

# A computationally directed screen identifying interacting coiled coils from *Saccharomyces cerevisiae*

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**Computational methods can frequently identify protein-interaction motifs in otherwise uncharacterized open reading frames. However, the identification of candidate ligands for these motifs (e.g., so that partnering can be determined experimentally in a directed manner) is often beyond the scope of current computational capabilities. One exception is provided by the coiled-coil interaction motif, which consists of two or more  $\alpha$  helices that wrap around each other: the ligands for coiled-coil sequences are generally other coiled-coil sequences, thereby greatly simplifying the motif/ligand recognition problem. Here, we describe a two-step approach to identifying protein–protein interactions mediated by two-stranded coiled coils that occur in *Saccharomyces cerevisiae*. Coiled coils from the yeast genome are first predicted computationally, by using the MULTICOIL program, and associations between coiled coils are then determined experimentally by using the yeast two-hybrid assay. We report 213 unique interactions between 162 putative coiled-coil sequences. We evaluate the resulting interactions, focusing on associations identified between components of the spindle pole body (the yeast centrosome).**

**W**hole-genome sequence information is now available for many organisms. Sequence alignments, pattern recognition algorithms, and empirical protein-folding algorithms, such as comparative structure modeling and threading, are providing valuable initial sequence annotations by finding protein motifs in predicted ORFs (1, 2). A major challenge is to determine, accurately and efficiently, biologically relevant partners for these protein motifs (e.g., the corresponding DNA, RNA, protein, or small-molecule ligands).

Here we report the identification of protein–protein interactions mediated by coiled coils that occur in *Saccharomyces cerevisiae*. Our focus is on two-stranded coiled coils that exist either as homodimers, when they pair with themselves, or as heterodimers, when they pair with different coiled-coil sequences. We use a two-step approach. First, the computer program MULTICOIL (3) is used to predict coiled-coil sequences in the translated genome. Second, interactions between these coiled-coil sequences are determined experimentally, by using the yeast two-hybrid assay (4–7). We use previously published spatial data (8) to further evaluate the observed interactions, focusing on associations between coiled-coil sequences from spindle pole body (SPB) (centrosome) proteins.

Coiled coils are an oligomerization motif consisting of two or more  $\alpha$  helices that wrap around each other with a slight left-handed superhelical twist (9–12). Sequences capable of forming coiled coils are characterized by a heptad repeat pattern, (abc-defg)<sub>n</sub>, in which residues at the **a** and **d** positions are often hydrophobic, and residues at the **e** and **g** positions are predominantly charged or polar (13, 14) (Fig. 1*a*). This repeating pattern, and the large number of known coiled-coil sequences, have led to the development of reliable statistics-based computer programs that recognize coiled-coil sequences (3, 14–16). More recently, computational algorithms have been developed that permit the prediction of coiled-coil structures in atomic detail (17, 18).

Coiled coils are particularly amenable to protein-association studies. First, because coiled-coil sequences generally interact

with other coiled-coil sequences, computational methods can be used to identify both interaction motifs and their candidate ligands. Second, coiled coils are often capable of folding independently (i.e., they are autonomously folding units) (19–21). Third, coiled coils demonstrate interaction specificity *in vitro*, and this specificity corresponds to known interactions *in vivo* (19, 20, 22, 23). Fourth, coiled coils are often capable of mediating specific interactions without the need for additional protein domains (20, 24, 25). Fifth, coiled coils occur in a large number and wide range of proteins of general interest, including structural proteins, motor proteins, transcription factors, and membrane fusion proteins (9, 12, 26, 27).

Our strategy of targeting the coiled-coil motif to identify protein–protein interactions has several advantages over more traditional approaches that use full-length proteins. First, it is more efficient. A targeted strategy identifies a greater number of interactions (i.e., per sequence screened) than a strategy that is not targeted to protein-association motifs. Second, the resulting data immediately identify regions responsible for protein associations. Using this information, one can disrupt a specific interaction without removing the rest of the ORF. For example, the insertion of one or more proline residues can abolish a coiled-coil association (14, 28), and mutations in coiled coils can be responsible for the creation of temperature-sensitive alleles (29, 30). Third, the use of a discrete oligomerization motif likely increases the specificity of a screen by eliminating domains that can interact indiscriminately. Finally, the exclusive use of coiled-coil domains in a screen limits the risk that an interaction will be masked by another protein region (see, for example, refs. 31–33) or will not be detected because a protein is misfolded. For these reasons, we have included in our interaction experiments only those regions of proteins that are predicted to contain coiled-coil sequences.

## Materials and Methods

**Coiled-Coil Prediction.** To estimate the number of full-length proteins in the yeast genome that contain coiled coils, we ran MULTICOIL using default settings, a probability cutoff of 0.01, and a window of 21 residues (3), on the translated yeast genome available at [ftp://genome-ftp.stanford.edu/pub/yeast/yeast\\_ORFs/orf\\_trans.fasta](ftp://genome-ftp.stanford.edu/pub/yeast/yeast_ORFs/orf_trans.fasta). The coiled-coil probabilities of sequences used in our screen (see Table 1, which is published as supplemental data on the PNAS website, [www.pnas.org](http://www.pnas.org)) were computed as described previously (3). As shown in Fig. 1*b*, the coiled-coil probabilities of these sequences are generally  $\geq 0.5$ .

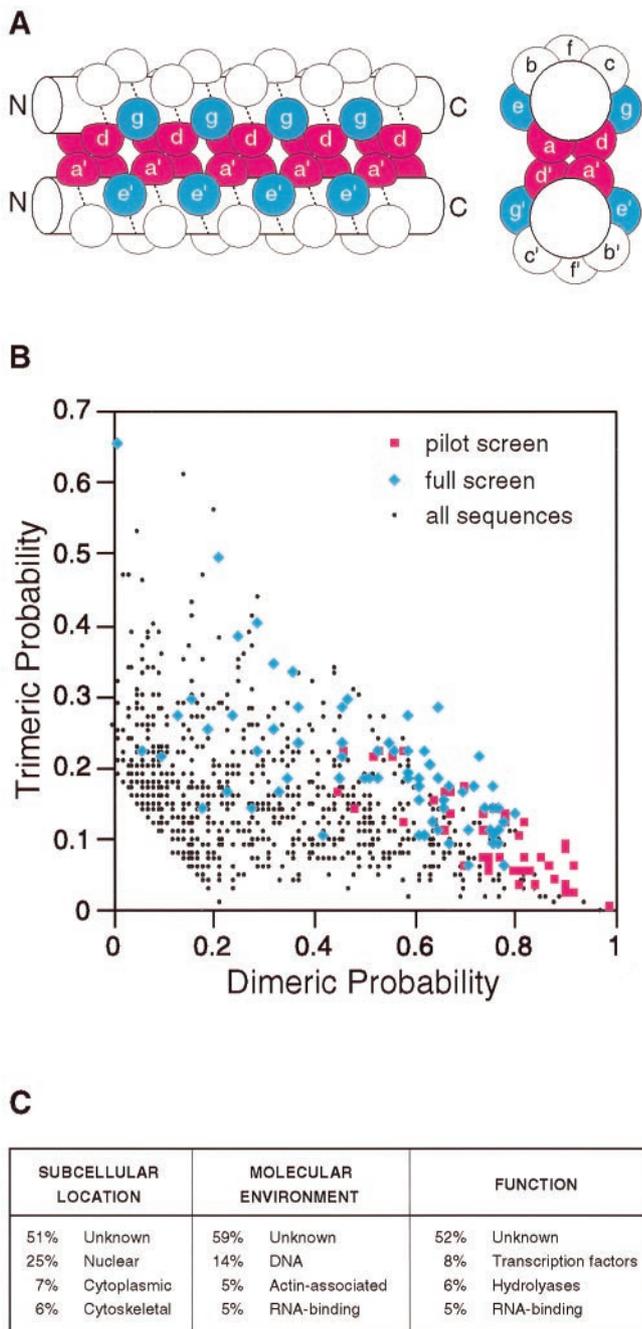
Abbreviations: SPB, spindle pole body; YPD, Yeast Proteome Database.

See commentary on page 12935.

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**Fig. 1.** Coiled coils and the yeast genome. (A) A Schematic representation of a two-stranded coiled coil showing its characteristic heptad repeat (abcdefg)<sub>n</sub>. Residues at the a and d positions (in red) are predominantly hydrophobic, whereas those at the e and g positions (blue) are frequently charged or polar (14). For simplicity, the superhelical twist of the two helices is not depicted. (B) The coiled-coil probabilities of translated yeast ORFs scoring  $\geq 0.2$  (dimer + trimer). The majority of sequences used in the pilot screen (red squares) have high dimeric and low trimeric probabilities. Sequences used in the directed screen (blue diamonds) show more variation. Sequence scores (3) for the remaining translated ORFs are shown as black dots. (C) Properties of translated yeast ORFs that are predicted to contain the two-stranded coiled-coil interaction motif. MULTICOIL identifies 490 sequences in the translated yeast genome with  $\geq 0.2$  probability of forming a two-stranded coiled coil. Data are derived from YPD, Ver. 9.46A (40).

**DNA Cloning and Two-Hybrid Assay.** Primers were designed by using the computer program PRIMER3 ([http://www-genome.wi.mit.edu/ftp/distribution/software/primer3\\_0.5.tar.gz](http://www-genome.wi.mit.edu/ftp/distribution/software/primer3_0.5.tar.gz)). PCR products were

amplified from genomic DNA (S288C) with *Pwo* (Roche Biochemicals) by using the manufacturer's recommended conditions and subcloned into pGAD-C1 and pGBDU-C1 (7) (see Supplementary Material, Table 1, [www.pnas.org](http://www.pnas.org)). All plasmids were sequenced to confirm they contained the desired insert. pGAD constructs were transformed into PJ69-4 $\alpha$ , pGBDU into PJ69-4A (7). Eleven pGBDU constructs are capable of strongly activating the ADE2 reporter gene by themselves: 64, 78, 104, 105, 117, 118, 124, 130, 138, 142, and 153. These constructs were not used further. pGAD and pGBDU plasmids were introduced into the same cell by mating yeast transformants on YPAD plates for 24 h at 30°C. Cells were then replica plated onto -Leu-Ura medium for 48 h and then onto -Leu-Ura-Ade plates (34). The growth of cells on -Leu-Ura-Ade medium was recorded after 4, 8, 12, 16, and 20 days. Typically, the rate of growth from experiment to experiment did not change. Approximately 30% of the colonies required 12 or more days to become visible on selective medium.<sup>‡</sup> To confirm that two plasmids permitted growth under selective conditions, the plasmids were cotransformed into PJ69-4A cells, and growth was monitored as described above. Our use of the ADE2 reporter gene provided the most stringent selection conditions of those we tested (ADE2, HIS3, or LacZ). In addition, the ADE2 reporter gene does not require inhibitors to "tune" the readout (e.g., 3-AT), and it provides a binary output (growth/no growth). The full screen was repeated three times. A comprehensive list of primers and experimental protocols is available from the authors.

**Gene Disruptions.** The single genomic copy of YDL074C in YPH278 was replaced by a copy of the HIS3 gene from *Saccharomyces kluyveri* by using a PCR-based deletion strategy (35). Gene disruption was confirmed by PCR and Southern blotting. Chromosome loss was monitored by using a colony-sectoring assay (36).

**Yeast Strains.** PJ69-4A: MATa trp1-901 leu2-3,112 ura3-52 his3-200 gal4 $\Delta$  gal80 $\Delta$  LYS2::GAL1-HIS3 GAL2-ADE2 met2::GAL7-lacZ (7). PJ69-4 $\alpha$  is isogenic to PJ69-4A but is of opposite mating type (gift of Brian Cali, Whitehead Institute).

## Results

**Identifying Putative Coiled Coils in *S. cerevisiae*.** The single-cell eukaryote *S. cerevisiae* was chosen for this study because its sequence is publicly available and its genome, containing approximately 6,000 translated ORFs, is moderately sized (37). Additionally, *Saccharomyces* is amenable to genetic and biochemical manipulations, and many processes that occur in yeast also occur in larger eukaryotes, making yeast a model system for the study of these organisms (38).

We used the computer program MULTICOIL (3) to predict putative coiled coils in *Saccharomyces*. MULTICOIL compares the pairwise amino acid frequencies in protein sequences to frequencies in known coiled coils and predicts the probability that these sequences will form two-stranded (dimeric) or three-stranded (trimeric) coiled coils. From the individual sequence probabilities predicted by MULTICOIL (3), we estimate there are approximately 300 proteins with two-stranded and 250 proteins with three-stranded coiled coils in yeast (Fig. 1b and legend). We expect 1 in every 11 proteins in *Saccharomyces* to contain a coiled-coil sequence: over half have no known function (Fig. 1c).

**A Pilot Screen.** To assess the feasibility of experimentally determining interactions between putative two-stranded coiled coils, we conducted a pilot screen. First, we identified the top 50

<sup>‡</sup>Structural studies of one coiled coil from a slow-growing strain showed that the sequence [construct 106 (5pc42p), bait and prey] forms a discrete homodimer (J.R.S.N. & P.S.K., unpublished results).

scoring dimeric sequences in *S. cerevisiae*. The 49 proteins that contain these sequences have 68 regions that are predicted to form coiled coils (some proteins have more than one putative coiled-coil sequence). Next, the DNA encoding each region was amplified and cloned into the two-hybrid vectors developed by James *et al.* (7) (Table 1, constructs 1–68), and interactions between these regions were identified by the mating-type two-hybrid assay (4–7).

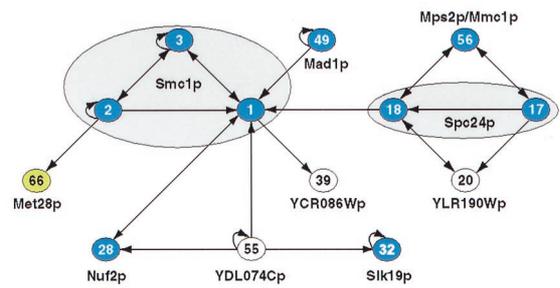
From 4,624 pairwise combinations of bait (DNA-binding-domain fusions) and prey (activation-domain fusions), we identified 42 interactions between 28 different sequences. Twenty of these interactions are homotypic, that is, they occur between identical coiled-coil regions. Three interactions are found between two separate regions of the same protein. The remaining 19 associations involve different proteins.

Sixteen of these nineteen heterotypic interactions form a nexus containing ten proteins (Fig. 2). Remarkably, six of the seven known proteins in the nexus share a common characteristic: they are involved in some aspect of chromosome segregation (Fig. 2 and legend). The seventh protein, Met28p, has no known role in chromosome segregation but does bind Cbf1p, a component of the centromere (39). The remaining three proteins are uncharacterized. Interestingly, however, deletion of the gene encoding one of these proteins (YDL074C), in a haploid strain, results in an approximately 10-fold increase in the rate of chromosome loss (J.R.S.N. and P.S.K., unpublished data), as measured by a colony sectoring assay (36).

**A Directed Screen.** The results from our initial pilot screen led us to perform a more directed screen, expanded to include putative coiled coils thought to be involved in mitotic chromosome segregation. To this end, we searched the Yeast Proteome Database (YPD) (40) under the subject headings mitosis, segregation, spindle pole body, and kinetochore. Next, we used MULTICOIL to predict coiled-coil sequences in these proteins. In this manner, we identified 45 potential coiled coils from 36 proteins to add to the screen. A further ten candidate coiled coils from five proteins (Spc105p, Tid3p, Spc29p, Spc25p, and Spc19p) were added after publication of a characterization of SPB components by Kilmartin, Mann, and coworkers (8). Finally, 39 putative coiled coils from 31 proteins were added to the screen, primarily based on their high MULTICOIL dimer scores, giving a total of 162 coiled-coil regions from 121 proteins, including the proteins used in the pilot screen (Table 1, Supplementary Material, www.pnas.org).

We used these 162 sequences in a mating-type two-hybrid assay and found 213 unique interactions, involving 100 coiled coils from 77 different proteins (Fig. 3 and Table 2, which is published as supplemental data on the PNAS website, www.pnas.org). Thirty-three interactions are homotypic, 5 occur between two different regions of the same protein, and 175 are heterotypic. Of the 77 proteins that interact in our screen, 59 have been previously described in the literature, and 18 are of unknown function. As might be expected given the directed nature of our final screen, over half of the 59 characterized interacting proteins are nuclear and, of these, roughly half are DNA associated.

**Evaluation of Experimental Results.** Protein–protein interactions provide information about the structural organization of cellular complexes and give insight into the functions of uncharacterized or partially characterized proteins. Interaction data obtained by using the two-hybrid assay, however, can be incomplete or inaccurate (see e.g., refs. 33 and 41–43 and below) and should therefore be interpreted with caution. For example, the two-hybrid assay does not provide information on the relative affinity of one coiled-coil strand for another. *In vivo*, coiled-coil strand affinities are likely to play an important role in determining



**Fig. 2.** Coiled-coil interactions identified in a pilot screen predominantly involve proteins that participate in chromosome segregation. Ten proteins included in the pilot screen make up the interaction nexus shown. Construct numbers are shown inside each circle (see Supplementary Material, Table 1, www.pnas.org). Constructs from the same protein are grouped inside gray ovals. Seven of the proteins in the nexus have been characterized: six (in blue) are involved in some aspect of chromosome segregation (40). The seventh protein, Met28p (in green), is not known to play a role in this process but has been shown in other studies to interact with Cbf1p, a protein necessary for proper chromosome segregation (39). Three of the proteins in this nexus (in white) have no known function (see also text). Arrows point toward the DNA-binding domain construct in each interaction. Double-headed arrows denote reciprocal interactions. Circular arrows indicate homotypic interactions.

partnering specificity. Ultimately, protein associations need to be confirmed by independent methods.

In the absence of additional experiments, one potential concern is that some of the associations we find might occur nonspecifically (i.e., are false positives) (25). Three lines of evidence, however, suggest that many of the interactions reported here are specific. First, although every protein region included in our screen is predicted to form an amphipathic  $\alpha$  helix, we detect only one interaction for approximately every 100 pairwise combinations tested. Second, of those putative coiled coils that do interact, most make only one or two interactions in our screen. Third, even minor amino acid changes can disrupt coiled-coil interactions *in vitro* and *in vivo* (22, 23, 44), implying that coiled coils associate with a high degree of specificity.

Indeed, by restricting our analysis to protein regions that are predicted to contain coiled coils, we likely miss a number of interactions: those that require noncoiled-coil domains. This may explain in part why our data recapitulate only 6 of the approximately 25 interactions reported in the YPD (40) between proteins in our screen (see Fig. 3 legend).

In addition, parallel homodimeric coiled coils are not easily detected by the two-hybrid assay. For example, we fail to detect the well-established homodimeric interaction of the Gcn4p coiled coil (11, 19) (construct 156). Most likely, this is because the DNA-binding domain used in the two-hybrid assay (from Gal4p) binds DNA as a dimer (see ref. 45), creating a high effective concentration of the attached coiled-coil sequences. Consequently, bait constructs have a reduced ability to associate, in the two-hybrid assay, with activation-domain constructs (prey) to form two-stranded coiled coils.

The more directed nature of our screen allows us also to overcome some experimental factors that may give rise to false negatives (42). For example, membrane proteins are not well suited for use in the two-hybrid assay, but the exclusive use of coiled-coil domains circumvents this problem. As noted by others (33, 42), mating bait to large pools of prey makes the two-hybrid assay more rapid but discriminates against cells that mate inefficiently, grow slowly, or are otherwise underrepresented when colonies are chosen for sequencing. By restricting our screen to sequences that are predicted to form coiled coils,

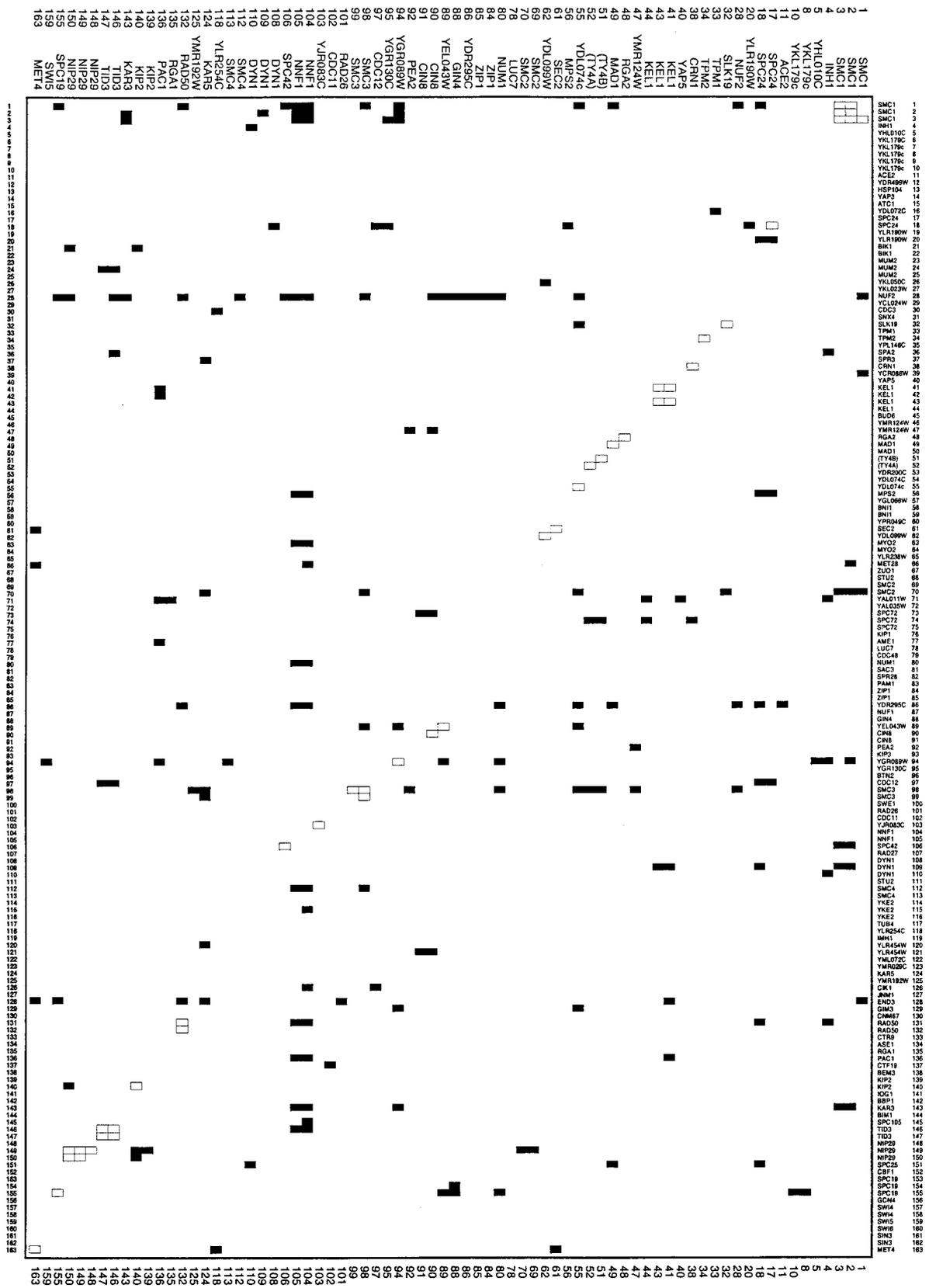


Fig. 3. Coiled-coil interactions identified in this study. pGAD (activation domain) constructs are listed (*Top*, discontinuous numbering), with pGBDU (DNA-binding domain) constructs (*Right*, contiguous numbering) (see also Table 1). The common names of the full-length proteins from which the constructs are derived are also listed. Because of space constraints, pGAD constructs making no interactions have not been included. Homotypic interactions are shown as open boxes, heterotypic interactions as closed boxes (see also Table 2). Although many of these interactions are likely to form

it was logistically feasible to combine bait and prey constructs in a pairwise manner. Additionally, we monitored colony growth for up to 20 days (see *Materials and Methods*), allowing us to identify many interactions that might otherwise have been missed. Our focused but more time-consuming strategy may generally account for the striking observation that none of the interactions reported here have been described in studies utilizing large pools (33, 42).

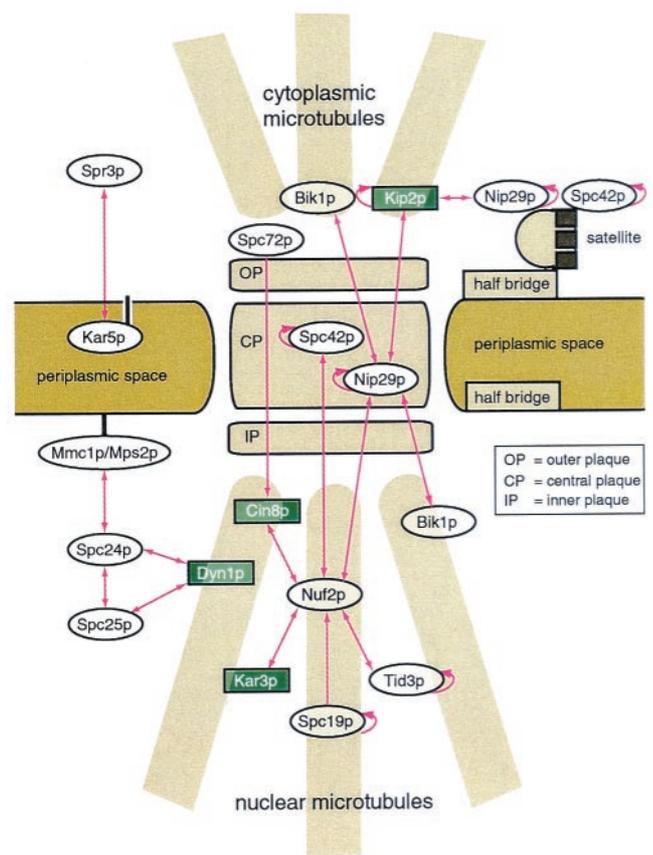
More broadly, although there are limitations to the two-hybrid assay, independent studies (see ref. 42) have shown that the technique can accurately identify protein–protein interactions, and six of the interactions reported here have been documented previously (see Fig. 3 legend) (40). Moreover, all of our plasmids contain the correct insert (see *Materials and Methods*) and are tolerated both by bacteria and yeast [some constructs encoding full-length yeast proteins are toxic (42)]. The reproducibility of our results was verified by repeating the screen three times. Thus, the associations reported here provide attractive targets for further biochemical and genetic studies.

**Coiled Coils at the SPB.** Cytoplasmic (astral) and nuclear (pole-to-pole, kinetochore, or nonkinetochore) microtubules are required for many processes during mitosis, meiosis, and cell fusion. These processes include the movement of chromosomes, nuclei, and organelles. In budding yeast, microtubules emanate from a 300- to 500-MDa complex (30, 46), alternately termed the microtubule-organizing center or the SPB (47). A related complex known as the centrosome is found in larger eukaryotes.

Genetic and biochemical techniques have identified  $\approx 50$  proteins that are either part of the SPB or are closely associated with it (refs. 8 and 40 and refs. therein). Twenty-four of these proteins are predicted by MULTICOIL to contain at least one coiled coil and, with the exception of Spc34p, were represented in our screen. Coiled coils from six of these proteins do not interact with any other sequence in our study. Sequences from the remaining 17 proteins make 51 interactions among themselves (Fig. 4). One of these 51 coiled-coil interactions has been documented previously: the homodimerization of Spc42p (30, 48).

These multiple interactions (Fig. 4) strongly suggest that coiled coils play a major role in organizing the SPB. Indeed, this and other studies indicate that coiled coils may influence several aspects of SPB structure, including assembly [e.g., Spc29p (30)], duplication [e.g., Spc42p (29)], and spatial organization [e.g., Nuf1p/Spc110p (49)]. Proteins containing coiled coils may also assist in anchoring the SPB to the nuclear and cytoplasmic microtubules. For example, coiled coils from six of the seven known microtubule-based motor proteins in yeast were included in our screen: coiled coils from four of these (Dyn1p, Kar3p, Kip2p, and Cin8p) interact with SPB components (Figs. 3 and 4).

Additionally, coiled coils may contribute to the regulation of SPB structure in at least two ways. First, phosphorylation is known to both stabilize and destabilize coiled-coil interactions (50–52). Several SPB components are phosphorylated (8, 53), including Spc42p (see Fig. 4), which is phosphorylated in a cell cycle-dependent manner (29, 30). Second, the coiled coils of Nip29p (constructs 148 and 149) can form both intra- and intermolecular interactions. The transition from one interaction state to another may represent a second method of regulating the SPB structure (see e.g., refs. 24 and 32).



**Fig. 4.** A schematic representation of interacting SPB components. The trilaminar SPB is shown embedded in the nuclear envelope. (*Top*) Cytoplasmic microtubules; (*Bottom*) nuclear microtubules. The half-bridge and satellite structures are depicted to the right of the SPB: both participate in SPB duplication. SPB components that interact via putative coiled coils in this screen are also shown. Protein localization is based on prior immunoelectron microscopy and immunofluorescence studies (8, 30, 46, 48, 56–58). Dyn1p is known to localize to the SPB and to cytoplasmic microtubules (59). As Dyn1p independently contacts two nuclear SPB components in this study, we have also positioned it in the nucleus. For clarity, multiple coiled-coil domains from single proteins have been omitted. Arrowheads point toward DNA-binding domain proteins. Double arrowheads indicate reciprocal interactions. Circular arrows indicate homotypic interactions. The numerous interactions depicted in this figure show how coiled coils may organize the SPB. Interactions between motor proteins (in green) and the SPB provide clues about how the SPB moves and is moved by microtubules.

**Future Direction.** Greater than 5% of all putative ORFs found in sequenced genomes are predicted to contain coiled coils (unpublished data), so many additional interactions between coiled-coil sequences remain to be identified. Although coiled coils are the simplest case for which computational tools are sufficiently sophisticated to identify an interaction motif and a set of candidate ligands, this is changing, and our understanding of other domains is rapidly increasing (see, e.g., refs. 54 and 55). A major challenge that remains is to identify cognate ligands for individual protein motifs. This is currently best done experimentally, and our results indicate that an approach that uses a discrete motif has many potential advan-

two-stranded coiled coils, some may form higher-order oligomers. Additionally, we cannot distinguish parallel from antiparallel coiled coils. Six of the interactions shown here have been previously documented (Smc1p/Smc2p; Smc1p/Smc3p; Met4p/Met28p; Met4p/Met4p; Kel1p/Kel1p; and Spc42p/Spc42p) (40).

tages over techniques that use full-length proteins. In the future, we anticipate that the use of computationally directed approaches will play a pivotal role in the identification of motif/ligand interactions.

**Note Added in Proof.** Recent experiments using imaging techniques (60) suggest that Nuf2p, Spc24p, and Spc25p are localized to the kinetochore (P. K. Sorger, personal communication). Thus, the interactions depicted in Fig. 4 suggest a link between spindle pole body and kinetochore proteins. This new information also provides insights into the Nuf2p, Spc24p, and Spc25p interactions detected in this study (see Table 2). For example, Nuf2p and Spc24p interact not only with microtubule-binding proteins (Cin8p, Kar3p, and Dyn1p), but also with members of the Smc

family. In addition, Spc25p and Smc1p interact with the checkpoint protein Mad1p (see also Fig. 2).

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