Taylor, 2007) to evaluate intermediary phenotypes by large-scale cell biological investigations (Abraham et al., 2004). The HCS approach involves quantitative single cell image analysis of fluorescent markers for specific pathways and structures in hundreds of different mutants. Recently our group has developed an experimental system that
combines SGA-based high-throughput strain manipulation and HCS to enable the visualization and quantitative measurement of specific morphological features for numerous single cells within a field (Vizeacoumar et al., 2009; Vizeacoumar et al., 2010).

The combination of SGA analysis with HCS (SGA-HCS) provides a powerful method for identifying and deciphering specific cellular functions and this approach has been applied previously to study the morphogenesis of the mitotic spindle. Specifically, integrating these systems has doubled the number of genetic interactions known for two genes \textit{BNI1} and \textit{BIM1} by SGA alone (Vizeacoumar et al., 2010). Now, I have exploited this phenomics SGA-HCS platform to explore a broad range of cellular pathways and developed a morphological profile for the ts mutant collection. In combination with manual scoring of images, automated image analysis has helped us to identify several mutants suggesting not only novel functions for several well-characterized genes but also revealing new roles for uncharacterized genes in well-established pathways. This has helped us to uncover novel functions for members of two essential protein complexes, namely cohesins and condensins, in mitotic spindle disassembly. Accordingly, the essential gene conditional array is useful to the yeast research community as a resource that complements the yeast nonessential deletion array.

Below I highlight the three major large-scale applications I have used in characterizing the temperature-sensitive mutant array to explore the pleiotropic roles of highly conserved essential pathways in yeast. In particular, the integration of data from SGA and HCS points to a novel role for two essential protein complexes, cohesins and condensins, in mitotic spindle disassembly.
3.3 Results

3.3.1 Identification of pathway components using a barcoded ts library

The yeast deletion mutant collection has been particularly useful for chemical-genetic analysis in which large sets of mutants are pooled and scored for sensitivity to a specific compound in competitive-growth assays (Giaever et al., 2004). Each of the deletion mutant strains carries two oligonucleotide molecular barcodes flanked by common primer sites, which enable a quantitative estimate of strain abundance using a barcode microarray or high-throughput sequencing readout (Pierce et al., 2007; Shoemaker et al., 1996; Smith et al., 2009). I reasoned that introducing similar barcodes into the ts mutant collection would provide a new reagent set for systematic chemical-genetic analysis, which can be examined for exaggerated growth defects at a semi-permissive temperature as well as for suppression of growth defects at non-permissive temperature.

To enable a barcode-based analysis of the ts collection, I took advantage of the barcoder approach to introduce molecular barcodes into a subset of ~400 ts strains such that the HO allele is replaced by a drug–resistance marker flanked by two unique oligonucleotide in these strains (Yan et al., 2008). As a proof-of-principle for suppression analysis, I examined suppression by zaragozic acid A (Squalestatin), an inhibitor of mammalian and fungal squalene synthase, both in vivo and in vitro (Baxter et al., 1992; Bergstrom et al., 1993). Squalene synthase (farnesyl diphosphate farnesyltransferase 1, FDFT1, encoded by the ERG9 gene in yeast) is an enzyme in the sterol synthesis pathway (Do et al., 2009; Menys and Durrington, 2003), catalyzing the reductive condensation of farnesyl diphosphate (FPP) to form squalene, the first specific intermediate in the sterol biosynthetic pathway (Figure 3-1).
Figure 3-1 Isoprenoid Pathway in *Saccharomyces cerevisiae*

The solid lines indicate single synthetic steps and the dashed lines indicate multiple synthetic steps in the ergosterol and protein prenylation pathways (Dimster-Denk et al., 1999; Kuranda et al., 2009; Song et al., 2003). HMG-CoA: 3-hydroxy-3-methylglutaryl-coenzyme A; HMGR: HMG-CoA reductase; FPP: farnesyl diphosphate; GGPP: geranylgeranyl diphosphate.
Squalene synthase, which is present in yeast, bacteria, plants, and humans (Okada et al., 2000; Robinson et al., 1993), is responsible for directing the flow of the metabolite FPP to either the sterol or the non-sterol branch of the pathway. Squalene synthase has received attention as a potential target for lipid lowering agents in humans (Charlton-Menys and Durrington, 2008). In contrast to the statins, which inhibit HMG-CoA reductase (HMGR), inhibitors of squalene synthase do not inhibit the biosynthesis of isoprenoids, including FPP and geranylgeranyl diphosphate (GGPP), two substrates for protein prenylation (Dimster-Denk et al., 1999; Kuranda et al., 2009). Efforts to identify novel inhibitors of squalene synthase have been limited by difficulties in configuring high-throughput assays for lipid-metabolizing, integral membrane proteins. The pooled subset of ts strains was grown at 36.5°C in the presence or absence of zaragozic acid A and then their corresponding molecular barcodes were quantified by PCR and hybridized to a microarray (GeneChip Genflex Tag 16K Array v2, Affymetrix). Two strains, bet2-1 and cdc43-2, were over-represented in the pooled population exposed to inhibitor indicating that zaragozic acid A suppressed temperature-sensitive growth defects associated with these mutant alleles (Figure 3-2A). Suppression of bet2-1 and cdc43-2 growth defects was also confirmed by liquid growth (Figure 3-2B) and spot dilution assays on solid media (Figure 3-2C).
Figure 3-2 Zaragozic Acid Rescues bet2-1 and cdc43-2 ts Phenotype

A. A pool of 440 barcoded ts mutants was grown in rich (YEPD) medium in the presence of 4 μM zaragozic acid A (ZA) or DMSO at 36.5°C. Genomic DNA was prepared from cells after 5 generations of growth. Molecular barcodes were amplified by PCR and hybridized to a microarray (GeneChip Genflex Tag 16K Array v2, Affymetrix). The x-axis represents the 440 ts strains ordered alphabetically by ORF name. The y-axis represents the log2 ratio of barcode hybridization intensity between the ZA treatment and the solvent (DMSO) treatment. Mutants with highest log2 ratios at the restrictive temperature were identified as suppressors.

B. The diluted cells were grown in YEPD with or without ZA in a 96-well plate at 36.5°C. The fitness was defined as the ratio of the doubling times of yeast exposed to the solvent DMSO or to ZA treatment. The error bars represent the standard deviation for four independent experiments.

C. Spot Assay. Ten-fold serially diluted cells of wild-type and ts stains were spotted on YEPD plates containing DMSO or 4 μM ZA. The plates were incubated for 2 days at 23°C or 36.5°C.
3.3.2 Genetic interactions for essential genes

3.3.2.1 Properties of essential genetic interactions

As observed in other studies (Davierwala et al., 2005; He and Zhang, 2006) and in my primary experimental results (Table 3), ts alleles tend to have more genetic interactions than deletion mutants, indicating that essential genes are hubs in the genetic interaction network (Figure 3-3A).

In contrast to genetic interactions observed among nonessential mutants, the set of essential genetic interactions show a much higher overlap between negative genetic interactions and protein-protein interactions (~0.8% for negative as compared to ~0.3% for positive) (Figure 3-3B). The higher overlap between protein-protein and negative genetic interactions likely reflects so-called “within pathway” genetic interactions which are predicted to be enriched for essential genes (Boone et al., 2007; Kelley and Ideker, 2005).
Figure 3-3 Properties of Essential Genetic Interactions

A. The distribution of genetic interaction network degree for essential ts mutant alleles and nonessential deletion mutants (see Materials and Methods for details).

B. The percentage of negative (red) and positive (blue) genetic interactions that overlap physically interacting protein pairs derived from affinity purification/mass spectrometry, yeast two hybrid and protein-fragment complementation assay studies (see Materials and Methods for details). Background (dashed line) represents the random expectation for a protein-protein interaction. Error bars represent 95% confidence intervals.
3.3.2.2 Functional impact of essential genetic interactions

Given that essential genes tend to be highly connected in the genetic interaction network (Figure 3-3A), the utility of essential versus nonessential genetic interaction screens was compared for characterizing the functions of other genes. Genes that share similar genetic interaction partners are typically members of the same pathway, protein complex, or biological process (Costanzo et al., 2010; Tong et al., 2004). Using this property, a function prediction approach was developed by simply assessing the interaction profile similarity of a gene of interest to genes annotated to known biological process. Each gene’s known annotations were withheld and then genes were ranked for their suspected involvement in each biological process. The performance of this approach in predicting the held-out functions of genes was evaluated by using different sets of SGA screens as a basis for the interactions profiles (ts alleles or DAmP alleles).

In particular, the information provided by screens of essential ts alleles was directly compared to screens of deletion alleles and DAmP alleles and their capacity to predict known annotations to the Gene Ontology was evaluated. Although the most accurate gene function predictions were obtained from the combination of essential and non-essential screens, for a comparable number of screens (92), ts alleles of essential genes provided greater predictive power than either deletions of nonessential genes or DAmP alleles of essential genes (Figure 3-4). For example, at a predictive performance of 30% precision and 25% recall (P30R25), interaction profiles based on ts allele screens were able to cover 4-fold more GO biological process terms compared to predictions based on either deletion mutants or DAmP alleles (Figure 3-4). In general DAmP alleles may lower the abundance of an essential gene product (Schuldiner et al., 2005), but if the reduced protein level does
Figure 3-4 Function Prediction Performance of Different Query Genes

Comparative functional evaluation of genetic interactions involving essential ts alleles (red), essential DAmP alleles (green), nonessential deletion alleles (blue), or essential and nonessential genes combined (black). True positive examples were defined as interacting gene pairs annotated to the same non-redundant GO term as described elsewhere (Myers et al., 2006). All other gene pairs, excluding uncharacterized genes, were considered negative examples. The number of true positives (TP), positive examples that were correctly predicted, and false positives (FP), negative examples that were incorrectly predicted were
measured for a range of similarity cutoffs. The precision \( \frac{TP}{TP+FP} \) at 25% recall
\( \frac{TP}{TP+FN} \) is plotted against the number of different GO terms for which the function
prediction performance met the corresponding threshold. The set of GO terms was restricted
to the non-redundant set defined in (Myers et al., 2006). For example, the DAmP query
collection (92 queries) can only achieve 30% precision at 25% recall on 3 different terms,
while an equal number of randomly selected ts alleles can achieve the same level of
precision on 13 different terms. Precision scores shown represent the median over 20
iterations, each with 92 randomly chosen queries for both ts alleles and deletions.
Predictions were made using a variant of K-nearest neighbors (see Materials and Methods
for details).

The profile of interactions across a set of query mutants is a powerful approach for
characterizing function because genes from the same pathway or protein complex often
exhibit similar patterns of genetic interaction (Costanzo et al., 2010; Tong et al., 2004). For
example, genes encoding components of the cohesin complex share similar patterns of
genetic interactions with those of the CTF19 kinetochore complex, suggesting they have
overlapping functions (Figure 3-5A). Moreover, cohesin genes show negative genetic
interactions with CTF19 complex genes (Figure 3-5B). Because our recent study defined a
role for the CTF19 complex components in the mitotic spindle disassembly pathway
(Vizeacoumar et al., 2010), the similarities in their genetic interaction profiles suggest a
similar role for cohesin proteins in this essential process.
Figure 3-5 Network Connecting Cohesin, Condensin, and Other Genes Involved in Spindle Dynamics

A. A network derived from correlation profiles between cohesin, condensin, and other genes involved in spindle dynamics. Nodes indicate genes and edges highlight the similarity in the pattern of genetic interactions shared between pairs of genes. Numbers represent Pearson correlation coefficients.

B. A genetic interaction network amongst cohesin, condensin, and other genes involved in spindle dynamics. Nodes represent genes and edges highlight genetic interactions. Negative (red) and positive (green) quantitative genetic interactions were measured as described previously (Costanzo et al., 2010). The edge opacity is proportional to the score strength.
3.3.3 Functional prediction of the essential genes from high-content screening

Combining SGA-based high-throughput strain manipulation with high-content screening enables functional discovery through the visualization and quantitative measurement of specific morphological features at a single cell level (Vizeacoumar et al., 2010). To exploit this general platform in the context of the ts collection, I constructed a panel of SGA query strains expressing fluorescent markers, representing six fundamental subcellular compartments or structures, including the nucleus, DNA damage foci, mitochondria, mitotic spindle, actin patches, and the plasma membrane (Figure 3-6). I introduced these reporters into an array of 769 ts strains and then imaged both the ts mutant strains and the wild-type control strains at 26°C and 32°C. I chose 32°C as a restrictive temperature because ~80% of the mutant alleles showed a growth defect at this temperature (Figure 2-4D). The customized journaling of MetaXpress software (Vizeacoumar et al., 2010) automates image analysis and measures several morphological features, generating a quantitative and unique cell morphology profile for each ts mutant strain. The cell morphology profile comprises features such as cell shape, budding index, organelle density, as well as 85 reporter-specific parameters (Table 5). Importantly, all computationally derived phenotypes were confirmed by visual inspection of the corresponding images (Table 6) generating a high confidence and systematic profile for each ts strain.
Figure 3-6 Fluorescent Reporters Introduced into the ts Collection

The left panels show the localization of six different GFP-tagged marker proteins in wild-type cells as well as computationally processed images. Representative mutants with defects in localization and/or abundance of a particular marker are shown on the right. Markers included a plasma membrane marker (Psr1p-GFP), a reporter of DNA damage (Ddc2p-GFP), a nuclear marker (Mad1p-NLS-RFP), a mitotic spindle reporter (GFP-Tub1), a mitochondrial marker (OM45p-GFP), and an actin reporter (Sac6p-GFP).
In principle, a cell morphology profile should provide a rich phenotypic signature revealing functional relationships between genes. For example, mutants exhibiting increased DNA damage foci, as measured by Ddc2p-GFP localization, were enriched for DNA replication ($P = 2.1 \times 10^{-10}$), pre-replicative complex assembly ($P = 1.4 \times 10^{-5}$), and/or DNA synthesis during DNA repair ($P = 2.0 \times 10^{-5}$). The number of gene-specific profiles indicative of morphological defects increased at elevated temperature further validated our approach (Figure 3-7).
Figure 3-7 Temperature Shift Causes Increased Phenotype

Increased temperature exacerbates ts allele phenotypes. The percentage of mutant alleles exhibiting a phenotype that deviated significantly from wild-type at both permissive (26°C, blue) and restrictive temperature (32°C, red) was plotted (P<0.05, Wilcoxon Rank Sum Test after Bonferroni correction) following normalization.
Comparison of cell morphology profiles uncovered gene-specific phenotypes suggesting unanticipated functions for well-characterized essential genes. For example, components of cohesin, condensin and the chromosomal passenger complex (CPC), shared similar morphological profiles (Figure 3-6). According to the genetic interaction profiles described above (Figure 3-5A), ts alleles of cohesin and condensin components shared similar interactions to the CTF19 components. Our lab has previously shown that the components of the CTF19 complex along with the CPC can regulate spindle disassembly and that these mutants exhibit hyper-elongated spindles as well (Vizeacoumar et al., 2010). The products of IPL1, SLI15 and BIR1 interact to form the Chromosomal Passenger Complex (CPC) that re-localizes dynamically in dividing cells to perform key mitotic roles (Buvelot et al., 2003; Pereira and Schiebel, 2003; Ruchaud et al., 2007a; Sullivan et al., 2001; Zeng et al., 1999). Specifically, I found that cohesin mutants, including smc3-1, mcd1-73, irr1-1, and smc1-2 (Figure 3-6), as well as condensin mutants, ycs4-1, smc2-8 and smc4-1 (Table 6) displayed a hyper-elongated fish hook spindle phenotype as visualized with a tubulin marker, GFP-Tub1p, suggesting that similar to the CPC both cohesin complex and condensin complex may be involved in spindle disassembly.

### 3.3.4 A role for cohesin/condensin in spindle disassembly

Cohesins bind the sister chromatids together during metaphase and are cleaved in a separase-dependent fashion during anaphase (Huang et al., 2005; Nasmyth and Haering, 2005). When cells are synchronized in G1 phase with α factor and then released into the cell cycle at a restrictive temperature, ts cohesin mutants activate the mitotic spindle
assembly checkpoint and arrest as large-budded cells with short or partially elongated spindles (Guacci et al., 1997). However, in our screen, when asynchronous cohesin ts mutants were shifted to restrictive temperature, I found two populations of cells: (1) large budded cells with short or partially elongated spindles and (2) a subpopulation of large budded cells with hyper-elongated fish hook spindles, which presumably represent cells exhibiting a post-checkpoint spindle disassembly defect (Figure 3-8).
Figure 3-8 Cohesin and Condensin Mutants Exhibit Abnormal Spindle Morphology

A sub-population of cohesin and condensin mutants exhibit abnormal spindle morphology. Spindle length and morphology were quantified in large-budded wild-type cells and strains harboring a cohesin (smc3-1) or condensin (smc2-1) mutant allele grown asynchronously at permissive (26ºC) and restrictive temperatures (32ºC). The fraction of cells exhibiting either short (left panel) or fish hook-shaped spindles (right panel) were quantified in three independent populations and error bars represent standard deviation across three independent experiments.
An extended fish hook spindle may result from changes in microtubule (Straight et al., 1998) or CPC complex dynamics (Buvelot et al., 2003; Vizeacoumar et al., 2010). To examine this more carefully, I monitored spindle association and midzone enrichment of CPC components (Ipl1p-GFP, Sli15p-GFP, and Bir1p-GFP) in a cohesin, smc3-1, and a condensin mutant, smc2-8. In wild-type cells, Ipl1p-GFP normally associates with short spindles ranging from ~1.5 to 2 microns in length and this localization persists until the spindle disassembles at which point Ipl1p localization is more prominent at the spindle midzone (Figure 3-9). Interestingly, in the cohesin mutants, Ipl1p-GFP, Sli15p-GFP and Bir1p-GFP associated with the kinetochore but failed to associate with the spindle until the spindle reached a length greater than 6 microns. In contrast, Ipl1p-GFP, Sli15p-GFP and Bir1p-GFP failed to localize to either the kinetochore or the spindle in condensin mutants (Figure 3-9 and Figure 3-10).
Figure 3-9 Localization of Ipl1p-GFP in Cohesin/Condensin Mutants

Ipl1p-GFP localization to the mitotic spindle was monitored in wild type as well as in the smc3-1, and smc2-8 mutants at the permissive (26°C) and restrictive temperatures (32°C). Cells were grown to mid log phase at the permissive temperature and shifted to the restrictive temperature for 60 minutes before imaging. Spindle morphology was monitored using an RFP-Tub1p marker. DIC images, fluorescent micrographs and merged images of representative single cells are shown.

Spindle length was also measured in these cells using a RFP-Tub1p marker. Wild-type and mutant cells were divided into three groups based on spindle length (2-4 μm, 4-6 μm, >6 μm). The percentage of cells exhibiting mis-localized Ipl1p-GFP was measured for each spindle length category. In each spindle length category 60-100 cells were counted at 26°C and 32°C. Error bars represent the standard deviation across three independent experiments.
A

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<td>smc2-8</td>
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B

![Graph showing the percentage of mislocalization with spindle length and temperature](image)

- **% of Mislocalization**
- **Spindle Length (μM)**: 2-4, 4-6, >6
- **Temperatures**: 26°C, 32°C
- **Lines**: Blue (26°C), Red (32°C)
Figure 3-10 Localization of Sli15p-GFP/Bir1p-GFP in Cohesin/Condensin Mutants

Localization of Sli15p-GFP (A and B) and Bir1p-GFP (C and D) to the mitotic spindle was monitored in wild-type as well as in the \textit{smc3-1} and \textit{smc2-8} mutants at the permissive (26°C) and restrictive temperatures (32°C). Cells were grown to mid log phase at the permissive temperature and shifted to the restrictive temperature for 60 minutes before imaging. Spindle morphology was monitored using an RFP-Tub1p marker. DIC images, fluorescent micrographs and merged images of representative single cells are shown.

Spindle length was also measured in these cells using a RFP-Tub1p marker. Wild-type and mutant cells were divided into three groups based on spindle length (2-4 μm, 4-6 μm, >6 μm). The percentage of cells exhibiting mis-localized Sli15p-GFP (B) and Bir1p-GFP (D) was measured for each spindle length category. In each spindle length category 60-100 cells were counted at 26°C and 32°C. Error bars represent the standard deviation across three independent experiments.
Vizeacoumar et al. showed that in the kinetochore mutant ($mcm21\Delta$), a member of the CTF19 complex, CPC components were mislocalized (Vizeacoumar et al., 2010). Since I observed that CPC components were mislocalized in the cohesin mutants, I hypothesized that CPC mislocalization in $mcm21\Delta$ is due to disruption of cohesin structure. Consistent with the genetic interactions between genes encoding cohesin and CTF19 kinetochore complex components (Figure 3-5A), I found that the normal distribution of cohesin around the spindle was lost in a $mcm21\Delta$ kinetochore mutant (Figure 3-11), suggesting that cohesin is required to stabilize the CPC on the spindle (Vizeacoumar et al., 2010; Yeh et al., 2008).
Figure 3-11 Cohesin Barrel Structure was Lost in the mcm21Δ Kinetochore Mutant

The top two panels show the distribution of Smc3p-GFP in wild-type cells. The cohesin barrel structure (shown in yellow arrows) was defined by the two oblongate lobes of fluorescence with an intervening dim region. A representative image of an mcm21Δ cell is shown in the bottom panel.
While the experiments suggest that cohesin and condensin may regulate the CPC components, one might speculate that the hyper-extended spindle might not represent a true anaphase-specific event and that it could be a continuation of prolonged metaphase and that these cells might still linger with elongated spindles. To test if the elongated spindles in smc3-1 and smc2-8 mutants are actually in true anaphase, I monitored the localization of the midzone bundling protein Ase1p and mitotic exit trigger Cdc14p.

Ase1p, which is required for spindle elongation and stabilization, is a member of a conserved family of midzone-specific microtubule-associated proteins (Schuyler et al., 2003). Mutation of ASE1 leads to delayed spindle disassembly (Juang et al., 1997) and loss of Ase1p results in premature spindle disassembly in mid-anaphase (Schuyler et al., 2003). Importantly, Ase1p enriches to the spindle midzone only in anaphase (Khmelinskii et al., 2007).

CDC14 encodes a protein phosphatase which is essential for mitotic exit and meiotic progression (Schild and Byers, 1980; Stegmeier and Amon, 2004; Taylor et al., 1997). Cdc14p dephosphorylates key mitotic targets leading to the coordinated inactivation of mitotic cyclins (Visintin et al., 1998), proper spindle disassembly (Khmelinskii et al., 2007; Khmelinskii and Schiebel, 2008), and completion of cytokinesis (Hall et al., 2008). Cdc14p gets released from the nucleolus only after anaphase onset, particularly to trigger mitotic exit. Mutants that have not initiated anaphase would still retain Cdc14p inside the nucleolus.

Therefore I monitored the localization of Ase1p-GFP and Cdc14p-GFP in cohesin/condensin mutants. The spindle bundling protein Ase1p was strictly co-localized to the spindle midzone in the smc3-1 and smc2-8 mutants (Figure 3-12A) and the release
of the mitotic trigger Cdc14p from the nucleolus was unaffected in the smc3-1 and smc2-8 mutants (Figure 3-12B). These observations are important because they suggest that cohesin and condensin mutants have initiated mitotic exit (Fridman et al., 2009; Khmelinskii et al., 2007; Pereira and Schiebel, 2003; Schuyler et al., 2003) and further suggests the fish hook spindle observed in these mutants is an anaphase-specific phenotype.
Figure 3-12 Localization of Ase1p-GFP/Cdc14p-GFP in Cohesin/Condensin

Mutants

Ase1p-GFP/Cdc14p-GFP localization to the mitotic spindle was monitored in wild-type as well as in the *smc3-1* and *smc2-8* mutants at the permissive (26°C) and restrictive temperatures (32°C). Cells were grown to mid log phase at the permissive temperature and shifted to the restrictive temperature for 60 minutes before imaging. Spindle morphology was monitored using an RFP-Tub1p marker. DIC images, fluorescent micrographs and merged images of representative single cells are shown.
An alternative explanation is that activation of the spindle assembly checkpoint might prevent the localization of CPC to the spindle midzone. Another test of whether the fish hook spindle and its associated mislocalization of the CPC components in the cohesin and condensin mutants is linked to the spindle assembly checkpoint is to determine whether these phenotypes are observed in the mutants of checkpoint proteins. *MAD1* encodes a component of the spindle assembly checkpoint (Hardwick and Murray, 1995; Li and Murray, 1991). The spindle assembly checkpoint delays the onset of anaphase in cells with defects in mitotic spindle assembly or in the attachment of chromosomes to the spindle microtubules (Hardwick, 1998). To see whether the CPC mislocalization in cohesin/condensin mutants is due to the spindle checkpoint activation or not, I checked Sli15p localization in *mad1Δ smc3-1* and Ipl1p-GFP localization in *mad1Δ smc2-8* (Figure 3-13). The results showed that the association of components of the CPC (Sli15p-GFP and Ipl1p-GFP) with elongated spindles (>6 microns) was observed in double mutants defective in a cohesin or condensin component and lacking a functional spindle assembly checkpoint (*mad1Δ smc3-1* and *mad1Δ smc2-8*) (Figure 3-13), indicating that CPC mislocalization observed in cohesin/condensin mutants is not due to spindle assembly checkpoint activation (Pinsky and Biggins, 2005; Stern and Murray, 2001).
Figure 3-13 Localization of Sli15p-GFP and Ipl1p-GFP in smc3-1 mad1Δ and smc2-8 mad1Δ Double Mutants

Exponential phase cells were grown at the permissive temperature (26°C) and shifted to the restrictive temperature (32°C) for 60 minutes before imaging. Spindle morphology was monitored using an RFP-Tub1 marker. DIC images, fluorescent micrographs and merged images of representative single cells are shown.
Although dephosphorylation of Sli15p by Cdc14p could trigger spindle association of the CPC (Pereira and Schiebel, 2003), our results so far have suggested that stabilization of the CPC to the spindle is regulated by cohesin and condensin. To confirm this, I monitored the localization of a phosphorylation-deficient CPC mutant (Sli15p-6A-GFP) which binds to the spindle throughout mitosis (Pereira and Schiebel, 2003) in the smc3-1 mutant background. Compared to the wild-type cells, where Sli15p-6A-GFP strictly binds the spindles, in the smc3-1 mutant, the association with spindles was reduced, at least until the spindle reached over ~4 to 6 microns, and the enrichment to the spindle midzone was delayed resulting in elongated fish hook spindles (Figure 3-14). Because Sli15p dephosphorylation prevents mitotic checkpoint reengagement during anaphase (Mirchenko and Uhlmann, 2010), this finding is consistent with our checkpoint mutant observations.
Cells were grown to mid log phase at the permissive temperature (26°C) and shifted to the restrictive temperature (32°C) for 60 minutes before imaging. Spindle morphology was monitored using an RFP-Tub1p marker. DIC images, fluorescent micrographs and merged images of representative single cells are shown.

Taken together, these results suggest that the observed defect in spindle midzone accumulation of the CPC complex and the fish hook spindle phenotype associated with cohesin and condensin complexes is not dependent on activation of the Cdc14p pathway but rather is an anaphase-specific event.
3.3.5 Physical interactions of the cohesin, the condensin and the passenger complexes

I next asked if the stabilization of CPC on the spindle by the cohesin and condensin components is by direct interaction of these components or bridged by an indirect interaction. Cohesin is concentrated at the pericentromeric region of the yeast chromosomes that overlaps with the spindle midzone (Vizeacoumar et al., 2010; Yeh et al., 2008) while condensin associates with the kinetochore, chromosomal arms and ribosomal DNA (rDNA) (Bachellier-Bassi et al., 2008; Freeman et al., 2000; Lavoie et al., 2004). To test for physical interactions between CPC components, cohesin, and condensin, Ipl1p-GFP, Sli15p-GFP, Bir1p-GFP, Smc3p-GFP and Smc4p-GFP were affinity purified from yeast extracts and subjected to LC-MS/MS (see Materials and Methods). These experiments recapitulated all previously reported interactions (Figure 3-15 and Table 7), and uncovered reproducible interactions between the CPC component, Bir1p-GFP, and members of the condensin complex, supporting a direct role for condensin components in stabilizing the CPC (Figure 3-15).
Figure 3-15 Physical Interaction Map for Cohesin, Condensin and CPC Complexes

Physical interaction network illustrating protein-protein interactions involving cohesin, condensin and CPC complexes. Nodes represent individual proteins and edges represent physical interactions. Proteins found in the same complex are grouped by ovals.
3.3.6 Condensin, cohesin and CPC complexes co-localize to the spindle

Physical, genetic and cell biological analyses of cohesin and condensin components suggest that these complexes may regulate the localization of the CPC. To understand the spatial association of these components, collaborating with Andrew Stephens and Julian Haase in Kerry Bloom Lab and Franco Vizeacoumar, I monitored the localization pattern of the CPC, cohesin and condensin complexes in wild-type cells. As reported previously (Yeh et al., 2008), cohesin forms a cylindrical barrel that surrounds Ipl1p (Figure 3-16). During metaphase, Ipl1p-GFP localized to the spindle axis and was surrounded by the cohesin barrel, as identified with Smc3p-GFP (Figure 3-16B). As the cells enter anaphase, Ipl1p-GFP was largely restricted within the cohesin-enriched region (Figure 3-16B), suggesting that cohesin restricts the movement of the CPC along the spindle. The condensin component, Smc4p-GFP, displayed a localization pattern similar to that of Ipl1p-GFP along the spindle axis, distinct from the spindle pole body marker, Spc29p-RFP (Figure 3-16A). Importantly, while both cohesin and condensin were necessary for the spindle localization of Ipl1p, the localization of cohesin and condensin proteins was unaffected in an ipl1-2 mutant (Figure 3-17), suggesting that cohesin and condensin complexes regulate the localization of the CPC and not vice versa. Thus, the co-localization of the CPC and condensin, within a cohesin barrel, is consistent with the physical interactions observed between the CPC and condensin and between condensin and cohesin.
Figure 3-16 Cohesin, Condensin and CPC Localization in WT Cells

A. Ipl1p-GFP, Smc3p-GFP and Smc4p-GFP localization was assessed with respect to the spindle pole body marker, Spc29p-RFP in a wild-type strain. Fluorescent micrograph images illustrate representative single cells. Blue arrow indicates the localization of condensin (Smc4p-GFP) between the spindle pole bodies (Spc29p-RFP) and white arrow indicates the localization of condensin (Smc4p-GFP) to rDNA.

B. Localization of Ipl1p-GFP and cohesin (Smc3p-RFP) in wild-type cells. Cells were grown to mid log phase before imaging. Fluorescent micrographs and merged images of representative single cells are shown. The top frames represent a budded cell with the transverse section of the mitotic spindle surrounded by the cohesin barrel.
Cells were grown to mid-log phase at the permissive temperature (26°C) and shifted to the restrictive temperature (32°C) for 60 minutes before imaging. Fluorescent micrographs and merged images of representative single cells are shown. In the lower panel, blue arrows indicate the localization of condensin (Smc4p-GFP) between the spindle pole bodies (Spc29p-RFP) and white arrows indicate the localization of condensin (Sme4p-GFP) to rDNA.
3.4 Discussion

In this chapter I described the validation and use of the ts collection in a number of different ways. First, I showed that the ts array is useful for exploration of chemical-genetic interactions because it enables screens for compounds that suppress defects in vital pathways. Second, in collaboration with Michael Costanzo, Anastasia Baryshnikova and Chad Myers, I demonstrated that the ts collection represents a key reagent set for genetic interaction analysis because essential genes tend to be highly connected hubs on the global genetic network. Third, in collaboration with Franco Vizeacoumar, I further validated the ts array as a key resource for quantitative phenotypic analysis by using a high-content screening protocol to score six different fluorescent markers, diagnostic for different subcellular compartments or structures, in hundreds of different mutants. Quantification of the marker behaviour at the single-cell level enabled integration of this data set to generate a morphological profile for each ts mutant to reveal both known and previously unappreciated functions for essential genes, including roles for cohesion and condensin genes in spindle disassembly.

*BET2* encodes the catalytic subunit of the type II geranylgeranyl transferase and *CDC43* encodes the beta subunit of geranylgeranyltransferase type I involved in prenylation (Figure 3-1). Previous studies show that when squalene synthase is inhibited by zaragozic acid A, squalene and post-squalene metabolites are decreased (Bergstrom et al., 1993), whereas FPP (Baxter et al., 1992) and farnesoic acid-derived metabolites (Bergstrom et al., 1993) are increased. Thus, increased levels of cellular FPP and GGPP, may rescue the *bet2-1* and *cdc43-2* ts phenotype at restrictive temperature. Further support for this hypothesis comes from previous studies showing that the lethality of the *bet2-1* mutant at 37°C was
suppressed by the overexpression of \textit{BTS1} (Jiang et al., 1995), which encodes 
geranylgeranyldiphosphate (GGPP) synthase (Figure 3-1).

Our analysis shows that a screen for rescue of the ts growth of a \textit{bet2-1} mutant can identify inhibitors of squalene synthase and may lead to a cell-based screen for squalene synthase inhibitors. I expect that any cellular mechanism to raise GGPP levels would be captured by a \textit{bet2-1} rescue screen, potentially providing novel compounds and/or targets for developing lipid lowering agents.

Combining SGA-based high-throughput strain manipulation with high-content screening enables functional discovery through the visualization and quantitative measurement of specific morphological features for numerous single cells within a field (Vizeacoumar et al., 2010). High content microscopy-based screening of the ts array has revealed new functions for multiple genes (Figure 3-6). For example, cellular profiling of our ts collection revealed a connection between the cohesin and condensin complexes and spindle disassembly. Spindle disassembly is a spatio-temporally regulated event such that disassembly of the mitotic spindle occurs only after separation of sister chromatids from the spindle midzone region. The findings are consistent with a model whereby the CPC decorates the spindle in part through interactions with condensin and cohesin within the vicinity of the centromere (Yeh et al., 2008). Loss of cohesin/condensin function results in the mislocalization of the CPC. I speculate that the CPC accesses the spindle midzone only after the cohesin is cleaved, such that cohesin cleavage and clearing of chromosome midzone regions are prerequisites for the mitotic spindle disassembly.

Our collection of ts alleles builds upon a non-overlapping set of ts strains constructed recently (Ben-Aroya et al., 2008). The combined collections cover the
majority of essential genes in the yeast cell and enable numerous systematic approaches for querying the pleiotropic roles of essential genes. For example, I have highlighted the use of the collection in chemical-genetic screens for small molecule suppressors of the ts phenotype, a general screen that may be of particular interest for yeast orthologs of human disease genes because in theory such small molecule suppressors may represent therapeutic leads.

Another powerful approach involving the ts collection will involve the mapping of genetic interactions by systematic dosage suppression analysis using high-copy libraries, such as a high-copy version of the molecular barcoded yeast ORF (MoBY-ORF) library (Ho et al., 2009; Magtanong et al., submitted) or the systematic library for comprehensive overexpression analysis (Jones et al., 2008). Dosage suppression network interactions are highly abundant and functionally relevant and, while some of these interactions overlap with protein-protein negative genetic interactions, most of these suppression edges are unique and offer the potential for expanding our global view of the functional wiring diagram of the cell (Magtanong et al., submitted).

3.5 Materials and Methods

3.5.1 Bar-coding temperature-sensitive strains

The ts alleles were introduced into the $MAT_\alpha$ query strain background by switching selectable marker and mating type (Tong and Boone, 2006). The $kanMX$-marked ts strains were transformed with a $natMX$ fragment, plated on YEPD, incubated at 22°C for 3-4 days, and replica-plated to YEPD+clonNAT at 22°C for 3-5 days to select for $nat^R$ colonies. Transformants were replica-plated to YEPD+G418 and
YEPD+clonNAT at 22°C to select strains resistant to clonNAT and sensitive to G418. Three \( \text{nat}^{R}-\text{kan}^{S} \) colonies were picked to test for ts phenotype at 22°C and 39°C. The resultant \( \text{nat}^{R}-\text{kan}^{S} \) ts strains were mated to the SGA starting strain. The diploids were sporulated at 22°C for one week. Strains were streaked for singles on haploid selection media and grown at 22°C for 2-5 days. The haploid strains were replica-plated on final selection plates and grown for 2-3 days. Colonies from final selection plates were streaked on a fresh final selection plate for singles. The \( \text{MAT}^{\alpha} \) ts query strains were verified and confirmed by fluorescence activated cell sorting (FACS), mating type test and colony PCR.

The resultant \( \text{natMX} \)-marked ts query strains were arrayed in 96-format on agar plates and mated to an array of barcoder strains, each carrying a unique \( \text{kanMX} \)-marked barcode cassette (Yan et al., 2008). Haploid bar-coded ts strains were selected using SGA methodology (Tong and Boone, 2006). The resultant individual bar-coded ts strains were streaked out to obtain single colonies on SGA final selection plate. A single colony from each strain was re-arrayed in 96-format.

3.5.2 Microarray analysis

The homozygous deletion pool containing ~4700 strains (Lee et al., 2005) and the ts pool consisting of 440 ts strains plus 162 control strains were prepared, frozen and stored as previously described (Pierce et al., 2007). Both pools were thawed and diluted in 700 µl of YEPD to \( \text{OD}_{600} \) of 0.06. The homozygous deletion pool and the ts pool were separately grown in YEPD with zaragozic acid A trisodium salt (ZA, Sigma-Aldrich) or with the solvent DMSO (at 2% final concentration) at 36.5°C. Cells were collected after
five generations using a Robot Liquid Handling System (The MultiPROBE II PLUS, PerkinElmer). Both pools were mixed together for genomic DNA preparation, PCR amplification of molecular barcodes, and microarray hybridization as previously described (Pierce et al., 2007; Yan et al., 2008). Data from both pools were then plotted independently. Microarray experiments were repeated three times.

3.5.3 Analysis of growth rates in liquid medium

Wild-type (BY4741) and ts strains were grown in YEPD for 24 hours at 23°C. The cultures were diluted to an OD$_{600}$ of 0.0625 and grown in 100 μl of YEPD with either the solvent DMSO or 4 μM of ZA in a 96-well plate. The 96-well plate was constantly shaken in a microplate reader (Tecan, GENios) at 36.5°C for 20 hours and the OD$_{600}$ was read every 15 minutes. The doubling time of strains was calculated according to the previously described method (St Onge et al., 2007). The fitness was defined as the ratio of the doubling time of strains grown in the presence of solvent alone (DMSO) relative to ZA treatment. The fitness assays were repeated four times.

3.5.4 Spot Assay

Wild-type and ts strains were grown overnight in YEPD. Cultures were serially diluted 10 fold in YEPD. An aliquot (2.5 μl) from each of the 10X serial dilutions of cells was spotted on YEPD plates containing either the solvent DMSO or 4 μM ZA. The plates were incubated for 2 days at 23°C or 36.5°C and photographed.
3.5.5 Degree Distribution Analysis

The total number of interactions, including both positive and negative, was measured for each query strain using the intermediate confidence cutoff on the genetic interaction network as described in Costanzo et al., 2010 ($|\varepsilon| > 0.08, p < 0.05$). The degree distribution was then plotted separately for deletion queries and ts queries.

3.5.6 Protein-protein interaction network overlap

To analyze the overlap between genetic interactions and the physical interaction network, a combined protein interaction network was constructed by taking the union of four recent high-throughput studies (Gavin et al., 2006; Krogan et al., 2006; Tarassov et al., 2008; Yu et al., 2008), which include interactions derived from affinity purification/mass spectrometry (Kaiser et al., 2008), yeast two hybrid (Suter et al., 2008) and protein-fragment complementation assay (Morell et al., 2009) studies. Positive and negative genetic interactions were defined using an intermediate confidence threshold ($|\varepsilon| > 0.08, p < 0.05$) as described in Costanzo et al., 2010.

3.5.7 Function prediction from comparison of genetic interaction data

Deletion array genes were classified into functional categories using a variation of k-nearest neighbors with leave one out cross-validation. Functional categories for classification were taken from a subset of the Gene Ontology annotations downloaded on April 29, 2010 (Ashburner et al., 2000). Classification was restricted to the non-redundant set of GO terms described in (Myers et al., 2006), and was performed independently on each GO term. Terms with fewer than 10 participating genes were
removed leaving 132 GO terms for prediction. The full set of 3,885 SGA array genes was reduced to 2,709 after removing genes with no annotations in the participating terms as these would provide no information for classification and would be impossible to correctly classify.

Each gene then received a score for every GO term in the following way. The $K (=5)$ largest similarity scores between the gene in question and members of the term in question were summed. Similarity scores were calculated as inner products between array gene profiles, using the selected subset of query genes. Genes were then ranked within each term according to this summed similarity with known term participants. This process was then repeated using different subsets of SGA queries to calculate similarities between array genes.

No more than one allele of each gene was used in either the DAmP or ts analysis, and the allele with the largest single mutant fitness defect was selected as representative. Additionally, only query strains that exhibited a single mutant fitness defect (fitness < 0.98) were allowed to participate in prediction. This restriction was imposed to remove both ts alleles that behaved like wild-type under the tested conditions as well as nonessential deletions with no functional impact. Since there were fewer DAmP alleles than alleles in the other classes of SGA queries, their performance was compared against an equal number (92) of randomly selected ts alleles and deletion mutants with average results being bootstrapped over 20 iterations.
3.5.8 Automated image acquisition and analysis

*MATα* query strains carrying different cellular markers were mated to the *ts* allele array, which contained 769 *ts* alleles representing 485 genes. *MATα* haploid *ts* strains expressing GFP and/or RFP fusion proteins were isolated using SGA technology (Costanzo and Boone, 2009; Tong and Boone, 2006), transferred into liquid selection media and cultured for 1-2 days. Automated imaging and image analysis were performed as described previously (Baryshnikova et al., 2010; Vizeacoumar et al., 2009; Vizeacoumar et al., 2010). To optimize cell density for automated image analysis, a liquid handling robot (Biomek FX Lab automation work station, Beckman Coulter) was used to dispense sample volumes based on the optical density of each strain. Image acquisitions were achieved using an ImageXpress 5000A fluorescence microscopy system (Molecular Devices) with 96-well format glass-bottomed plates (MMI Greiner M plates) at 26°C and 32°C. Cytomat automated incubators (Thermo Fisher Scientific Inc.) were linked to the ImageXpress system to increase throughput.

3.5.9 Confocal microscopy and image quantitation

Images were captured using the Quorum WaveFX Spinning Disc Confocal System (Quorum Technologies, Guelph, ON). The Z-axis images were converted into a single composite image by using the brightest pixel over all images in the Z-stack for every position in image. This maximum pixel projection technique produced a 2-D representation of the GFP fusion proteins within the cell from the 3-D data set.
3.5.10 Analysis of biologically relevant mutants using the HCS dataset

To ensure that the identified HCS phenotypes are due to genetic mutations, instead of uneven distribution of cell shape, size or cell-cycle stages between mutant and wild-type cell population, the relevant measurements of cellular organelles were scaled into relative morphometric features in comparison with the global cell geometry. For example, for the spindle marker, fiber length was normalized by cell length; for the mitochondria marker, mitochondria count was then scaled into mitochondria density by normalizing cell area. After the normalization procedure, for each of the morphometric features, the values between the mutant cell population and the wild-type cell population were compared. The statistical comparison was made based on Wilcoxon Rank Sum test ($P < 0.05$ after Bonferroni correction).

3.5.11 Affinity purification and mass spectrometry

Cells expressing GFP- or TAP-tagged proteins (1 L) were collected at mid-logarithmic phase (A600 = ~0.6), and lysed using glass bead beating as described (Gingras et al., 2005). TAP purification was performed as previously described (Breitkreutz et al., 2010). For GFP AP-MS, lysates (approximately 150-200 mg protein) were incubated for 2 hours at 4°C with 25 μl GFP-Trap magnetic particles (Chromotek). Beads were then subjected to one rapid wash in 1 ml lysis buffer and one wash in 20 mM Tris pH 8 containing 2 mM CaCl₂. On-bead trypsin digestion was performed, followed by LC-MS/MS on a Thermo Finnigan LTQ using the protocols and parameters described previously (Breitkreutz et al., 2010). Sequence database searching using Mascot 2.2.1 and the S. cerevisiae complement of RefSeq release 21 (both forward and reverse entries) was
performed, and hits with scores above 35 corresponded to a protein false discovery rate of <4%, determined using a target-decoy strategy (Perkins et al., 1999). A stringent set of filters were applied to remove likely false positives: 1) all proteins detected in any of three parallel purifications from untagged yeast were removed from the dataset; 2) proteins detected with a frequency of ≥15% across 800 purifications were removed; 3) only those proteins detected with at least 2 unique peptides in two biological replicates for any given bait were included in the analysis (their interactions with any of the members of the network are shown).
CHAPTER FOUR

Summary and Future Directions

4.1 Summary of thesis

Synthetic genetic array (SGA) analysis automates the systematic construction of double mutants in yeast, enabling a comprehensive and high-throughput analysis of genetic interactions (Tong et al., 2001). Because essential genes cannot be deleted in yeast haploid strains, one of the ways to apply SGA analysis to essential genes is to construct yeast strains harboring conditional alleles in essential genes. The goals of my thesis work were to: (1) construct a collection of yeast strains carrying temperature-sensitive (ts) conditional alleles of essential genes in a background compatible with automated genetics; (2) confirm and characterize the ts mutant collection; (3) validate and demonstrate the biological utilities of the ts mutant collection.

My newly constructed collection of ts mutants consists of 795 ts strains, covering 501 (~45%) of the 1,101 essential yeast genes, with ~30% of the genes represented by multiple alleles. This is the largest collection of ts yeast mutants constructed to date. The integrity of more than 99% of the ts alleles was confirmed using a PCR-based strategy and ~ 99% of the ts mutants were rescued by introduction of the wild-type version of the relevant gene.

I validated and demonstrated the usefulness of the ts collection in a number of different ways. First, in collaboration with colleagues at the University of Gothenburg, the ts mutant collection was characterized by high-resolution profiling of the temperature sensitivity of each ts strain in liquid growth assays. This analysis revealed that genes involved in similar biological pathways showed highly similar growth profiles. Second, I
used the ts array to explore genetic interactions involving genes whose interactions had been previously explored using either the yeast deletion mutant \((bni1Δ::natMX)\) or another type of conditional allele \((rfc5-1::natMX)\). This analysis confirmed that the ts array should be a valuable additional tool available to yeast geneticists for exploring gene function. Third, I collaborated with members of Chris Bulawa’s lab in FoldRx Pharmaceuticals Inc. to show that the ts mutant array is useful for exploration of chemical-genetic interactions because it enables the screening of compounds that may suppress defects in vital pathways. Fourth, I collaborated with members of the Boone, Andrews and Zhang labs to use the ts array in quantitative phenotypic analysis to assess defects in the subcellular localization of six fluorescent markers, diagnostic for different subcellular compartments or structures. Quantification of the marker behaviour at the single-cell level enabled integration of this data set to generate a morphological profile for each ts mutant. This analysis revealed previously unappreciated functions for essential genes, including those involved in chromosome cohesion and condensation.

### 4.2 Future Directions

#### 4.2.1 Exploring functions for uncharacterized yeast genes using the ts mutant collection

Essential genes function as hubs in the genetic interaction network (Chapter 3)(Davierwala et al., 2005; He and Zhang, 2006). This property means that exploration of genetic interactions involving essential genes ought to be particularly useful for dissection of the function of the many remaining uncharacterized yeast genes (Pena-Castillo and Hughes, 2007). The ts mutant collection provides an important complement
to the deletion collection (and other resources) that should enable the comprehensive assessment of gene function in a model eukaryotic cell. In addition, I found that the phenotypic analysis of strains carrying ts alleles in essential genes provides greater predictive power about gene function than comparable analysis of either strains carrying deletions in nonessential genes or DAmP alleles of essential genes (Chapter 3).

For example, a subset of deletion mutants showed few or no genetic interactions when crossed with the nonessential deletion array (Tong et al., 2004). It may be fruitful to assess genetic interactions involving these mutants using the ts mutant collection that I constructed. In addition, the general properties of genetic interactions involving essential genes have not been thoroughly explored, since no appropriate strain collection had been constructed. It is now possible to systematically explore genetic interactions involving essential genes by crossing a ts mutant in a particular essential gene to the entire ts collection

4.2.2 Discovery of new protein interaction partners for yeast essential genes using systematic genetics

Dosage suppression studies have been productively used as a genetic means to identify protein-protein interactions. For example, genes encoding the positive regulatory subunits, Cln1p and Cln2p, were discovered as dosage suppressors of cdc28-1, a temperature-sensitive allele of the essential gene encoding the cyclin-dependent kinase Cdc28p (Reed et al., 1989). One application of the ts array that I constructed is systematic dosage suppression analysis. To enable this application, our lab developed a high-copy (2µ-based) plasmid library in which each plasmid contains a DNA insert composed of a
single yeast ORF with its native upstream and downstream genomic sequences, along with a *kanMX* marker flanked by two unique 20-nucleotide molecular barcode tags (Magtanong et al., submitted). As proof-of principle, Leslie Magtanong, a former graduate student in our lab, transformed 41 different ts mutants from my ts array with the high-copy plasmid library and quantified the barcodes associated with colonies that were able to grow at the semi-restrictive temperature. Leslie’s analysis identified 150 dosage suppression interactions for 32 essential genes including 13 query genes that had no previously known dosage suppressors (Magtanong et al., submitted). Mapping a dosage suppression genetic interaction network for the entire spectrum of essential genes could extend to the majority of nonessential genes on the dosage suppression network by creating ts alleles of each gene within the context of a synthetic lethal background (Costanzo et al., 2010).

In addition to identifying new protein interaction partners by dosage suppression studies, another genetic way to find interacting components in a protein complex is by using nonallelic noncomplementation (Stearns and Botstein, 1988). Nonallelic noncomplementation occurs when two recessive mutations in two different genes do not complement one another even though a wild-type copy of each gene is present (Yook et al., 2001). This is also known as unlinked noncomplementation (Stearns and Botstein, 1988), second-site noncomplementation (Halsell and Kiehart, 1998), or extragenic noncomplementation (Vinh et al., 1993). Conditional mutants in *TUB2* (*tub2-501*) and *TUB3* (*tub3-1*), two yeast tubulin genes, were isolated by unlinked noncomplementation of a *tub1-1* strain (Stearns and Botstein, 1988). Thus, my large ts mutant collection can be
used to identify functionally related genes by nonallelic noncomplementation, which will expand the global functional wiring diagram of the cell.

### 4.2.3 Discovery of drug targets for essential genes using the barcoded ts mutant collection

Conditional mutations in essential genes ought to be equally useful compared to deletion collection since yeast strains carrying deletion mutations in nonessential genes have been useful tools in drug discovery (Hillenmeyer et al., 2008). For example, I found that treatment of *bet2-1* and *cdc43-2* mutants with zaragozic acid could rescue the ts phenotype of these strains (Chapter 3). Identification of ts mutants that are suppressed by zaragozic acid A could provide a cell-based screen for inhibitors of squalene synthase, a potential target for lipid lowering agents (Charlton-Menys and Durrington, 2008). Screens for compounds that suppress defects in other ts mutants could lead to the discovery of new treatment strategies for human diseases. For example, we could screen human disease-associated genes with homologues to essential yeast genes such as *SPT14*, *ERG11*, and *HEM2* (Foury, 1997).

### 4.2.4 Phenotypic analysis of the ts mutant collection using multiple fluorescent reporter genes

To explore genetic interactions in more detail, our group developed methods to systematically assess cell biological phenotypes in arrays of yeast mutants (Vizeacoumar et al., 2010). I collaborated with Franco Vizeacoumar and Jingjing Li to explore the changes in the subcellular localization of a number of fluorescent reporter proteins in my
ts mutant collection. We discovered a functional relationship between cohesin and condensin components, the CPC and the CTF19 kinetochore complex (Chapter 3). Our large scale phenotypic analysis identified an elongated spindle phenotype in the cohesion and condensin mutants suggesting a novel role for these components in spindle disassembly. By extending this approach to the entire ts mutant array, one can examine multiple cell biological compartments, and this should provide mechanistic insight to virtually any cellular function. In addition, unexpected pleiotropic functions of multiple genes can be uncovered. For example, I would explore the potential role of Cdc48p in DNA damage repair. A functional connection between Cdc48p, which is encoded by an essential gene, and DNA damage and repair pathways was suggested by large-scale assessment of synthetic lethal interactions (Costanzo et al., 2010). We used high content screening experiments to assay the formation of DNA damage foci in the ts mutant collection, and discovered an increased fraction of cells with focus formation in cdc48 mutants. Furthermore, high-resolution profiling of the temperature sensitivity of ts alleles showed that cdc48-2 had similar growth behavior to mutants with ts mutations in DNA repair genes (Figure 2-4E). Cdc48p is an ER resident protein with a major role in protein folding. However, our assay suggests a novel function for this protein in DNA damage and repair pathway. Components required for DNA damage pathway may be recruited from the cytosol to the nucleus and such components might go through a quality control for proper folding in the ER in a Cdc48p dependent fashion. In addition to confirm the increased focus formation in cdc48 mutants using different reporters (e.g., Rad52p-GFP), we also can detect the sensitivity of cdc48 mutants to DNA damage agents (e.g., MMS, HU, UV). To further understand the role of Cdc48p, it will be best to map its complete
physical and genetic interaction profile and test if any of these components share the same phenotype as that of Cdc48p.

Our lab is currently exploring a wide range of cell biological reporters for different cellular structures and components such as spindle pole, kinetochore, septin, peroxisomes etc. (Table 8). While experiments can be done in the deletion array, a major advantage of the ts mutant collection is the smaller size of the array as opposed to the deletion array. From our genetic interaction studies, it seems clear that the ts array has at least six times higher predictive power in gene function. Utilization of such an array for cell biological experiments, would thus be a powerful way to define gene function.

4.2.5 Spatio-temporal regulation of spindle disassembly

From our detailed analysis we have shown how cohesin and condensin complexes regulate spindle disassembly through the chromosomal passenger complex. However, it is still unclear how the chromosomal passenger proteins regulate spindle disassembly and the mechanism of this signaling remains unknown. Previous work suggests that Ipl1p might play a potential role in this process (Vizeacoumar et al., 2010). Ipl1p localizes to the trailing end of the disassembling spindle, starting from a midzone localization (Buvelot et al., 2003). Ipl1p was initially identified for its role during metaphase where it regulates the biorientation of the spindle (Biggins and Murray, 2001; Biggins et al., 1999). The simplest explanation would be that Ipl1p can signal the detachment of the incorrectly attached kinetochore from the microtubule and ensure that it is attached from two opposing microtubules from the opposite poles. This “scissoring” activity of Ipl1p might very well be utilized as a cellular mechanism to disassemble the spindle.
Consistent with this idea, increased kinase activity of Ipl1p has been detected in late anaphase (Buvelot et al., 2003; Vizeacoumar et al., 2010). The kinase activity of Ipl1p may activate microtubule depolymerizers to efficiently break the spindle. Another potential substrate for Ipl1p to initiate spindle disassembly might be Kar3p, which also localizes to the spindle midzone and has microtubule depolymerizing activity. It would be interesting to assess whether Kar3p is a substrate of Ipl1p and identify its associated components to understand how Ipl1p can signal spindle disassembly.
References


