

A protein interaction map of the mitotic spindle

Jonathan Wong,^{*} Yuko Nakajima,^{*} Stefan Westermann,^{*} Ching Shang,^{*} Crystal Goodner,[†]
Pantea Houshmand,^{*} Stanley Fields,[†] David Drubin,^{*} Georjana Barnes,^{*} and Tony
Hazbun[‡]

^{*}Dept. of Molecular and Cell Biology, University of California, Berkeley, CA 94720,
USA.

[†]Howard Hughes Medical Institute, Departments of Genome Sciences and Medicine,
University of Washington, Box 355065, Seattle, WA 98195-5065, USA.

[‡]Department of Medicinal Chemistry and Molecular Pharmacology, Bindley Bioscience
Center and Purdue Cancer Center, Purdue University, West Lafayette, IN 47907, USA.

Address correspondence to D. D. E-mail: drubin@berkeley.edu

Telephone: 510-642-3692, Fax: 510-643-0062.

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Abstract

The mitotic spindle consists of a complex network of proteins that segregates chromosomes in eukaryotes. To strengthen our understanding of the molecular composition, organization, and regulation of the mitotic spindle, we performed a system-wide two-hybrid screen on 94 proteins implicated in spindle function in *Saccharomyces cerevisiae*. We report 604 predominantly novel interactions that were detected in multiple screens, involving 303 distinct prey proteins. We uncovered a pattern of extensive interactions between spindle proteins reflecting the intricate organization of the spindle. Furthermore, we observed novel connections between kinetochore complexes and chromatin-modifying proteins and used phosphorylation site mutants of *NDC80/TID3* to gain insights into possible phospho-regulation mechanisms. We also present analyses of She1p, a novel spindle protein that interacts with the Dam1 kinetochore/spindle complex. The wealth of protein interactions presented here highlights the extent to which mitotic spindle protein functions and regulation are integrated with each other and with other cellular activities.

Introduction

The faithful inheritance of chromosomes is essential for the propagation of organisms. Central to this process in eukaryotes is the mitotic spindle, an elaborate array of microtubules and associated proteins that positions and segregates chromosomes during cell division. The fundamental nature of this dynamic structure is reflected by the significant number of components that are shared by humans and many simpler organisms including *Saccharomyces cerevisiae*. The proteins involved in spindle function not only encompass tubulin, motor proteins, and other microtubule-associated proteins, but also the microtubule-organizing centers, kinetochore complexes, chromatin-associated proteins, regulatory kinases and phosphatases, and the anaphase-promoting complex. The dependence of cell division on the mitotic spindle makes its disruption both a cause of diseases and a target for anti-cancer treatments.

Although our understanding of the mitotic spindle has increased significantly in recent years, our knowledge of the mechanisms that intricately choreograph chromosome segregation remains incomplete. Different models, each consistent with available observations, have been proposed to explain spindle dynamics, chromosome capture by microtubules, force generation on chromosomes, and checkpoint function (Mogilner et al., 2006). Achieving a holistic understanding of mitosis at the molecular level would be aided by an in-depth interaction network map of the proteins involved. Such a map would facilitate elucidation of the functions and organization of spindle proteins, and of their roles within the greater context of the cellular environment.

Systems approaches such as systematic two-hybrid screens are necessary to reveal the myriad patterns of protein interactions that underlie complex processes. In yeast, genome-wide approaches including systematic tandem affinity purification

(Gavin et al., 2006; Krogan et al., 2006), synthetic genetic arrays (Tong et al., 2004), and two-hybrid screens (Uetz *et al.*, 2000; Ito *et al.*, 2001) have provided a wealth of data from which models of functional interactions and pathways can be generated. However, the scale of genome-wide assays necessitates a high level of stringency and uniform experimental conditions to maximize their efficiency. An advantage of a study that focuses on an individual cellular process is that a denser interaction map can be created due to the additional experimental flexibility and customizability. By intensively probing the mitotic machinery in our two-hybrid study, we generated a high-resolution map of protein interactions within the mitotic spindle and with proteins not conventionally considered to be part of the spindle.

Materials & Methods

Strains and growth conditions

The plasmids and yeast strains used in this study are listed in Supplemental Material 5. Yeast strains were grown in either YP (yeast extract/peptone) or minimal medium supplemented with 2% glucose and appropriate nutrients. Geneticin (G418; GIBCO BRL) was used at a concentration of 0.4 mg/mL.

For C-terminal tagging with three tandem GFPs, the *SHE1* ORF was subcloned into the BamHI site of pYS47 (Sun et al., 2007), using primers oJW131 (CGCGGATCCCAAGATCTAAAGTACACAGATCG) and oJW132 (CGCGGATCCCCGCCAAATAGGTCTATCACT), to generate pJW15. The orientation of the ORF was confirmed by digesting pJW15 with AflIII and AgeI. pJW15 was linearized with AatII and transformed into a wild-type yeast strain. Transformants were selected on minimal medium plates lacking histidine. The diploids were sporulated to isolate haploids expressing She1-3GFP.

The C-terminal tagging of genes with mRFP (a generous gift from Roger Tsien, University of California, San Diego, La Jolla, CA) was performed as described (Longtine et al., 1998).

Two-hybrid screen

Genome-wide two-hybrid screens were performed as described by the Yeast Resource Center (<http://depts.washington.edu/~yeastrc/>). Briefly, each prospective bait gene was amplified from the genomic DNA of DDY1102 by PCR and a unique restriction site was added at each terminus of the amplified fragment. The genes were then cloned into the vector pOBD2 or pBDC for fusion of the Gal4p-DBD to the N-terminus or C-

terminus, respectively (Uetz *et al.*, 2000; Millson *et al.*, 2003). After verification of the cloning by restriction digest, the plasmids were transformed into PJ69-4a for mating with ~6000 strains hosting the Gal4p-AD-fused genome-wide array and subsequent screening (Hazbun *et al.*, 2003). Each bait was then re-screened against an array of 732 preys that exhibited interactions in the initial screen. Both screens were performed in duplicate. Verification of the Gal4p-AD-fused strains was performed by sequencing 20 strains, indicating that the array strain identities were correctly positioned. Graphical representations of protein interaction networks were created with Cytoscape software (<http://www.cytoscape.org>) unless otherwise noted. Comparison of the spindle two-hybrid data set with the database of physical interactions hosted by the Saccharomyces Genome Database was also performed with Cytoscape software.

Microscopy

Indirect immunofluorescence microscopy on intact yeast cells was performed as described (Ayscough and Drubin, 1998). The rabbit anti-GFP (Torrey Pines Biolabs) and YOL1/34 anti- α -tubulin antibody (Accurate Chemical and Scientific Corporation) were used at dilutions of 1:2000 and 1:500, respectively. Fluorescein- or rhodamine-conjugated anti-IgG heavy chain secondary antibodies (Jackson ImmunoResearch) were used at 1:100 dilution. Fluorescein- or rhodamine- conjugated goat anti-rat secondary antibodies (ICN Biomedicals/Cappel) were used at 1:500 dilution. Images were obtained on a Nikon TE300 microscope equipped with an 100x/NA 1.4 objective and an Orca-100 camera (Hamamatsu) controlled by Image Pro Plus software (Phase-3 Imaging Systems).

Live cell imaging was performed on log-phase cells grown at 25°C. Cells were adhered to Concanavalin A-coated (Sigma) coverslips and sealed into 50 μ L of minimal

medium with vacuum grease (Dow Corning) on a glass slide. Fluorescent images were obtained on an Olympus IX81/71 microscope using an 100X/NA 1.4 objective and a Orca-ER camera (Hamamatsu) controlled by Metamorph software (Universal Imaging). Image processing was performed with Image J software (<http://rsb.info.nih.gov/ij/>).

Results

Two-hybrid overview

We conducted a yeast two-hybrid screen of proteins implicated in mitotic and/or meiotic spindle function in *Saccharomyces cerevisiae*. For this screen, we selected 113 proteins that are either components of the spindle, regulate its activity, or are directly required for its wild-type function during mitosis and/or meiosis (Supplemental Material 1). In addition to proteins that comprise the spindle structurally, we included proteins in mitotic regulatory pathways such as the Cdc14-early-anaphase-release (FEAR) and spindle checkpoint pathways to gain insight into mitotic protein regulation. The genes encoding these proteins were cloned into “bait” vectors containing the DNA-binding domain (DBD) of the Gal4p transcription factor. Of the 113 genes cloned as baits, 19 fusion constructs were either strongly self-activating or lethal when transformed into *Saccharomyces cerevisiae* and were not used further (Supplemental Material 1). The remaining clones were screened in duplicate against an array of ~6000 “prey” yeast strains expressing individual open-reading frames (ORFs) fused to the Gal4-activation domain (AD). Pair-wise interactions were scored as multiple hits if they were detected in duplicate, or as single hits if they were detected in only one of the two trials. To further saturate our data set and to retest the single hit interactions, we re-screened the spindle baits in duplicate against a mini-array of 732 preys encompassing the majority of prey interactants from the initial screen. The data set was filtered for dubious ORFs, transposon and viral genes, and common false positives including drug-resistance genes and positive transcriptional regulators using annotations from the *Saccharomyces Genome Database* (<http://www.yeastgenome.org>). From the remaining data, 857 interactions that occurred exclusively between nuclear and non-nuclear

proteins as annotated by the Gene Ontology project (GO) (<http://www.geneontology.org/>) were not analyzed further (Supplemental Material 2). The possibility exists that some of these represent *bona fide in vivo* interactions by proteins that shuttle between the nucleus and cytoplasm, or whose localizations have not been thoroughly characterized.

1526 protein interactions covering 730 distinct prey proteins were tallied after filtering. Of these pair-wise combinations, 604 (39.6%) were detected by multiple screens. These multiple-hit interactions comprise the core data set of this study (Figure 1 and Supplemental Material 3). 303 (41.5%) of the prey proteins interacted with multiple bait proteins. Although they may represent weak or indirect, but otherwise meaningful interactions, the single hit data were segregated from the main data set and were not analyzed further in this investigation (Supplemental Material 2). The number of multiple hit interactions per bait construct ranged from 1 to 52 with a mean of 7.4.

Intersection with published databases

To evaluate the novelty of the data obtained in this study, we compared our 604 multiple-hit interactions with those in a database of physical protein interactions available in the Saccharomyces Genome Database. A direct comparison with previously published yeast two-hybrid data including two comprehensive genomic studies (Uetz *et al.*, 2000; Ito *et al.*, 2001) revealed only 58 interactions in common (Supplemental Material 4). Thus, >90% of the interactions detected were novel. The greater number of interactions for the mitotic proteins used in this study, compared to those reported in previous studies, could be partially attributed to differences in experimental design. In the genome-wide studies, multiple baits were pooled and tested against a complementary pool of preys, whereas each bait in this study was

tested individually against every protein in the prey library. We also compared our results with interactions reported in the database of physical interactions detected by tandem affinity capture and mass spectrometry (MS) (Rigaut *et al.*, 1999; Puig *et al.*, 2001). Although the methodologies of protein-protein interaction detection are different, and the affinity capture technique is predicted to preferentially detect stable complexes (Gavin *et al.*, 2002), the 65 protein-protein interactions shared by our study and the affinity capture-MS datasets was slightly higher than the overlap with the two-hybrid database. The higher level of intersection of our data set with data derived from an independent and orthogonal method argues for the validity of our data set. Importantly, the vast majority of interactions presented here represent novel findings.

Evaluation of Dam1 complex interactions

To further evaluate the comprehensiveness of this study, we closely analyzed the protein network generated for subunits of the well-defined Dam1 kinetochore complex. Purified from yeast, the Dam1 complex consists of ten essential proteins (Dam1p, Duo1p, Dad1p, Spc19p, Spc34p, Ask1p, Dad2p, Dad3p, Dad4p, and Hsk3p) that localize to the kinetochore and spindle microtubules (Cheeseman *et al.*, 2001a; Cheeseman *et al.*, 2001b; Li *et al.*, 2002). Each subunit purifies stoichiometrically in the complex, and multiple Dam1 complexes can oligomerize into stable ring structures that may be required to form stable attachments between chromosomes and spindle microtubules (Miranda *et al.*, 2005; Westermann *et al.*, 2005). Additional biochemical and yeast two-hybrid studies had also previously identified many of the subunits of the complex (Figure 2A) (Uetz *et al.*, 2000; Ito *et al.*, 2001; Ikeuchi *et al.*, 2003; Shang *et al.*, 2003). Of the two comprehensive yeast two-hybrid projects, one study (Ito *et al.*, 2001) reported 18 interactions between seven subunits, compared to the eight interactions between the

same seven subunits identified by the other study (Uetz et al., 2000). The Spc19p – Spc34p reciprocal interactions reported in the latter study were isolated from those of the other subunits, such that they could not be inferred to be part of the larger Dam1 complex. Some proteins, such as Ask1p, had only a single interaction with another member of the complex. Whether Ask1p was an integral component of the Dam1 complex or a protein with a separate function was obscured.

In contrast, our study identified 35 interactions between all ten subunits of the Dam1 complex (Figure 2B). The pattern of interconnectivity was apparent even though several two-hybrid constructs were missing from the analysis. The Ask1-bait construct was non-functional. Also, since interactions of the Dam1-bait construct were published previously, this bait was excluded from our study (Shang et al., 2003). Finally, Hsk3p, Dad3p, and Dad4p were only screened as baits since the construction of the prey library predated their identification as ORFs. Despite these limitations, every subunit had at least three two-hybrid interactions with other Dam1 complex components. The extensiveness of the interaction network connecting these ten proteins strongly supports the conclusion that they form a stable structure *in vivo*.

Interactions among subunits of defined protein complexes

As with the Dam1 complex, multiple interactions were detected among subunits of other biochemically well-defined kinetochore complexes. Fifteen protein-protein interactions were detected within the 11-subunit Ctf19 complex. There was no enrichment of connections among proteins of the proposed COMA (Ctf19p-Okp1p-Mcm21p-Ame1p) sub-complex (De Wulf et al., 2003), although it should be noted that the Ame1p and Mcm21p bait constructs were non-functional. Of the six interactions within the Ctf19 complex involving COMA subunits, three were with non-COMA

subunits. Within the four-member Mtw1 and Ndc80 kinetochore complexes, there were four and three internal interactions respectively. The ratio of internal two-hybrid interactions to the number of subunits for these kinetochore complexes was significantly lower than was obtained for the Dam1 complex. This is partially attributed to complications with non-functional Dsn1p and Nsl1p bait constructs and self-activation of the Ndc80p-AD fusion protein. In addition, the elaborate network of interactions within the Dam1 complex might include indirect linkages. Nevertheless, the two-hybrid data appeared consistent with available biochemical data regarding the composition of previously annotated kinetochore complexes, and provides further biological validation for the specificity of our screen.

Patterns of interactions between kinetochore and chromatin-associated proteins

Considered in its entirety, the wealth of yeast two-hybrid data forms a dauntingly complex network (Figure 1). Whether this reflects the occurrence of indirect interactions or the reality of a multitude of protein-protein interactions possible under a variety of environmental and temporal conditions within a dividing cell is unclear. To simplify the overall interaction network, an interaction map was generated in which well-characterized processes and multi-subunit complexes were represented as single nodes (Figure 3). Multiple hits between a protein and different subunits within a particular complex increase confidence that the interactions are relevant biologically. The simplified interaction network reveals high connectivity among proteins implicated in spindle function, especially at the level of chromosome attachment to the spindle.

A high level of connectivity exists between kinetochore components and other chromatin-associated proteins (Table 1). One example of this is Mif2p, a homolog of the mammalian CENP-C inner kinetochore protein (Meluh and Koshland, 1995, 1997). It

co-purifies with the Mtw1 kinetochore complex and histone proteins including the centromeric histone H3 variant, Cse4p/CENP-A (Westermann et al., 2003). Our data registered single-hit interactions of Mif2p with Hta2p and Htb1p, possibly indicating an indirect or transient connection between this inner kinetochore protein and these nucleosome subunits. Mif2p also interacted with Rlf2p, a subunit of chromatin assembly factor I (CAF-I), whose redundant function with the histone regulatory genes (HIR) pathway is required in the deposition of Cse4p at centromeres (Sharp et al., 2002). Interactions of Mif2p with the Mtw1 complex and the CBF3 inner kinetochore complex (Ndc10p, Ctf13p, Cep3p, and Skp1p), with which it shows synthetic phenotypes, were not detected (Meluh and Koshland, 1995). The Gal4p fusion domains might interfere with Mif2p's binding site for other kinetochore proteins. Finally, Mif2p specifically associated with two subunits of the ubiquitous and highly conserved casein kinase 2, Cka2p and Ckb2p. Intriguingly, Mif2p is a phosphoprotein *in vivo* whose phosphorylation is essential for its function (Westermann et al., 2003). Altogether, these data reinforce a model in which a Mif2p-Cse4p-containing nucleosome interaction bridges the chromosome and peripheral kinetochore elements.

Strikingly, a significant number of DNA-associated proteins exhibited multiple interactions with kinetochore components. One highly represented class of interactors was chromatin remodeling factors. These ATP-dependent complexes generally serve to modulate nucleosome positioning, integration, and removal from chromatin for processes such as gene transcription and repairing DNA damage (Shen *et al.*, 2000; Mohrmann and Verrijzer, 2005). Both the Ino80 and SWI/SNF chromatin remodeling complexes exhibited multiple interactions with kinetochore proteins, but not with Cse4p or Mif2p inner kinetochore proteins. Subunits of the Ino80 complex had two interactions with the Mtw1 complex, a central kinetochore element, and the SWI/SNF

chromatin remodeling complex had five interactions with the Ndc80/Hec1 complex, an outer kinetochore element. A third class of chromatin remodeler, the abundant RSC complex, was not appreciably represented in our interaction network. Its absence is consistent with the locus and operational specificity exhibited by chromatin remodeling complexes despite their shared function, and suggestive of specific protein interactions rather than a general connection to a cellular process (Chai et al., 2005). While the anchorage of central and outer kinetochore complexes to centromeres is believed to be mediated by inner kinetochore proteins, it is possible that their deposition is facilitated by the re-positioning of centromeric and neighboring nucleosomes. If the Ino80 and SWI/SNF complexes function redundantly in this process, this role may have so far gone undetected.

Another class of enzymatic chromatin structure modifiers that was highly represented in our screen was the histone acetyltransferases/deacetylases. These histone-modifying proteins help to regulate gene transcription, gene silencing, DNA replication, and DNA repair via modification of lysine residues on the amino-terminal tails of histones (Kurdistani and Grunstein, 2003). Our protein interaction network exhibited connectivity between this class of histone modifiers and central/outer kinetochore elements. The SAGA acetyltransferase complex had two interactions with the Spc25p subunit of the Ndc80/Hec1 kinetochore complex. Ahc2p, a proposed subunit of SAGA, interacted with two subunits of the Dam1 complex and with Nkp2p, a protein in the Ctf19 complex. These interactions with acetyltransferases were complemented by a similar pattern of interactivity with histone deacetylases. Both Pho23p and Sds3p of the Rpd3 deacetylase complex interacted with Spc25p. In addition, Pho23p also interacted with Dad4p of the Dam1 complex and Nkp2p. Another histone deacetylase, Hda2p, interacted with two subunits of the Ndc80/Hec1

complex and Hsk3p of the Dam1 complex. These interactions are indicative of a specific relationship between histone acetyltransferases/deacetylases and particular components of the kinetochore.

The detection of interactions between kinetochore proteins and the transcriptional Mediator complex was particularly intriguing. Mediator is a 20+ subunit coactivator that can be biochemically divided into head, middle, and tail domains (Biddick and Young, 2005). It has been shown to recruit RNA polymerase II (RNAPII) to promoters and can physically bridge transcriptional activators with RNAPII via its tail and head/middle domains respectively (Kim *et al.*, 1994; Bhoite *et al.*, 2001). Pgd1p/Med3p, a subunit of the tail domain, had three interactions with subunits of the Dam1 kinetochore complex and one interaction with Nkp2p of the Ctf19 kinetochore complex. In addition, Srb7p, Med8p, and Med11p associated with Dam1 complex subunits. Nkp2p also interacted with Srb7p of the middle domain. The ability of Gal4p, the transcriptional activator used in the two-hybrid fusion constructs, to bind to the tail domain of Mediator was considered as a possible source of false positive results (Park *et al.*, 2000). However, the specific affinity of multiple bait constructs of the Dam1 complex for Pgd1p argues against the possibility of the non-specific recruitment of Mediator and the RNAPII holoenzyme to Gal4p binding sites. Additionally, the Pgd1p-prey fusion construct is not a common false positive in other screens using the same prey library.

She1p and other MAPs interact with the Dam1 and Aurora kinase complexes

One protein that warranted further investigation was She1p, a mostly uncharacterized protein that exhibited interactions with Duo1p and Spc34p of the Dam1 outer

kinetochore complex, and that was previously demonstrated to interact with Dam1p (Shang et al., 2003). It also interacted with the yeast INCENP homolog, Sli15p, which, in conjunction with the Aurora B kinase, Ipl1p, phosphorylates Dam1p, Spc34p, and Ask1p of the Dam1 complex (Cheeseman et al., 2002). The specificity of She1p interactions with the Dam1 complex and its effector, taken together with their common localization to nuclear microtubules, strongly suggests a previously unrecognized function for this protein in mitosis (Hofmann et al., 1998; Huh et al., 2003).

To further validate novel protein-protein interactions detected by the yeast two-hybrid system and to obtain clues to possible functions, we employed fluorescent microscopy to localize fluorescently-tagged proteins within live cells. She1p was C-terminally tagged with three tandem GFPs for increased fluorescence. She1-3GFP localized to the mitotic spindle at all stages of spindle assembly, and to nuclear microtubules during G1 (Figure 4A). She1-3GFP co-localized with RFP-tagged versions of both Duo1p and Sli15p along the spindle, as predicted by the two-hybrid data (Figure 4, B and C). The intensity of She1-3GFP staining was uniform along the length of the spindle, consistent with the localization pattern of the Dam1 complex, and the localization pattern of the Ipl1 complex prior to late anaphase (Hofmann *et al.*, 1998; Biggins *et al.*, 1999). However, She1p did not concentrate at the spindle midzone with Ipl1p-Sli15p during late anaphase, and therefore does not appear to share the Ipl1 complex's roles in stabilizing the midzone and spindle disassembly (Buvelot et al., 2003; Pereira and Schiebel, 2003). We also found that She1p localizes to the bud neck throughout mitosis (Figure 4A). She1p is recruited to the bud site early during bud formation and persists through the large-budded stage. Cross-sectional images show brighter staining at the edges of the bud neck compared to the middle, indicative of a ring-shaped structure. Thus, fluorescent microscopy revealed that She1p localizes to

the same mitotic structure as its two-hybrid interacting partners, and additionally localizes to the bud neck, which is shown here for the first time.

In addition, subunits of the Dam1 complex exhibited multiple interactions with other MAPs. Bim1p and Stu2p are plus-end tracking proteins implicated in microtubule stability and elongation. Both of these proteins interacted with the Spc34p and Duo1p subunits. We also detected two-hybrid interactions between Bim1p and the Aurora kinase proteins, Ipl1p and Sli15p, and with She1p. Finally, Bim1p and Stu2p interacted with each other in our screen. This intricate web of interconnectivity between these microtubule-associated and kinetochore-localized proteins strongly implies a shared mechanistic function, presumably at the microtubule plus ends.

Phosphorylation state dependency of Ndc80p interactions

The mitotic machinery is tightly regulated to ensure faithful chromosome segregation. Protein modifications such as peptide cleavage, phosphorylation, ubiquitination, and sumoylation have a central role in signaling for progression through the cell cycle. Modification-dependent protein-protein interactions are one way in which these signals might be recognized. Although the yeast two-hybrid method has widely been used to detect interactions between proteins, its capacity to screen for modification-dependent interactions has not been well utilized. We tested two phospho-mutant forms of Ndc80p (Hec1/Tid3p), part of the KMN network of kinetochore proteins that is required to form a stable attachment with microtubules, for altered two-hybrid interactions (Kotwaliwale and Biggins, 2006). The N-terminus of Ndc80p/Hec3, including Ser100 in budding yeast Ndc80p, is phosphorylated *in vitro* by the Aurora B kinase (Cheeseman et al., 2002; Cheeseman et al., 2006; DeLuca et al., 2006) and may electrostatically modulate the protein's affinity for microtubules (Wei et al., 2007).

We performed a two-hybrid screen of Ndc80p harboring the native Ser100, or S100A or S100D mutations, against the entire yeast genome (Figure 5). The majority of Ndc80p-interacting proteins exhibited specificity for one or two of the Ndc80p forms. The S100A mutant had significantly fewer protein-protein interactions than the other forms, but these included interactions with two other kinetochore proteins, Dam1p and YDR532c, suggesting that phosphorylation of Ser100 is not required for the association of these kinetochore components. Although the wild-type and S100D forms of Ndc80p had a comparable number of interactions with other proteins, there were notable differences in their yeast two-hybrid interaction maps, including their ability to bind to YDR532c and Kar3p, a kinesin. Using the yeast two-hybrid method, we were able to efficiently create an interaction profile for mutants mimicking different modifications of Ndc80p and to categorize the interactors based on their preferential affinity for a particular form of the bait protein.

Discussion

A two-hybrid screen of proteins implicated in spindle function uncovered 604 protein-protein interactions

Understanding how the mitotic spindle functions depends on the identification of the proteins involved in its composition and regulation, and on determining how each protein is positioned within a basic organizational framework. The budding yeast *Saccharomyces cerevisiae* is amenable to such an undertaking due to the availability of an annotated genome and procedures for systematic studies. While genome-wide screens for protein-protein interactions have been conducted previously and in principle should have uncovered most of the interactions involving the mitotic spindle, the large scale of such studies required compromises in their execution and scoring that likely lead to a significant number of false negative results.

To investigate spindle-mediated chromosome segregation in depth, we have conducted a two-hybrid screen that focuses on that cellular process. Each bait construct averaged almost twice as many interactions as were found in previous large scale screens (Uetz *et al.*, 2000; Ito *et al.*, 2001), supporting the idea that these screens were not saturating. By individually testing each pair-wise protein-protein combination in a focused study, we could detect a greater number of spindle-related physical interactions.

The improved coverage achieved here can be attributed to the more focused scope of our study, which allowed for pairwise testing of all possible bait interactions. By focusing on a single cellular process, we could expend more effort to optimize and troubleshoot individual screens. Some baits that exhibited no interactions were re-cloned with the DBD fused to the opposite end of the protein. Other baits required

scaling of selection conditions to balance the suppression of false positives with the avoidance of false negatives. These factors allowed our two-hybrid investigation to uncover new protein-protein interactions within the budding yeast spindle.

The spindle two-hybrid screen successfully identified known complexes

To gauge the effectiveness of this investigation, it is useful to compare the physical interaction network generated here for spindle protein complexes to those generated in other studies. A number of investigations have identified interactions between subunits of the Dam1 kinetochore complex, making these proteins useful as a comparative template for this study. The spindle two-hybrid screen presented here detected over twice as many pair-wise interactions between subunits of the Dam1 complex as other previously published two-hybrid screens (Uetz *et al.*, 2000; Ito *et al.*, 2001). All ten subunits identified by biochemical purification (Cheeseman *et al.*, 2002; Miranda *et al.*, 2005; Westermann *et al.*, 2005) were also detected by this study, attesting to the sensitivity of this genomic survey. The integrity of this protein complex is made apparent by the multiple interactions made by each subunit with other subunits of the Dam1 complex. This analysis was facilitated in part by the recent identification of many new short ORFs in the yeast genome, including *DAD3* and *DAD4* of the Dam1 complex, that were not included in older genomic libraries. In the case of the Dam1 kinetochore complex, this two-hybrid study proved to be more sensitive than previous efforts, which raises the expectation for the detection of novel spindle interactions.

The spindle two-hybrid screen also identified She1p as a novel binding partner of the Dam1 complex. Other than being localized to the mitotic spindle (Huh *et al.*, 2003), this non-essential protein is largely uncharacterized. We demonstrated by fluorescence microscopy that She1p co-localizes with Duo1p and Sli15p on the mitotic

spindle, but does not share with these proteins the enriched localization at the spindle poles where kinetochores cluster during anaphase. This suggests that the role of She1p may be related to the spindle integrity function of the Dam1 complex rather than to its kinetochore function. We further found that She1p localizes to the yeast bud neck in a ring-shaped structure, but that it does not appear to interact with the Dam1 complex or Ipl1 complex in that area. Its localization to two structures essential for cell division suggests a novel function, the nature of which awaits further investigation.

In addition, a local network of interactions connected She1p, the Dam1 complex, and the Ipl1 complex with the microtubule plus-end tracking proteins (+TIPs) Stu2p and Bim1p. It has been proposed that a combination of Stu2p, Bim1p, and a third +TIP, Bik1p, act together and, partially redundantly, to modulate kinetochore-microtubule dynamics (Wolyniak et al., 2006). This control is important because a newly-captured chromosome bound to the lateral surface of a microtubule can become detached if the microtubule shrinks beyond the attachment point (Tanaka et al., 2005). The formation of an end-on attachment between a kinetochore and microtubule plus-end is postulated to be mediated by the Dam1 complex. The ability of these various proteins to physically interact may be indicative of a cooperative function for the establishment and/or maintenance of end-on attachments.

Discovery of novel interactions between chromatin-associated proteins and spindle proteins

The 604 pair-wise interactions mapped by the spindle two-hybrid screen presented an opportunity to reveal heretofore-undiscovered mechanisms important for spindle function. By congregating the interaction network nodes based on biochemically characterized physical associations and on participation in specific, narrowly defined

cellular processes, patterns signifying the novel convergence of nuclear processes were observed. One of the most striking convergences was that of kinetochore proteins with chromatin-associated proteins, which has implications for the formation of kinetochores on newly replicated chromosomes. While the current data support a model wherein the yeast kinetochore is organized around the association of Mif2p to centromere-specific nucleosomes containing Cse4p in a CBF3-dependent manner (Westermann et al., 2007), our results suggest that the establishment of kinetochores on centromeric DNA might be more complex.

That remodeling of DNA is important for kinetochore loading has previously been shown by the dependence of chromosome segregation fidelity on the function of either of two redundant chromatin remodeling pathways: the CAF-I and HIR pathways (Sharp et al., 2002). It is possible that regulation of the underlying centromeric chromatin structure is an integral part of kinetochore function prior to and/or after its establishment. In support of such a possibility, null mutants of subunits of the NuA4 acetyltransferase have synthetic genetic interactions with kinetochore alleles, exhibit sensitivity to the microtubule-destabilizing drug benomyl, and display elevated levels of mini-chromosome mis-segregation (Krogan et al., 2004). Also, the *Schizosaccharomyces pombe* histone deacetylase Mis16, and its human homologs, RbAp46/48, are required for loading of Cnp1/CENP-A/Cse4p onto centromeres and for prevention of hyper-acetylation of centromeric histones (Hayashi et al., 2004). The two-hybrid data reported here raise the possibility that histone acetyltransferases/deacetylases may have an even more extensive role in kinetochore function than just remodeling the centromere for protein deposition.

Subunits of the Mediator complex, a transcriptional activator, were also implicated in spindle function by their interactions with spindle components, especially

Dam1 complex subunits. The lack of similarly dense interaction networks with other transcriptional complexes suggests that the interactions between spindle proteins and the Mediator complex are specific. The possibility of a functional connection between Mediator and the kinetochore is bolstered by previous studies of Cse2p, a Mediator subunit originally identified by its requirement for chromosome segregation fidelity. *cse2* mutants exhibited chromosome non-disjunction and mitotic arrest, which were synergistically exacerbated in combination with point mutations in centromeric DNA (Xiao et al., 1993; Xiao and Fitzgerald-Hayes, 1995). In its role as a transcriptional activator, Mediator has been shown to have histone-acetyltransferase activity, leading to chromatin remodeling (Lorch et al., 2000). Whether the role of Mediator subunits in chromosome segregation is linked to their transcriptional function or results from an independent function is not known.

The observation that chromatin remodeling proteins, histone acetyltransferases/deacetylases, and the Mediator transcriptional activator had specific physical interactions with the spindle machinery suggests a previously unrecognized functional relationship. The observations that only a small subset of the known classes of transcriptional helpers exhibited interactions in this screen, that their physical associations were specific to particular kinetochore complexes, and that they were not common false positives all indicate that these proteins were not activating the two-hybrid assay with their transcription-regulating properties. Whether their chromatin-modifying abilities are essential for their function with spindle proteins, and whether these proteins form the same complexes used in transcriptional regulation when interacting with spindle proteins, is not known. While it is possible that these interactions have revealed a novel function for these chromatin-associated proteins, this network of interactions is also consistent with the hypothesis that specific chromatin

effectors play a role in the establishment and/or maintenance of kinetochores. By possessing the abilities to associate with both DNA and kinetochore proteins, these chromatin effectors are well-positioned to execute a variety of possible tasks including the recruitment, establishment, and/or maintenance of kinetochores. If functional redundancy exists amongst these proteins, it would explain how their mitotic functions have escaped characterization thus far.

Using a yeast two-hybrid screen to investigate the role of protein modification

In addition to screening for interactions between wild-type proteins in vegetative cells, we wanted to test how protein modifications may be studied by detecting associated alterations to their two-hybrid interaction profiles. To this end, we screened wild-type Ndc80p and two mutants of Ndc80p that mimicked the phosphorylated and unphosphorylated state of Ser100. Interestingly, most of the proteins found in this screen interacted preferentially with one of the two mutants (S100D). A notable exception was that Dam1p strongly interacted with all forms of Ndc80p tested. It was previously shown that Ndc80p binds to a S to A phospho-mutant of Dam1p, but not to the corresponding S to D phospho-mutant (Shang et al., 2003). These results suggest that the interaction between Dam1p and Ndc80p is regulated by the phosphorylation state of the former protein, and that Ndc80p's phosphorylation state controls other interactions.

YDR532c, which forms a complex with Spc105p (KNL-1) of the KMN network (Nekrasov et al., 2003), stands out as being the only protein that exhibits a strong interaction with the S100A variant of Ndc80p, but not the S100D variant. This result is consistent with the model of phosphorylation by Aurora B/Ipl1p weakening the integrity of the kinetochore-microtubule interface. In contrast, Kar3p, a member of the

kinesin-14 family implicated in the transport of newly captured chromosomes along microtubules (Tanaka et al., 2005), interacts only with the S100D variant of Ndc80p. This is significant because, in nocodazole-treated cells, Kar3p co-localizes with Ndc80p specifically on chromosomes detached from the mitotic spindle (Tytell and Sorger, 2006). Although it is unclear whether Ipl1p becomes activated in nocodazole-treated cells, this modification-specific interaction might be a mechanism for the localization of Kar3p to kinetochores inactivated by Ipl1p.

Although the genomes of several model organisms have been systematically screened using the yeast two-hybrid method, there are compelling reasons to use this technique for smaller, focused screens. Besides detecting potential binding partners for a protein, yeast two-hybrid screens can also be used to investigate the roles of protein modifications. Using this technique, we report the discovery of novel protein-protein interactions and effects of protein phosphorylation that provide insights into the mechanistic workings of the mitotic spindle.

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Figure 1. The mitotic spindle protein interaction network. Nodes representing proteins that were used as bait constructs are shown in green. Proteins that were not used as baits are shown in yellow.

Figure 2. Comparison of intra-Dam1 complex interactions detected from genome-wide and focused two-hybrid screens. Protein interaction networks of subunits within the Dam1 complex derived from yeast two-hybrid studies and an *in vitro* expression experiment were generated with Cytoscape network visualization software. The bait construct for Ask1p (shown in red) was lethal to yeast and could not be screened. (A) The interactions reported from previous comprehensive two-hybrid screens identified seven of the ten Dam1 complex subunits. (B) The network of interactions found by this study identified all ten subunits of the Dam1 complex. The very high number of interactions detected between subunits is consistent with their association as a protein complex.

Figure 3. A simplified spindle protein interaction network. This simplified network includes proteins with demonstrated spindle or chromosome functions as well as uncharacterized proteins that interact with multiple spindle proteins. Proteins that belong to the same complex or functional process are grouped into single nodes. Abbreviations: complex (cmplx), anaphase-promoting complex (APC), casein kinase I (CK1), casein kinase II (CKII), chromatin assembly factor I (CAF-I), chromatin remodeling complex (CRC), cyclin-dependent kinase (CDK), histone acetyltransferases (HATs), histone deacetylases (HDACs), microtubule-associated proteins (MAPs), polymerase II (Pol II), protein phosphatase I (PP1), protein phosphatase 2A (PP2A).

Figure 4. She1-3GFP localizes to the mitotic spindle and the bud neck. (A) Localization of She1-3GFP during metaphase and anaphase. (B) She1-3GFP (green) co-localizes with Duo1-RFP (red) on the mitotic spindle. (C) She1-3GFP (green) co-localizes with Sli15-RFP (red) on microtubules. Bar, 4 μ m.

Figure 5. Comparison of protein interaction maps of Ndc80p/Tid3p phospho-mutants. The protein interaction maps shown here summarize the results of yeast two-hybrid screens performed using wild-type Ndc80p, and phospho-mutants that mimic the phosphorylation and dephosphorylation of serine 100, as baits. The color of the nodes corresponds with their GO Process classification. The protein interaction maps shown here were generated with OSPREY [<http://biodata.mshri.on.ca/osprey>] (Breitkreutz et al., 2003).

Table 1. Notable interactions between spindle proteins and chromatin-associated proteins

Bait Name	Bait Description	Prey Name
SWI/SNF Chromatin remodeling complex interactions		
SPC25	Ndc80 kinetochore complex	SNF6
ESP1	Separase	SNF6
SMC1	Cohesin complex	SWI1
DAD4	Dam1 kinetochore complex	SWI1
CNN1	Kinetochore	SWI1
SPC24	Ndc80 kinetochore complex	SWI1
SPC25	Ndc80 kinetochore complex	SWI1
TID3	Ndc80 kinetochore complex	SWI1
NUF2	Ndc80 kinetochore complex	SWI3
Ino80 chromatin remodeling complex		
MTW1	Mtw1 kinetochore complex	IES3
NNF1	Mtw1 kinetochore complex	NHP10
NUF2	Ndc80 kinetochore complex	NHP10
SAGA histone acetylation		
SPC25	Ndc80 kinetochore complex	ADA2
NKP2	Ctf19 kinetochore complex	AHC2
DAD1	Dam1 kinetochore complex	AHC2
HSK3	Dam1 kinetochore complex	AHC2
CEP3	CBF3 complex	SGF73
SPC25	Ndc80 kinetochore complex	SGF73
APC1	Anaphase-promoting complex	TAF9
RPD3 deacetylase		
SPC25	Ndc80 kinetochore complex	DEP1
NKP2	Ctf19 kinetochore complex	PHO23
DAD4	Dam1 kinetochore complex	PHO23
SPC25	Ndc80 kinetochore complex	PHO23
CEP3	CBF3 complex	RXT3
SPC25	Ndc80 kinetochore complex	SDS3
Other deacetylases		
MCM22	Ctf19 kinetochore complex	CPR1
HSK3	Dam1 kinetochore complex	HDA2
NUF2	Ndc80 kinetochore complex	HDA2
SPC24	Ndc80 kinetochore complex	HDA2
Mediator complex		
CIN8	Kinesin	CSE2
DAD2	Dam1 kinetochore complex	MED11
CIN8	Kinesin	MED4
SPC25	Ndc80 kinetochore complex	MED6
SPC105	Kinetochore	MED7
HSK3	Dam1 kinetochore complex	MED8
NKP2	Ctf19 kinetochore complex	PGD1
DAD1	Dam1 kinetochore complex	PGD1
DAD2	Dam1 kinetochore complex	PGD1
HSK3	Dam1 kinetochore complex	PGD1
MCD1	Cohesin complex	SRB7
NKP2	Ctf19 kinetochore complex	SRB7
DAD4	Dam1 kinetochore complex	SRB7

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Figure 1

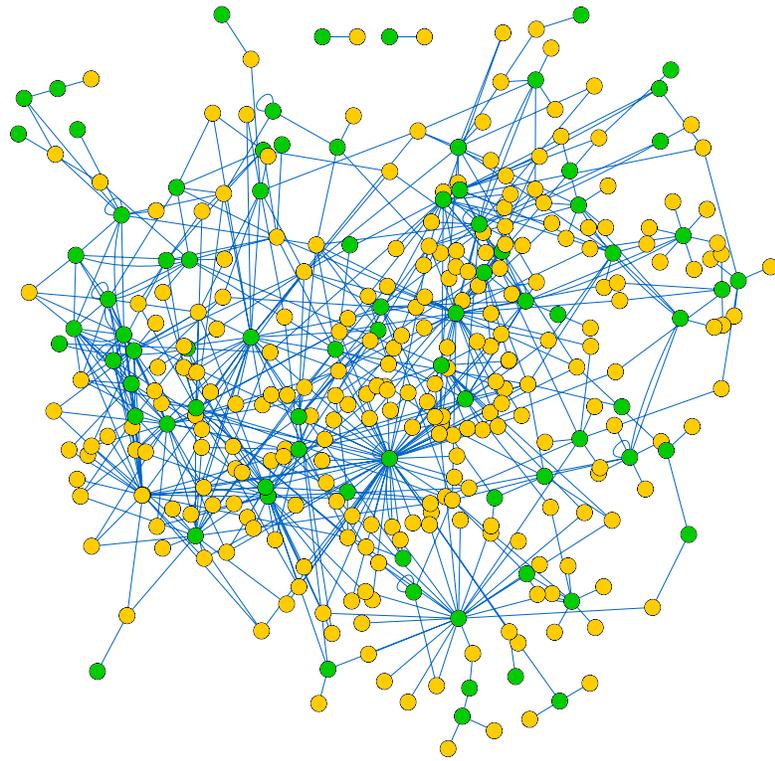


Figure 2

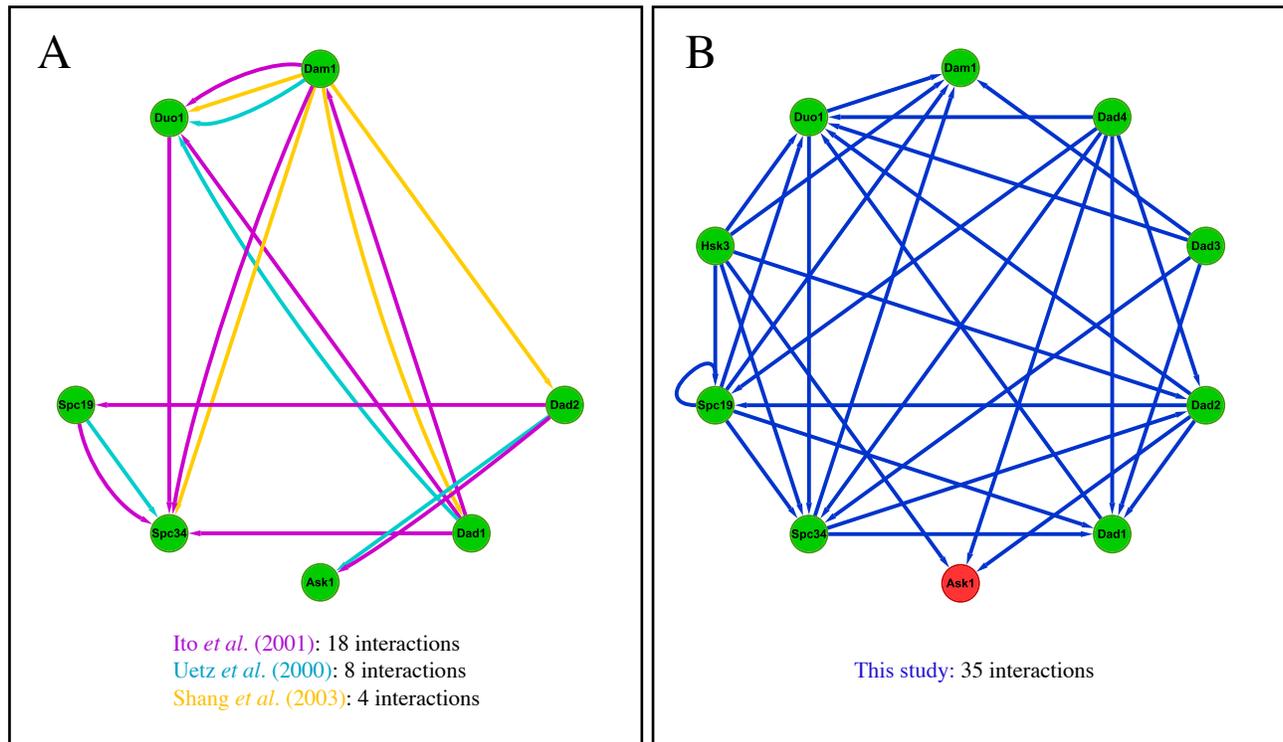


Figure 3

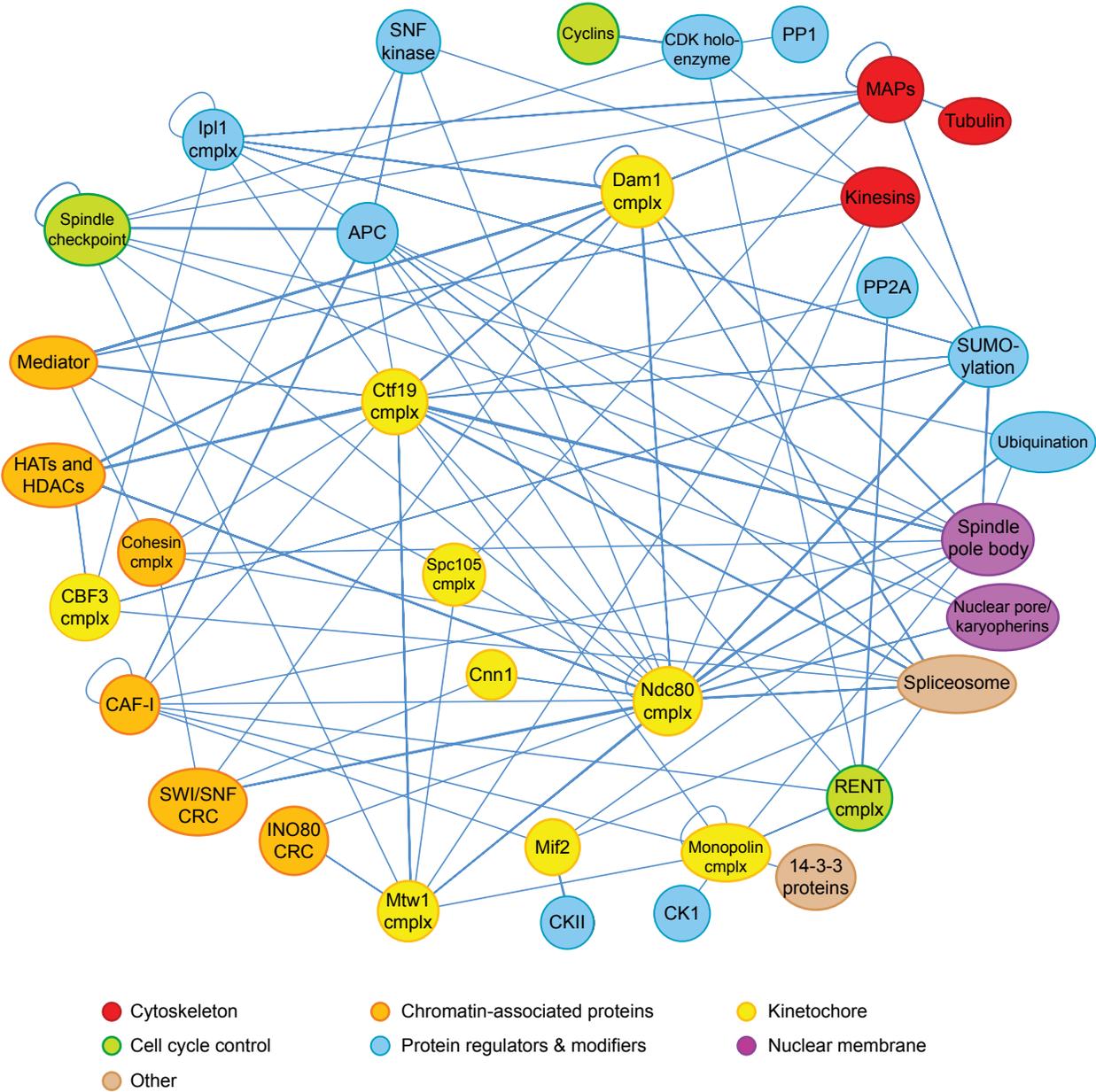


Figure 4

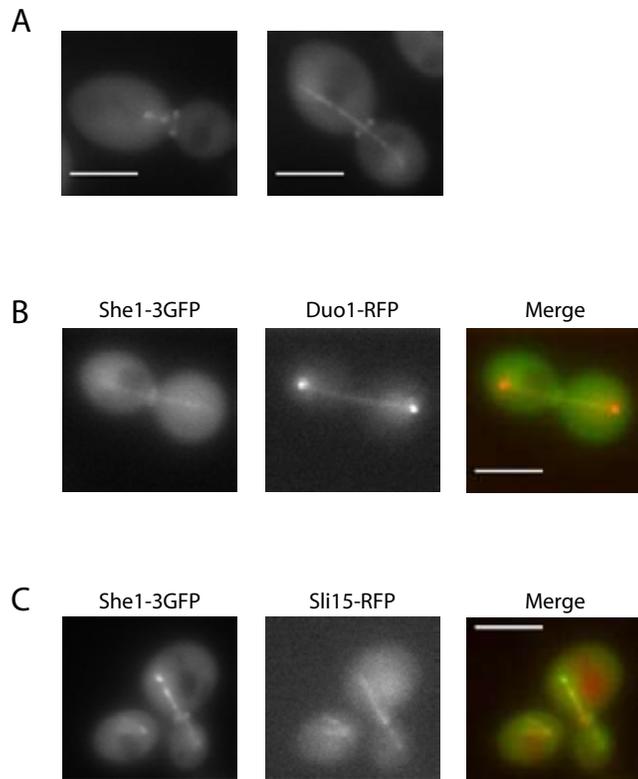


Figure 5

