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Diverse Functions of Spindle Assembly Checkpoint Genes in *Saccharomyces cerevisiae*

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ABSTRACT

The spindle assembly checkpoint regulates the metaphase-to-anaphase transition from yeast to humans. We examined the genetic interactions with four spindle assembly checkpoint genes to identify nonessential genes involved in chromosome segregation, to identify the individual roles of the spindle assembly checkpoint genes within the checkpoint, and to reveal potential complexity that may exist. We used synthetic genetic array (SGA) analysis using spindle assembly checkpoint mutants *mad1*, *mad2*, *mad3*, and *bub3*. We found 228 synthetic interactions with the four spindle assembly checkpoint mutants with substantial overlap in the spectrum of interactions between *mad1*, *mad2*, and *bub3*. In contrast, there were many synthetic interactions that were common to *mad1*, *mad2*, and *bub3* that were not shared by *mad3*. We found shared interactions between pairs of spindle assembly checkpoint mutants, suggesting additional complexity within the checkpoint and unique interactions for all of the spindle assembly checkpoint genes. We show that most genes in the interaction network, including ones with unique interactions, affect chromosome transmission or microtubule function, suggesting that the complexity of interactions reflects diverse roles for the checkpoint genes within the checkpoint. Our analysis expands our understanding of the spindle assembly checkpoint and identifies new candidate genes with possible roles in chromosome transmission and mitotic spindle function.

THE spindle checkpoint is a conserved surveillance mechanism that responds to spindle defects and arrests cells in mitosis (Amon 1999; Lew and Burke 2003). The checkpoint is evolutionarily conserved from yeast to humans and is believed to be essential for regulating fidelity of chromosome segregation and genome stability. Mutations in spindle checkpoint genes can predispose cells to cancer (Cahill *et al.* 1998). There are two branches of the checkpoint in budding yeast that are referred to as the spindle assembly and the spindle orientation checkpoints and it appears that only the spindle assembly checkpoint is conserved from yeast to man (Lew and Burke 2003). The spindle assembly checkpoint responds to misaligned chromosomes where there is a failure to achieve bipolar attachment of kinetochores on spindles. The checkpoint is believed to be activated by either a lack of tension or a lack of microtubule occupancy at kinetochores and responds by inhibiting the metaphase-to-anaphase transition (Waters *et al.* 1998; Stern and Murray 2001; Lew and Burke 2003). The checkpoint is extremely sensitive and a single unattached kinetochore can induce a cell to arrest in mitosis (Li and Nicklas 1995; Pangilinan and Spencer 1996). Five nonessential genes, *MAD1*, *MAD2*, *MAD3*, *BUB1*, and *BUB3*, and one essential gene, *MPS1*, originally defined the spindle assembly checkpoint (Hoyt *et al.* 1991; Li and Murray 1991; Weiss and Winey 1996). Mutants of all the nonessential genes in this pathway in yeast have similar phenotypes; they do not arrest the cell cycle in the presence of microtubule depolymerizing drugs (Hoyt *et al.* 1991; Li and Murray 1991).

Popular models for how the spindle checkpoint regulates mitosis propose that the spindle checkpoint genes define a signal transduction pathway that responds to misaligned chromosomes and transmits an inhibitory signal to the cell cycle machinery (Miller and Cross 2001; Yu 2002; Howell *et al.* 2004). Genetic evidence shows that the kinetochore plays an essential role in the spindle assembly checkpoint and is presumably at the top of the signal transduction pathway (Gardner *et al.* 2001; Janke *et al.* 2001; McCleland *et al.* 2002). All of the checkpoint proteins localize to kinetochores in mammalian cells and localization is correlated with spindle checkpoint activity (Amon 1999; Lew and Burke 2003). The precise roles of the spindle assembly checkpoint proteins in transmitting an inhibitory signal are not known. There are several biochemical interactions among checkpoint proteins and the

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interactions are presumed to be integral to checkpoint signaling. For example, Mad1 and Mad2 are constitutively associated in a complex (Chen *et al.* 1999; Luo *et al.* 2002). One popular model is that Mad1 brings Mad2 to unattached kinetochores where an interaction with kinetochores catalyzes the exchange of Mad2 into a Mad2–Cdc20 complex (Luo *et al.* 2002; Sironi *et al.* 2002). Cdc20p is an activator of the anaphase-promoting complex (APC) and elegant genetic experiments have shown that Cdc20 is the target of the checkpoint within the cell cycle machinery (Hwang *et al.* 1998). The precise mechanism of how APC^{Cdc20} is inhibited is unclear. Cdc20 is found in two separable complexes *in vivo*, an abundant complex containing Mad2 and a much less abundant complex containing Mad2, Mad3, and Bub3 (Chen *et al.* 1999; Sudakin *et al.* 2001; Poddar *et al.* 2005). Both complexes are essential for checkpoint activity in yeast. *In vitro* studies using extracts from human cells have identified an analogous complex of checkpoint proteins as a potent inhibitor of the APC termed the mitotic checkpoint complex (MCC) containing Mad2, BubR1 (the Mad3 homolog), and Bub3 bound to Cdc20 (Sudakin *et al.* 2001; Tang *et al.* 2001; Yu 2002; Poddar *et al.* 2005). There are conflicting data about regulated assembly of MCC. Mad2 interactions with Cdc20, including MCC assembly, are cell cycle regulated in yeast independently of the checkpoint and the kinetochore (Fraschini *et al.* 2001; Poddar *et al.* 2005). MCC forms constitutively in HeLa cells but forms in Xenopus extracts in response to checkpoint activation (Sudakin *et al.* 2001; Chen 2002). The assembly of MCC and the proposed importance of the complex as the ultimate inhibitor of the APC predict that Mad2, Mad3, and Bub3 have similar roles in the spindle assembly checkpoint.

Recent evidence has emerged supporting additional roles for checkpoint proteins outside of mitotic regulation. Chromosome loss rates differ among spindle checkpoint mutants, suggesting that Bub1 and Bub3 may have additional roles in kinetochore or spindle function (Warren *et al.* 2002). BubR1 has been implicated in aging and fertility in mice (Baker *et al.* 2004). Mad2 has been implicated in the DNA damage checkpoint (Garber and Rine 2002). Both Mad2 and BubR1 have roles as mitotic timers in mammalian cells independent of their roles in the spindle checkpoint (Meraldi *et al.* 2004). A recent study suggests that there is complexity within the spindle assembly checkpoint. Mad1 and Mad2 are believed to respond to microtubule occupancy as well as to tension at kinetochores, while Mad3 responds only to microtubule occupancy and is not required for the response to tension (Lee and Spencer 2004). Furthermore, there are distinct roles for some of the checkpoint genes in meiosis (Shonn *et al.* 2000; Cheslock *et al.* 2005).

One way to better understand the functions of individual checkpoint genes is by analysis of their genetic interactions. Synthetic lethality between two mutants occurs when a mutation of either gene individually yields viable cells but mutating both in the same cells causes lethality or compromised growth. Genes encoding proteins that function in parallel pathways or with overlapping functions within an essential pathway can exhibit synthetic genetic interactions (Hartwell 2004). A systematic method for identifying synthetic lethality in yeast using the collection of deletion mutants of nonessential genes has been described and is called synthetic genetic array analysis (SGA) (Tong *et al.* 2001, 2004). Screens have been performed using 132 query strains to identify a total of 4000 interactions and the data are publicly available (Tong *et al.* 2004). Synthetic genetic interactions are enriched among groups of genes that encode proteins involved in the same gene ontology (GO) processes or similar subcellular localizations (Tong *et al.* 2004). By this means, we can extrapolate and assign functions for genes exhibiting synthetic genetic interactions with our genes of interest or assign additional roles to those genes. The spindle assembly checkpoint is ideal for SGA. Most of the checkpoint genes are nonessential but respond to a process, chromosome segregation, which is required for viability. Many of the genes that encode components of the mitotic spindle and the kinetochore are nonessential and some of the genes are nonessential because the spindle checkpoint buffers the cells in the absence of the proteins (Tong *et al.* 2001, 2004). Therefore, a complete SGA analysis using the spindle checkpoint mutants as queries should identify all nonessential genes required for chromosome transmission via the mitotic spindle. The popular model of the spindle checkpoint as a signal transduction cascade predicts, in its simplest form, that the spindle assembly checkpoint mutants should share an overlapping spectrum of genetic interactions. In addition, SGA should reveal unique functions of the individual checkpoint genes.

We used SGA and the collection of deletion mutations for all nonessential genes in yeast to define the synthetic genetic interactions of four genes of the spindle assembly checkpoint. We identified a large number of shared interactions among *mad1*, *mad2*, and *bub3* query strains with mutants encoding kinetochore and spindle components. The interacting mutants include three that define genes of unknown function, suggesting new spindle or kinetochore components. Surprisingly, large numbers of synthetic lethal interactions are either shared between pairs of genes or unique to each gene, suggesting that spindle checkpoint proteins have nonoverlapping functions. In contrast to the large number of interactions we found with *mad1*, *mad2*, and *bub3*, we found very few synthetic lethal interactions with *mad3*. We propose that Mad1, Mad2, and Bub3 have multiple roles in spindle function and checkpoint signaling and that Mad3 functions uniquely within the spindle assembly checkpoint.

TABLE 1

Strains and genotypes

Strain	Relevant genotype	Study
BY4741	*MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	EUROSCARF
BY4742	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*	EUROSCARF
BY4743	*MATα/MAT***a** *his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 lys2Δ0/LYS2 MET15/met15Δ0 ura3Δ0/ura3Δ0*	EUROSCARF
2446-7-4	*MAT***a** *can1Δ his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 mfa1Δ::P_{MFA1}-his5*$^+$	This study
3174-3-2	*MATα can1Δ his3Δ1 leu2Δ0 ura3Δ0 lys2Δ0 mfa1Δ::P_{MFA1}-his5*$^+$ *mad1*::NAT	This study
3172-32-3	*MATα can1Δ his3Δ1 leu2Δ0 ura3Δ0 lys2Δ0 mfa1Δ::P_{MFA1}-his5*$^+$ *mad2*::NAT	This study
2447-1-2	*MATα can1Δ his3Δ1 leu2Δ0 ura3Δ0 lys2Δ0 mfa1Δ::P_{MFA1}-his5*$^+$ *mad3*::NAT	This study
2446-1-2	*MATα can1Δ his3Δ1 leu2Δ0 ura3Δ0 lys2Δ0 mfa1Δ::P_{MFA1}-his5*$^+$ *bub3*::NAT	This study

MATERIALS AND METHODS

Strains and plasmids: Strains constructed for this study were isogenic with BY4741, BY4742, and BY4743 (EUROSCARF, Frankfurt, Germany) and are listed in Table 1. The deletion set of haploid (*MAT***a**) mutants was purchased from EUROSCARF and arrayed from a 96-well format to a 384 format. The strains were frozen in a 384 format and revised from frozen stocks once per month. Query strains were derived from the heterozygous diploid deletion collection and we switched the marker from Geneticin (G418) resistance to nourseothricin (NAT) resistance by a one-step gene replacement to convert the KanMX4 cassette to NatMX4 as described previously (Goldstein and McCusker 1999; Tong *et al.* 2001). The strains were sporulated, haploids were recovered after tetrad dissection, and the appropriate progeny were mated to strain 2446-7-4 to construct query strains by standard tetrad genetics (Burke *et al.* 2000). All deletions were confirmed by PCR and plasmid complementation. The *mfa1*::P_{MFA1}-*his5*$^+$ was constructed in strain BY4741 by PCR-mediated one-step gene replacement of the open reading frame of *MFA1* with the open reading frame of the *Schizosaccharomyces pombe his5*$^+$ gene, using KanMXHIS3 plasmid as a template (Longtine *et al.* 1998). Including the *his5*$^+$ gene in the query strain improves the efficiency of SGA (Daniel *et al.* 2006, this issue).

SC medium was made as described and supplemented with the appropriate amino acids (Burke *et al.* 2000). All chemicals were purchased from Sigma (St. Louis, MO) unless otherwise noted. Medium for haploid selection and for scoring synthetic lethality was supplemented with 2 mm 3-aminotriazole and the appropriate selective drugs. The purpose of including the 3-aminotriazole was to suppress the leaky expression from the *MFA1* promoter that can confound SGA as described elsewhere (Daniel *et al.* 2006, this issue).

Synthetic genetic array analysis: SGA was performed manually using a modification of the method used by Tong *et al.* (2001). Query strains with gene deletions marked with the NAT resistance were mated to the haploid deletion library (EUROSCARF) containing deletions of ~4700 nonessential genes marked with G418. The library was manually pinned to a lawn of cells from the query strain using 384 floating pins (VP Scientific, cat. no. VP384F) and selected on solid minimal medium supplemented with histidine, leucine, and uracil in NUNC omni trays (Fisher Scientific, Pittsburgh) and incubated at 25° for 24–48 hr. Diploids were sporulated on solid medium containing 1% potassium acetate and supplemented with 1 mm uracil, 1 mm leucine, and 1 mm histidine. Haploid spores were germinated on SC–his media containing 60 μg/ml L-canavanine, 2 mm 3-aminotriazole, and 350 μg/ml Geneticin (GIBCO, Grand Island, NY) (SC–his + CAN + G418). Germinated colonies were pinned to both SC–his + CAN + G418 plates and SC–his media containing 60 μg/ml canavanine, 2 mm 3-aminotriazole, 350 μg/ml Geneticin, and 100 μg/ml nourseothricin (Werner BioAgents) (SC–his + CAN + G418 + NAT).

Candidate colonies scored as synthetic lethal in the primary screen were picked from the SC-his + CAN + G418 plates, resuspended in 200 μl liquid SC–his + CAN in 96-well culture dishes, and incubated at 25° overnight. Tenfold serial dilutions of the cultures were pinned to SC–his + CAN (control) and SC–his + CAN + G418 + NAT solid media, using an inoculating manifold (Dan-Kar). This constituted the secondary screen.

Random spore analysis was conducted by resuspending colonies from sporulation plates in 200 μl sterile water and spreading them on petri plates containing SC–his + CAN solid media supplemented with 2 mm 3-aminotriazole. These were allowed to germinate for 3–5 days at 25°. The colonies were then replica plated to SC–his + CAN (control), SC–his + CAN + G418 (G418), SC–his + CAN + NAT (NAT), and SC–his + CAN + G418 + NAT (G418/NAT). All of the above media contained 2 mm 3-aminotriazole. We calculated the ratio of colonies on each type of media and compared it to the expected ratio of 2:1 for G418:G418 + NAT and 2:1 for NAT:G418 + NAT for colonies not exhibiting synthetic lethality, and data were analyzed by a chi-square test of significance, $P \leq 0.05$. Twenty tetrad dissections were performed on 20 sporulated diploids that we scored as showing synthetic lethality and found that interactions correlated directly with the results of the random spore analysis. We used random spore analysis as our final means of confirming synthetic genetic interactions. The random spore data presented in supplemental Tables 1–4 (http://www.genetics.org/supplemental/) are filtered to remove genes linked to *MFA1*, *CAN1*, and the query gene marked with *NAT*.

Pins were sterilized by a series of washes between pinning. They were first sonicated in a water bath (Sinosonic Industrial) containing 500 ml of dH_2O and mild detergent for 1 min to remove yeast cells. They were consecutively washed in 150 ml of water, 150 ml 10% bleach, 150 ml water, 150 ml 10% bleach, 150 ml water, 200 ml water, and 250 ml water. The pins were soaked in each bath for 1 min. This was followed by an ethanol bath (95% ethanol) and flaming.

Data analysis: Networks were visualized using Osprey 1.2.0 (Breitkreutz *et al.* 2003). Function assignments were done using the Saccharomyces Genome Database GO Term Mapper (http://db.yeastgenome.org/cgi-bin/GO/goTermFinder).

RESULTS

Many of the genes encoding proteins that function in the mitotic spindle or the kinetochore are nonessential

as are the genes that encode most spindle checkpoint proteins (Ortiz *et al.* 1999; Lew and Burke 2003; McAinsh *et al.* 2003; Pot *et al.* 2003; Measday and Hieter 2004). Chromosome segregation is an essential process and we reasoned that this was an ideal situation for analyzing synthetic genetic interactions that arise because of complimentary functions within an essential pathway. We examined the synthetic genetic interactions of the checkpoint genes to identify genes involved in spindle and kinetochore function. In addition, we wished to identify alternative functions among the spindle checkpoint genes. We anticipated that genes involved in spindle function would be identified by shared synthetic genetic interactions with all of the spindle assembly checkpoint mutants. The interactions unique to each gene should provide insight into its independent functions. We used four of the six spindle assembly checkpoint mutants in query strains. We excluded *MPS1* because it is essential and required for spindle pole body duplication (Lauze *et al.* 1995). We were concerned that the overlapping role in spindle function would confound the analysis. We excluded *BUB1* because spores containing the *bub1* allele derived from heterozygous diploids have reduced and variable fitness, which confounds the analysis (Hoyt *et al.* 1991).

All interactions identified in this study were confirmed by quantitative meiotic (random spore) analysis (supplemental Tables 1 and 2 at http://www.genetics.org/supplemental/). Double mutants were scored as synthetic lethal if they generated no double mutants. In some cases the strains produced a very small number of double-mutant spores. For example, in a cross of *mad2* and *cik1* the random spore analysis had 88 G418-resistant colonies, 65 NAT-resistant colonies, and no colonies resistant to both drugs. In the cross between *mad2* and *cbf1* there were 77 G418-resistant colonies, 64 NAT-resistant colonies, and 2 colonies resistant to both drugs. Both cases were scored as synthetic lethal (chi-square test, $P < 0.05$). The two resistant colonies arise due to mechanisms such as gene conversion or disomy that can produce apparent double mutants (Daniel *et al.* 2006, this issue). In some cases the appropriate number of colonies were resistant to both drugs but they were small (less than one-tenth the size of wild type) and were scored as synthetic sick (reduced fitness) (Figure 1). All screens were conducted at least three times. Overall, 91% of the interactions we detected were synthetic lethal and 9% showed reduced fitness.

We did not consider synthetic fitness mutants in any of the screens where the double mutants showed a slight growth disadvantage. We performed our screens by pinning the ordered arrays manually without the aid of robotics. Often colony sizes varied after pinning simply by differences in manual manipulations. We could not reliably score small differences in colony size and when we included them it vastly increased the number of false positives in our screens and made the screens unmanageable. Furthermore, small differences in growth rate have a dramatic effect on cell number. For example, we determined that under our conditions of growth, the parent strain BY4741 has a doubling time of 2.2 hr. Two colonies that grow for 48 hr can differ in the number of cells by a factor of 10 with an 18-min difference in doubling time. We chose stringent criteria for what we

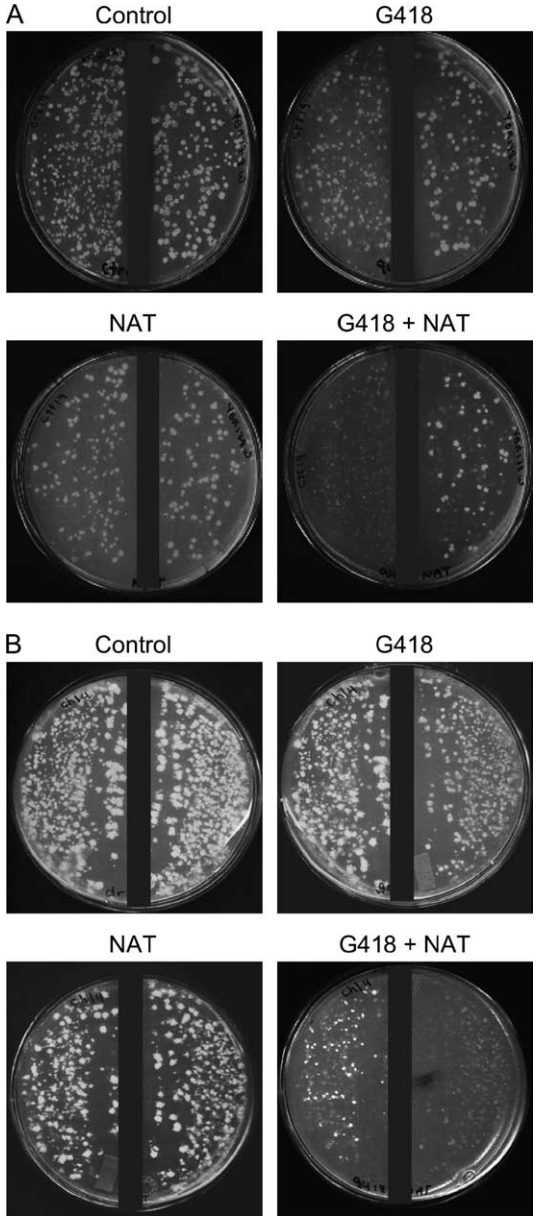


Figure 1.—Synthetic lethality and synthetic fitness identified by random spore analysis. (A) Colonies from haploid spores selected after meiosis were plated on nonselective medium (Control) and then replica plated to plates containing the indicated drugs. On the left is a cross demonstrating synthetic lethality and on the right are cells from a cross that does not show any synthetic interaction. (B) Colonies from haploid spores selected after meiosis were plated on nonselective medium (Control) and then replica plated to plates containing the indicated drugs. On the left are colonies from a cross showing synthetic fitness and on the right are colonies from a cross demonstrating synthetic lethality.

defined as synthetic lethal because we eventually want to understand the double-mutant phenotype and therefore the effect on the cell cycle has to be measurable.

The synthetic lethal interactions that we identified are shown in Figure 2 and are discussed in detail below. We were concerned about the potential for false negatives in the SGA screen (Tong *et al.* 2004). It is possible that the "unshared interactions" we identified were simply not scored in the other screen. Therefore, all interactions that were identified in Figure 2 using any checkpoint mutant were directly tested against the three other checkpoint mutants by constructing the double heterozygotes and analyzing synthetic lethality by quantitative random spore analysis.

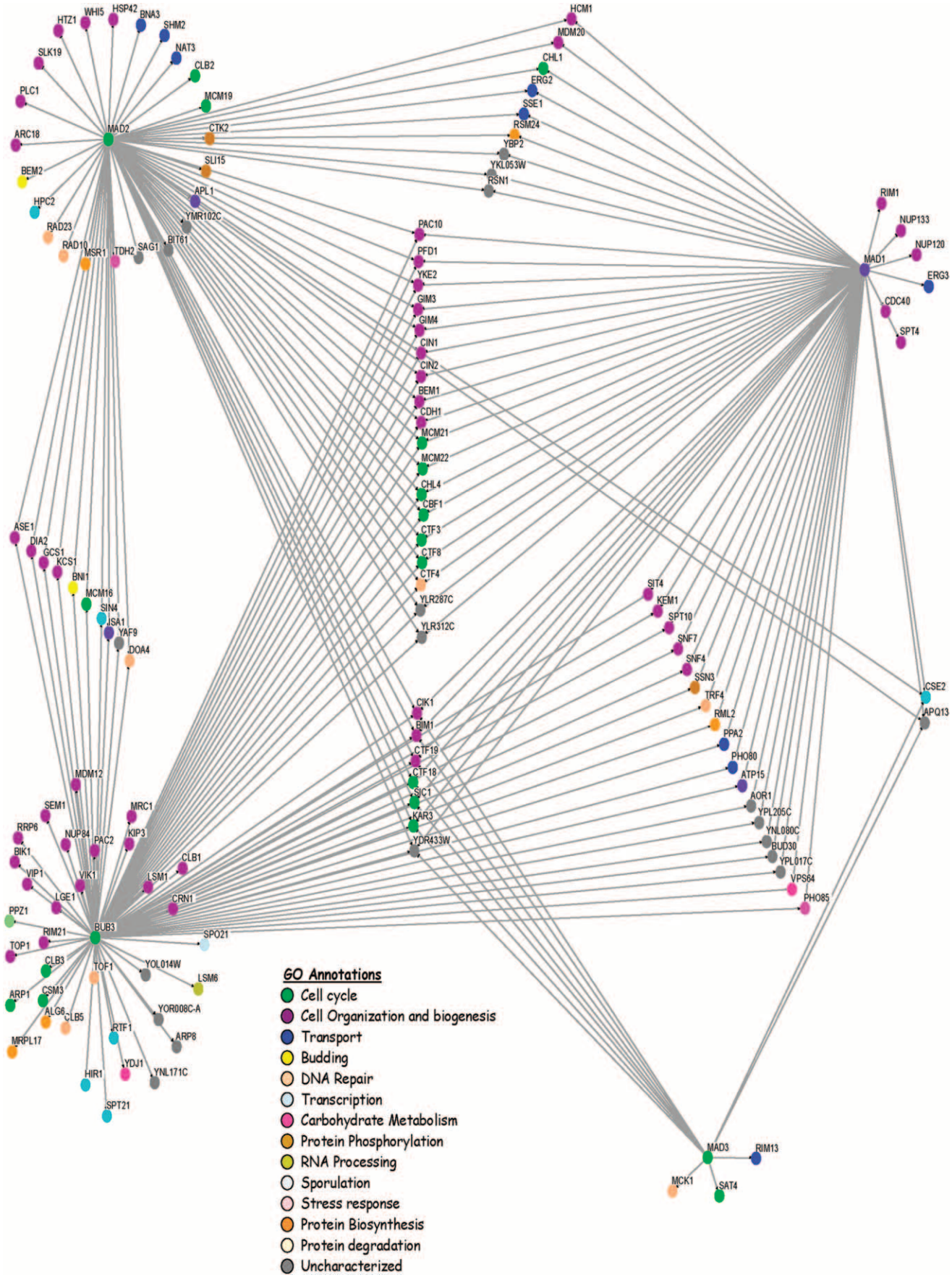
***MAD1* and *MAD2* have similar but not identical roles:** Spindle assembly checkpoint proteins Mad1 and Mad2 are constitutively present in a complex that localizes to kinetochores when the checkpoint is active (Chen *et al.* 1999; Chung and Chen 2002). All of Mad1 and about half of Mad2 are present in this complex (Chung and Chen 2002; Poddar *et al.* 2005). We expected that *mad1* and *mad2* mutants would have a very similar spectrum of genetic interactions. Sixty mutants exhibited synthetic lethality with *mad1*, 97% of which were synthetic lethal and 3% were synthetic sick (Figure 2). We recovered 69 mutants that exhibited synthetic lethality with *mad2*, 80% of which were synthetic lethal. There was a large overlap of interactions between *mad1* and *mad2*. Thirty-six mutants displayed synthetic genetic interactions with both checkpoint genes. These constituted 60% of the interactions with *mad1* and 52% of the interactions with *mad2*. The large overlap of interactions confirms that *MAD1* and *MAD2* are functionally related. However, many of the interactions were not shared by both genes. This suggests that Mad1 and Mad2 have divergent roles within the spindle checkpoint or participate in separate processes within the cell.

We examined the functions of the interacting genes using the GO processes database. Thirty-three percent of the shared interactions were genes that encoded proteins involved in chromosome segregation, 26 times more than expected by chance ($P = 3.3 \times 10^{-14}$). Twenty-eight percent encoded proteins involved in microtubule-based processes, 21 times more than expected by chance ($P = 3.3 \times 10^{-11}$). Seven of the genes encode known kinetochore proteins (*CTF3*, *CTF19*, *CHL4*, *CBF1*, *MCM21*, *MCM22*, and *BIM1*), four are involved in sister-chromatid cohesion (*CTF8*, *CTF18*, *CTF4*, and *CHL1*), and five are part of the prefoldin complex involved in tubulin folding (*GIM3*, *GIM4*, *YKE1*, *PAC10*, and *PFD1*). In addition, there were seven genes of unknown function.

Thirty-three of the *mad2* interactions were not shared with *mad1* and 24 of the *mad1* interactions were not shared with *mad2*. We analyzed the GO annotation of the genes whose synthetic genetic interactions were not shared by *mad1* and *mad2* to better understand their independent roles. Surprisingly, a subset of the unshared interactions, synthetic lethal with *mad2*, was genes that encoded kinetochore or spindle components. *MCM19*, *MCM16*, and *SLK19* encode outer kinetochore components, and *ASE1* and *SLI15* encode spindle components that are part of the microtubule cytoskeleton. This suggests that Mad1p and Mad2p either may have different roles in monitoring spindle function or may independently function within the kinetochore or the spindle. Fifteen percent of the mutants synthetic lethal with *mad2* but not with *mad1* encode transcriptional regulators and chromatin remodeling proteins, which may reflect an indirect interaction or may indicate that Mad2p has a role in these processes.

***MAD1*, *MAD2*, and *BUB3* have common genetic interactions with genes encoding proteins involved in spindle assembly and kinetochore function:** Eighty-seven mutants exhibited synthetic genetic interactions with *bub3*. All of them were synthetic lethal interactions and none were synthetic fitness. Twenty-five of these genes (29%) also showed synthetic genetic interactions with *mad1* and *mad2*, confirming that the genes function in a common pathway (Figure 2). Forty-four percent of these genes were annotated by the GO process as being involved in cytoskeleton organization and biogenesis, 11 times more than expected by chance ($P = 1.1 \times 10^{-11}$). These included genes encoding five members of the prefoldin complex, β-tubulin folding proteins, a cortical capture protein, and proteins involved in spindle establishment and maintenance. Forty percent of the genes that exhibit synthetic genetic interactions with *mad1*, *mad2*, and *bub3* encode proteins involved in cell cycle and chromosome segregation, 31 times more than expected by chance ($P = 5.3 \times 10^{-13}$). Most of the mutants representing genes encoding kinetochore components that exhibited synthetic genetic interactions with *mad1* and *mad2* were also synthetic lethal with *bub3*. *CTF19* and *MCM21*, both of which encode nonessential members of a protein complex that mediates kinetochore microtubule binding, and *MCM22*, *CTF4*, and *CHL4* encode outer kinetochore components (McAinsh *et al.* 2003; Measday and Hieter 2004). We did not find any large subset of genes exhibiting synthetic genetic interactions with *mad1*, *mad2*, and *bub3* that were involved in processes unrelated to spindle assembly and kinetochore function. We conclude that no other processes utilize the spindle assembly checkpoint exclusively to regulate the cell cycle. However, 3 of the 25 genes synthetic lethal with *mad1*, *mad2*, and *bub3* encoded proteins of unknown function. These uncharacterized genes may represent novel proteins required for proper kinetochore or spindle function.

Interestingly, 11 interactions were shared between *mad1* and *mad2* that were not shared with *bub3*. One gene (*CHL1*) has a role in chromosome segregation and another gene (*HCM1*) encodes a forkhead


HTZ1
WHI5
HSP42
BNA3
SHM2
SLK19
NAT3
CLB2
PLC1
MCM19
ARC18
MAD2
CTK2
BEM2
SLI15
HPC2
APL1
YMR102C
RAD23
RAD10
MSR1
TDH2
SAG1
BIT61
HCM1
MDM20
CHL1
ERG2
SSE1
RSM24
YBP2
YKL053W
RSN1
PAC10
PFD1
YKE2
GIM3
GIM4
CIN1
CIN2
BEM1
CDH1
MCM21
MCM22
CHL4
CBF1
CTF3
CTF8
CTF4
YLR287C
YLR312C
RIM1
NUP133
NUP120
MAD1
ERG3
CDC40
SPT4
ASE1
DIA2
GCS1
KCS1
BNI1
MCM16
SIN4
LSA1
YAF9
DOA4
SIT4
KEM1
SPT10
SNF7
SNF4
SSN3
TRF4
RML2
PPA2
PHO80
ATP15
AOR1
YPL205C
YNL080C
BUD30
YPL017C
VPS64
PHO85
CSE2
APQ13
CIK1
BIM1
CTF19
CTF18
SIC1
KAR3
YDR433W
MDM12
SEM1
MRC1
RRP6
NUP84
PAC2
KIP3
BIK1
VIP1
VIK1
CLB1
LSM1
LGE1
CRN1
PPZ1
BUB3
RIM21
SPO21
TOP1
CLB3
TOF1
YOL014W
LSM6
ARP1
CSM3
ALG6
CLB5
YOR008C-A
RTF1
ARP8
MRPL17
YDJ1
YNL171C
HIR1
SPT21
MAD3
RIM13
MCK1
SAT4
GO Annotations
Cell cycle
Cell Organization and biogenesis
Transport
Budding
DNA Repair
Transcription
Carbohydrate Metabolism
Protein Phosphorylation
RNA Processing
Sporulation
Stress response
Protein Biosynthesis
Protein degradation
Uncharacterized

transcription factor that regulates the expression of Spc110, an essential component of the spindle pole body, the yeast equivalent of the centrosome (Zhu and Davis 1998). These observations were unexpected because we assumed that all mutations in nonessential genes that function in chromosome segregation would respond identically with respect to spindle checkpoint mutants. Ten interactions were uniquely shared between *mad2* and *bub3*, 5 of them also have GO process annotations of cell organization and biogenesis, and 3 were annotated as cell cycle. Eighteen interactions were shared exclusively between *mad1* and *bub3*, 8 of them were annotated as cell organization and biogenesis, 5 were involved in phosphate metabolism, and 5 were genes of unknown function. In every case, the shared interactions between pairs of spindle checkpoint genes contain a subset of genes that could affect spindle structure and function. This includes genes that are known to function in the spindle, which is highly suggestive of additional complexity within the spindle assembly checkpoint. Interestingly, there were 24 unique interactions with *mad1* when we compared *mad1* to *mad2*; however, 18 of them were shared between *mad1* and *bub3* (Figure 2). This suggests that Mad1 and Bub3 function in a common process outside the spindle checkpoint or that they function together within the spindle or the checkpoint but respond to a subset of spindle perturbations. If the latter is correct, then the synthetic genetic interactions that are shared between *mad1* and *bub3* may identify additional novel genes encoding spindle proteins or affecting spindle function. The same applies to the interactions that are shared between *mad1* and *mad2* and between *mad2* and *bub3*.

Thirty-nine percent of *bub3* genetic interactions, 33% of *mad2* genetic interactions, and 10% of the *mad1* genetic interactions were unique and not shared by the other members of the spindle assembly checkpoint. The genetic interactions unique to *bub3* include GO processes involving cell cycle transitions, DNA replication checkpoint, and meiosis. One of the mutants is *spo21*, suggesting that Spo21 functions in mitosis as well as in meiosis. Thirty-eight percent of the mutants with synthetic genetic interactions unique to *bub3* identify genes involved in cell cycle regulation. The B-type cyclins Clb1, Clb3, and Clb5 are involved in the latter half of the cell cycle and Bik1p and Arp1p are involved in mitotic anaphase B. Our data differ significantly from the 45 genetic interactions for *mad1*, *mad2*, and *bub3*, as described in the publicly available data (http://biodata.mshri.on.ca/yeast_grid/servlet/SearchPage). There are several reasons for the differences (see DISCUSSION) but they underscore that data sets from large genomewide screens provide an important view of the spectrum of interactions and a great deal of additional information can be obtained by repeating the analysis for specific genes of interest.

***MAD3* shares very few interactions with the other spindle assembly checkpoint genes:** Deletion of any of the spindle checkpoint genes results in cells that fail to arrest the cell cycle in the presence of spindle damage. Biochemical analysis of APC inhibitors in mammalian cells has identified complexes of Mad2, BubR1 (Mad3), and Bub3 as potent inhibitors of APC^{Cdc20} (Sudakin *et al.* 2001). We expected that the Mad2, Mad3, and Bub3 genes would share substantial overlap in the spectrum of synthetic lethal interactions. Surprisingly, we found only 12 synthetic genetic interactions with *mad3* and 75% of them were synthetic lethal (Figure 2). Eight of the genes (67%) encode proteins annotated by GO process ontology as being involved in the mitotic cell cycle, 22 times more than expected by chance ($P = 3.3 \times 10^{-10}$). Most of these are microtubule motors and components of the spindle pole body or chromosomes. The 12 interactions with *mad3* contrast with *mad1*, *mad2*, and *bub3*, which had an average of 72 interactions each. The paucity of interactions was not due to the severity of the *mad3* mutation. The precise deletion of the entire *MAD3* gene was confirmed by PCR analysis and the benomyl sensitivity of the deletion mutant was fully complemented by a YCp *MAD3* plasmid (data not shown). Furthermore, we used linkage as an internal control to confirm that the synthetic lethal effects were due to the mutation in *MAD3*. In a cross with *mad3::NAT* in the query strain, *mad3::G418* should score as synthetic lethal since they are alleles and it is impossible to be haploid with both *mad3::NAT* and *mad3::G418*. Genes that are tightly linked to *MAD3* (YJL013C) will appear as synthetic lethal because of linkage. All 12 nonessential linked genes (within 10 cM of *MAD3*) failed to generate double mutants, confirming that the synthetic lethality was due to *mad3*. Only 7 of the genes that exhibited synthetic genetic interactions with *mad1*, *mad2*, and *bub3* were shared with *mad3* (Figure 2). In addition, three interactions were not shared by any other spindle assembly checkpoint gene, suggesting that Mad3 may have a role separate from the spindle checkpoint.

Most genes in the network affect chromosome transmission or microtubule function: The network of interactions among the spindle checkpoint genes was surprising given that there were a large number of interactions shared between pairs of checkpoint mutants and unique interactions for each of the genes. One interpretation is that genes or pairs of genes have alternate

Figure 2.—Synthetic genetic interactions of spindle checkpoint mutants *mad2*, *mad1*, *bub3*, and *mad3*. Nodes represent the indicated mutants and edges. All interactions shown were directly tested for synthetic lethality with each query gene, using random spore analysis. The GO process annotation of each interacting gene is denoted by the node color and is described in the key.

functions outside of the spindle checkpoint. Genes affecting chromosome transmission or the microtubule cytoskeleton interact with the spindle checkpoint; therefore, we applied two assays to determine if mutants identified in the network affected either process. We isolated isogenic *MAT***a**/*MAT*α diploid strains homozygous for deletion mutations in both alleles for every gene in the network and tested chromosome stability by a mating test. *MAT***a**/*MAT*α diploids are sterile but can become fertile (*MAT***a** or *MAT*α) by loss of chromosome III to generate a monosomic strain. In addition, cells can remain diploid but become fertile by either mitotic recombination or gene conversion events to render *MAT* homozygous. Diploids that generate mating-competent cells at high frequency have reduced fidelity of chromosome transmission. We used a simple patch-mating assay to determine the proportion of mating diploids. We spread a small number of cells of each genotype onto a patch and mated them to a lawn of *MAT***a** *arg4* or *MAT*α *arg4* cells overnight. We printed the lawns onto plates to select diploids and scored mating by the presence of papillae. Chromosome loss, mitotic recombination, and gene conversion are stochastic events, and if they happen early in the growth of a colony, then patches of cells could appear to have an increased frequency of generating mating diploids simply by chance. We used five independent colonies of each homozygous mutant to make patches and scored mutants as affecting chromosome transmission if all five patches showed an increased frequency of maters. An example is shown in Figure 3A. Wild-type cells generated few mating diploids in contrast to the *mcm19* mutant lacking a kinetochore protein and a mutant lacking YLR287C, a gene of unknown function. Overall, we found that 78 mutants in the network (61%) affect chromosome transmission.

We also assayed the mutants for their sensitivity to the benzimidazole drug benomyl, which affects microtubule function. The wild-type diploid strain is significantly more resistant to 10 μg/ml benomyl than a *mad2* mutant as well as a mutant lacking YOR008C-A, a gene of unknown function (Figure 3B). We found that 51 mutants from the network (40%) were benomyl sensitive. The data summarizing the chromosome transmission assay and the benomyl sensitivity are combined and shown graphically in Figure 4. Red nodes represent mutants that are benomyl sensitive, blue nodes represent mutants with altered fidelity of chromosome transmission, and green nodes are mutants with both phenotypes. Gray nodes are mutants that scored negatively in both assays. Overall, 77% of the mutants affect chromosome transmission or microtubule function. Many of the mutants that have unique interactions with checkpoint mutants affect chromosome transmission or microtubule function. The same is true for interactions that are shared between any two checkpoint mutants This suggests that most of the interactions identified in the network are due to the spindle checkpoint and not novel functions of the spindle checkpoint proteins in some other cellular process. The exception is *mad1*, which has six unique interactions but none affecting chromosome transmission or benomyl sensitivity. Mad1 may have an additional role outside of the spindle checkpoint.

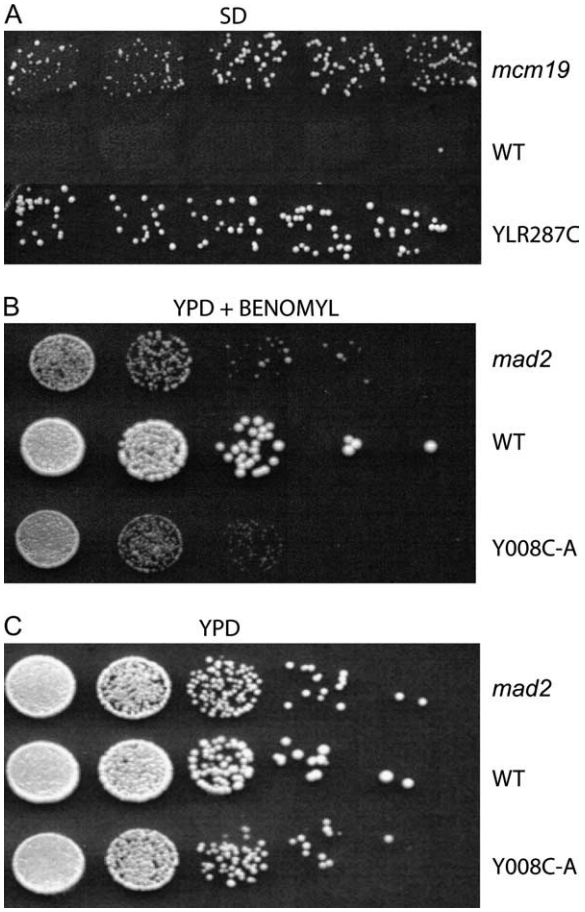
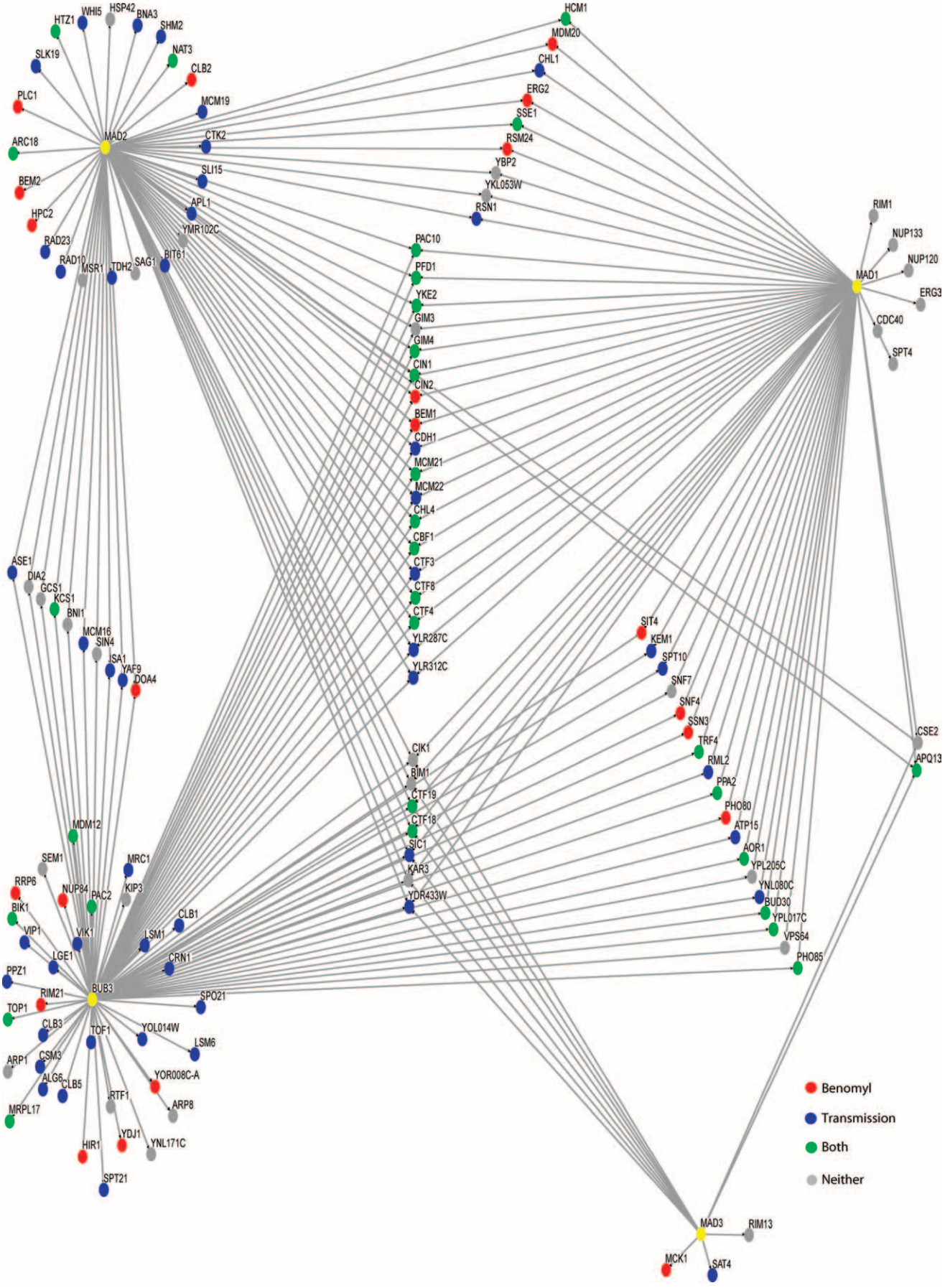


Figure 3.—Assays for chromosome transmission and spindle integrity. (A) Chromosome transmission. Five independent colonies of the indicated diploid genotype were patched onto a YPD plate and mated overnight to a lawn of *MAT***a** *arg4* cells and then printed onto an SD plate to select triploids. The papillae represent cells in the patch that successfully mated. (B) Spindle integrity. Serial 10-fold dilutions of cells of the indicated genotype were spotted onto a YPD plate containing 10 μg/ml benomyl. All strains grew equally well on YPD plates.

Figure 4.—Chromosome transmission and spindle integrity effects within the network. Mutants with enhanced benomyl sensitivity are indicated by red nodes, mutants with elevated frequency of producing maters are indicated by blue nodes, and mutants that both are benomyl sensitive and have enhanced benomyl sensitivity are indicated by green nodes. Gray nodes indicate mutants that neither were benomyl sensitive nor produced an elevated frequency of maters. Yellow nodes are the spindle checkpoint query mutants.


HCM1
MDM20
CHL1
ERG2
SSE1
RSM24
YBP2
YKL053W
RSN1
RIM1
NUP133
NUP120
MAD1
ERG3
CDC40
SPT4
PAC10
PFD1
YKE2
GIM3
GIM4
CIN1
CIN2
BEM1
CDH1
MCM21
MCM22
CHL4
CBF1
CTF3
CTF8
CTF4
YLR287C
YLR312C
CSE2
APQ13
SIT4
KEM1
SPT10
SNF7
SNF4
SSN3
TRF4
RML2
PPA2
PHO80
ATP15
AOR1
YPL205C
YNL080C
BUD30
YPL017C
VPS64
PHO85
CIK1
BIM1
CTF19
CTF18
SIC1
KAR3
YDR433W
HTZ1
WHI5
HSP42
BNA3
SHM2
NAT3
CLB2
SLK19
PLC1
MCM19
ARC18
MAD2
CTK2
SLI15
BEM2
APL1
HPC2
YMR102C
RAD23
BIT61
RAD10
MSR1
JDH2
SAG1
ASE1
DIA2
GCS1
KCS1
BNI1
MCM16
SIN4
ISA1
YAF9
DOA4
MDM12
SEM1
MRC1
RRP6
NUP84
PAC2
KIP3
BIK1
CLB1
VIP1
VIK1
LSM1
LGE1
CRN1
PPZ1
RIM21
BUB3
SPO21
TOP1
CLB3
TOF1
YOL014W
LSM6
ARP1
CSM3
YOR008C-A
ALG6
CLB5
RTF1
ARP8
MRPL17
YDJ1
HIR1
YNL171C
SPT21
MAD3
RIM13
MCK1
SAT4
Benomyl
Transmission
Both
Neither

DISCUSSION

SGA is a powerful tool to detect genetic interactions using the deletion set of nonessential genes in yeast (Tong *et al.* 2001, 2004; Huang *et al.* 2002; Jorgensen *et al.* 2002; Sarin *et al.* 2004). We have used SGA to study the process of chromosome segregation with the expectation that some of the nonessential genes that encode proteins that participate in the process of chromosome segregation would be essential in the absence of the spindle checkpoint. The goals of this study were to use spindle checkpoint mutants in SGA query strains to identify all synthetic lethal interactions with the spindle assembly checkpoint. We found that *mad1*, *mad2*, and *bub3* showed a large overlap in the spectrum of synthetic lethal interactions. We also found unique interactions as well as interactions shared between subsets of the spindle assembly checkpoint mutants. Surprisingly, we found very few interactions when we used *mad3* as the query mutant.

The large number of shared interactions between *mad1*, *mad2*, and *bub3* includes genes with known roles in microtubule, kinetochore, or sister-chromatid cohesion. This suggests that there are no other cellular processes that exclusively use the spindle checkpoint to regulate mitosis. In addition, there were genes of previously unknown function, and the synthetic lethality with all three checkpoint mutants suggests a role in chromosome segregation. In contrast to *mad1*, *mad2*, and *bub3*, which had an average of 72 interactions, *mad3* had only 12. Why were there so few genetic interactions with *mad3*? Either Mad3 plays a minor role in the spindle checkpoint or the spectrum of interactions with *mad3* is indicative of the response of the spindle assembly checkpoint in general. There is little evidence supporting the possibility that Mad3 plays a lesser role in the checkpoint. The spindle assembly checkpoint mutants are not distinguishable on the basis of benomyl sensitivity, and *mad3* is equally as sensitive as *mad1* and *mad2* and *bub3* (Lee and Spencer 2004). There may be a lower rate of chromosome loss in a *mad3* mutant when compared to other spindle assembly checkpoint mutants but the differences depend on the genetic background (Warren *et al.* 2002). The human ortholog of Mad3 is BubR1 and is believed to be one of the most important inhibitors of the APC (Sudakin *et al.* 2001; Tang *et al.* 2001). BubR1 (Mad3) is part of a complex purified from HeLa cells that includes Bub3, Mad2, and Cdc20 (MCC) that is a potent inhibitor of the APC (Sudakin *et al.* 2001). This complex also forms during mitosis in yeast, independently of kinetochores, and is required for maintaining a metaphase arrest by the checkpoint (Fraschini *et al.* 2001; Poddar *et al.* 2005). Therefore, the genetic and biochemical evidence suggests that Mad3 is an integral component of the spindle assembly checkpoint.

One report suggests that Mad3 has a limited role in the spindle assembly checkpoint (Lee and Spencer 2004). There is a mitotic delay in *cdc6* mutants that fail to initiate DNA replication and proceed through mitosis with unreplicated DNA. One model is that in the absence of sister chromatids there is a lack of tension on chromosomes, which activates the spindle checkpoint. A *cdc6 mad2* double mutant does not have a mitotic delay, suggesting that Mad2 is required for the cells to respond to a lack of tension; however, a *cdc6 mad3* double mutant retains the delay, showing that Mad3 is not required (Lee and Spencer 2004). These observations have been interpreted in terms of a popular model that the spindle checkpoint monitors two distinct events: microtubule occupancy at the kinetochore and tension between sister chromatids. Synthetic lethal interactions with *mad1* and *mad2* mutants that are not shared by the *mad3* mutant have been interpreted to mean that the interacting genes encode proteins required for producing tension between sister chromatids (Lee and Spencer 2004). The corollary is that none of these interacting genes is required for microtubule occupancy in the kinetochore. Although this is formally possible, we think that this is the unlikely explanation on the basis of our data. We have a much larger data set and have identified 27 interactions that are shared between *mad1* and *mad2* but not *mad3*. Many of these are genes involved with kinetochore or microtubule function, including genes that encode the prefoldin complex for post-translational tubulin folding. This latter class of mutants should be limited for tubulin within the cell and it seems unlikely that cells limited for tubulin would manifest the limitation by having reduced tension across kinetochores as opposed to limited occupancy. Furthermore, we identified interactions between *mad1*, *mad2*, or *bub3* that were not shared with *mad3* with nine mutants that lack kinetochore proteins (*cbf1*, *chl4*, *ctf3*, *mcm16*, *mcm19*, *mcm21*, *mcm22*, *sli15*, and *slk19*), and although the precise roles of each of these kinetochore proteins are unknown, it seems probable that at least one of them affects microtubule binding to kinetochores.

The other possibility is that the paucity of interactions with *mad3* represents the true response of the spindle checkpoint. Other studies have reported slight differences in synthetic fitness with spindle checkpoint mutants including *mad3* (Lee and Spencer 2004; Tong *et al.* 2004). This may reflect the true response of kinetochore and spindle mutants to a loss of the spindle checkpoint. If this is true, then why are there so many more severe genetic interactions with *mad1*, *mad2*, and *bub3*? One possibility is that the interactions reflect additional functions of Mad1, Mad2, and Bub3 in the kinetochore or the spindle. All three proteins localize to kinetochores and it has been assumed that this reflects their role in checkpoint signaling (Lew and Burke 2003; Gillett *et al.* 2004). However, another possibility is that the proteins have a role in chromosome segregation. It has been suggested that Bub3 may have an additional role in kinetochore function distinct from its role in the

spindle checkpoint (Warren *et al.* 2002). Perhaps all three checkpoint proteins, Mad1, Mad2, and Bub3, have roles in kinetochore function. If this were true, then the severity of genetic interactions in *mad1*, *mad2*, and *bub3* may be due to three deficiencies. For example, a kinetochore mutant such as *mcm21* may be synthetic lethal with *mad2* because the double mutant is missing both kinetochore functions of Mcm21 and Mad2 in addition to missing the spindle checkpoint. The *mcm21 mad3* mutant is only slightly compromised and shows reduced fitness because Mad3 functions only in the spindle checkpoint.

A recent study reported a large-scale synthetic lethal screen using a *mad1* query strain and a different technique based on competitive growth and microarray hybridization called synthetic lethal analysis by microarrays (SLAM) (Lee and Spencer 2004). Sixteen mutants were identified that required Mad1 for robust viability. Four of the double mutants were synthetic lethal and the rest had reduced fitness. All 16 of the candidates were tested for interactions with *mad2* and *mad3* mutants. The conclusion was that *mad1* and *mad2* had identical interactions and *mad3* had fewer and the effects were less severe. We used a completely different approach and identified 60 interactions with *mad1*, including 10 of 16 of the mutants reported by SLAM. The 6 reported in the SLAM study that we did not identify have reduced fitness and would have been missed in our screen. Overall, our study agrees with theirs but our 228 total interactions represent much more extensive analysis of the spindle assembly checkpoint. However, our richer data set allows us to conclude that *mad1* and *mad2* do not have identical interactions.

One important difference between our data and the SLAM data is that we included *bub3* and identified interactions that were common to different pairs of spindle checkpoint genes. The interactions that we detect among pairs of spindle checkpoint genes are specific and do not represent mutants that interact widely within any SGA and show up in many screens (Tong *et al.* 2004). In the publicly available database 1026 of the strains from the haploid deletion set showed genetic interactions. The genes that we identified that had interactions common to different pairs of spindle checkpoint genes had an average of 21 interactions each in the publicly available SGA data set. The probability that the interactions we detected with pairs of spindle checkpoint mutants occurred by chance is extremely low. For example, for the genes that interact only with *bub3* and *mad2*, we calculate that the probability that this is happening by chance is $<1.3 \times 10^{-22}$. There are similar low probabilities that the interactions detected between *mad1* and *mad2* and between *mad1* and *bub3* occur by chance. Furthermore, interactions were dominated by a small number of GO annotations. The interactions shared between *bub3* and *mad2* included genes with known roles in the kinetochore (*MCM16*) and cytoskeleton (*ASE1*, *ARC18*, *BNI1*) and half of the genes identified by these interactions are annotated by similar GO annotation processes. Why are there interactions between *mad2* and *bub3* that are not shared by *mad1*? One possibility is that this reflects some common function that Mad2 and Bub3 have within the spindle or kinetochore. The other half of the mutants that interact with both *mad2* and *bub3* do not share the common GO annotations. This may reflect a common function for Mad2 and Bub3 outside the spindle checkpoint although there is no hint from any of the GO annotations as to what cellular process that may be. The majority of genes in the interaction network affect chromosome transmission or benomyl sensitivity. This includes many genes that interact uniquely with the spindle checkpoint or with pairs of checkpoint genes. We favor the possibility that most of the genes in the interaction network impinge upon spindle function in ways that have not been described previously.

The large data set of SGA interactions included one of the genes that we used as a query gene, *mad2* (Tong *et al.* 2004). We found 69 interactions with *mad2* while the publicly available data set contains 31 interactions. Part of the reason for our increased success is that we improved the basic SGA screen by including a fusion of the promoter of *MFA1* to the *S. pombe his5*$^{+}$ gene as the haploid reporter, which eliminates gene conversion between the reporter and the *his3Δ1* allele and thereby reduces false negatives. Four of the 31 genes that interact with *mad2* from the large-scale SGA were essential genes that we did not have in our collection of deletion mutants and therefore were not tested. Of the remaining 27, there were 22 in common or >80% overlap in our respective screens. The remaining 5 interactions scored as synthetic fitness and would have been missed by us. Our improvements, which eliminate some of the false negatives in an SGA screen coupled with our screens being repeated three times, could account for why the publicly available SGA screen failed to detect the remaining 42 interactions. However, there is excellent agreement between the two SGA screens.

Our analysis differs substantially from the publicly available data on synthetic lethal interactions (Tong *et al.* 2004). There are 45 genetic interactions for *mad1*, *mad2*, and *bub3*, as described in the publicly available data. There are several reasons for the differences. One is that we recovered many more interactions because we used a more extensive screen even though we did not score many synthetic fitness interactions. Another reason is that we directly tested every interaction that we found with the other checkpoint mutants. In addition, *mad2* was used as a query in the publicly available SGA. Other interactions were detected because the data were generated using many query genes that encode cytoskeleton proteins and therefore are expected to show synthetic lethality with the spindle checkpoint. The overlap in some of the data is fortuitous. For example, *cin1*,

cin2, and *pac2* are synthetic lethal with *mad1* and *mad2*. There is overlap because all three were individually synthetic lethal with *mad1* when they were used as query genes but none were synthetic lethal with *mad2*. However, all three were found as synthetic lethal when *mad2* was used as a query. There was no attempt to reconcile why *mad2* was not identified when *cin1*, *cin2*, and *pac2* were used as query genes. Nor was there an attempt to directly reconcile potential differences, so the interactions that appear unique are tentative. The differences are very important for understanding the details of how the spindle assembly checkpoint is organized and how it functions. This underscores the need for interested researchers to carefully investigate interactions and to not rely solely on publicly available data sets.

Our SGA data change the fundamental way in which one thinks about the spindle checkpoint genes. The major function of the spindle checkpoint is to monitor chromosome segregation. However, the simple expectation that all of the genes function in a single linear signal transduction pathway must be replaced by a more complex model in which either subsets of spindle assembly checkpoint genes respond to different perturbations or they have a more direct role in chromosome segregation. Further work is needed to dissect the increasing complexity of the spindle assembly checkpoint genes, which will be greatly aided by additional genetic and genomic tools as they become available.

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