The mitotic spindle is required for loading of the DASH complex onto the kinetochore

Yumei Li,[1] Jeff Bachant,[1] Annette A. Alcasabas,[1] Yanchang Wang,[1] Jun Qin,[1,2] and Stephen J. Elledge[1,3,4,5]

[1]Verna and Marrs McLean Department of Biochemistry and Molecular Biology, [2]Department of Cell Biology, [3]Department of Molecular and Human Genetics, [4]Howard Hughes Medical Institute, Baylor College of Medicine, Houston, Texas 77030, USA

A role for the mitotic spindle in the maturation of the kinetochore has not been defined previously. Here we describe the isolation of a novel and conserved essential gene, *ASK1*, from *Saccharomyces cerevisiae* involved in this process. *ask1* mutants display either G_2/M arrest or segregation of DNA masses without the separation of sister chromatids, resulting in massive nondisjunction and broken spindles. Ask1 localizes along mitotic spindles and to kinetochores, and cross-links to centromeric DNA. Microtubules are required for Ask1 binding to kinetochores, and are partially required to maintain its association. We found Ask1 is part of a multisubunit complex, DASH, that contains ~10 components, including several proteins essential for mitosis including Dam1, Duo1, Spc34, Spc19, and Hsk1. The Ipl1 kinase controls the phosphorylation of Dam1 in the DASH complex and may regulate its function. We propose that DASH is a microtubule-binding complex that is transferred to the kinetochore prior to mitosis, thereby defining a new step in kinetochore maturation.

[*Key Words:* Ask1; spindle; kinetochore; chromosome segregation]

Received November 2, 2001; revised version accepted November 27, 2001.

Mitosis is the period of the cell cycle during which chromosomes are segregated to daughter cells through the process of anaphase. The ability to accurately distribute replicated chromosomes is critical for the proper transfer of genetic material from one generation to the next. Errors in this process can lead to developmental defects, cancer, or death. Cells ensure the fidelity of this process through multiple layers of control that operate during mitosis.

The physical movement of chromosomes during anaphase is carried out by the mitotic spindle. The mitotic spindle is a bipolar microtubule-based structure that emanates from two organizing centers, called centrosomes or spindle-pole bodies (SPB), and connects to chromosomes (Hoyt and Geiser 1996; Winey and O'Toole 2001). The mitotic spindle binds to newly replicated chromosomes (called sister chromatids), but chromosome segregation does not take place until cohesion along the sister chromatids is eliminated. The elimination of cohesion is a highly regulated step that is catalyzed by a protease, Esp1, which is activated by the destruction of its inhibitor, Pds1 (Cohen-Fix et al. 1996; Ciosk et al. 1998; Uhlmann et al. 1999).

Anaphase does not occur until all chromosomes form a bipolar attachment with the spindle. A centromere-based mitotic checkpoint system referred to as the spindle checkpoint (Amon 1999) monitors spindle–chromosome interactions and blocks anaphase until all sister chromatids have successfully attached to spindle microtubules in a bipolar fashion (Wang and Burke 1995; Pangilinan and Spencer 1996). In budding yeast, at least six genes, *MAD1–MAD3*, *BUB1*, *BUB3*, and *MPS1* (Hoyt et al. 1991; Li and Murray 1991; Hardwick and Murray 1995; Hardwick et al. 1996; Farr and Hoyt 1998), are required for the spindle checkpoint to prevent anaphase. When active, the spindle checkpoint prevents anaphase by inhibiting the ubiquitin-mediated degradation of anaphase inhibitor Pds1/Cut2 (Cohen-Fix et al. 1996; Funabiki et al. 1996; Yamamoto et al. 1996; Hwang et al 1998; Kim et al. 1998).

How the spindle forms, recognizes, and binds to chromosomes is currently a topic of extensive research. Several proteins are known to be associated with spindles and play important functions in spindle assembly and dynamics. These proteins include motor proteins, such

[5]Corresponding author.
E-MAIL seledge@bcm.tmc.edu; FAX (713) 798-8717.
Article and publication are at http://www.genesdev.org/cgi/doi/10.1101/gad.959402.

as Kar3, Kip1, and Cin8 (Hoyt et al. 1992; Roof et al. 1992; Saunders and Hoyt 1992), and other microtubule associated proteins, such as Ase1 (Pellman et al. 1995) and Esp1 (Jensen et al. 2001).

The spindle microtubules attach to chromosomes through the kinetochore, a specialized structure consisting of the centromeric DNA and its associated proteins (Lechner and Ortiz 1996; Pidoux and Allshire 2000). In budding yeast, the centromere (*CEN*) is a short sequence consisting of three well-conserved elements important for centromere function, CDEI, CDEII, and CDEIII. CDEIII and at least part of CDEII are indispensable for centromere function (Hegemann and Fleig 1993). Several kinetochore proteins have been identified that bind to a specific centromere region. Cbf1 binds to CDEI and is involved in the fidelity of chromosome transmission (Cai and Davis 1990). Ndc10 (Doheny et al. 1993; Goh and Kilmartin 1993; Jiang et al. 1993), Ctf13 (Doheny et al. 1993), Cep3 (Lechner 1994; Strunnikov et al. 1995), and Skp1 (Connelly and Hieter 1996) bind to CDEIII as a multisubunit protein complex (CBF3). In addition, Cse4 (Meluh et al. 1998), a homolog of CENP-A, is hypothesized to be involved in the formation of the specialized centromere nucleosome. Mif2 (Meluh and Koshland 1995, 1997), a homolog of CENP-C, interacts with *CEN* DNA in a CDEIII-dependent manner. Other proteins, such as Ctf19, Mcm21, and Okp1 are also important for the formation of the kinetochore (Hyland et al. 1999; Ortiz et al. 1999).

Once the kinetochore is attached by microtubules, it is likely that mechanisms exist to alter its properties. One piece of evidence supporting this hypothesis is the presence of a particular phosphorylation event recognized by the 3F3/2 antibody localized specifically to the kinetochores of unattached chromosomes (Gorbsky and Ricketts 1993). Further evidence comes from the presence of the Mad2 protein on these same chromosomes, presumably signaling to prevent anaphase entry (Chen et al. 1996). It is unknown how the kinetochore is altered by microtubule attachment. Presumably, there is a signaling event, possibly a structural change, that alters the kinetochore once a microtubule is bound. Other changes may also accompany binding such as strengthening of the attachment itself pursuant to segregation and possibly signaling from the kinetochore to the spindle to regulate spindle dynamics in *cis*. In this study, we describe the identification of a novel protein, Ask1 (associated with spindles and kinetochores), required for maintaining proper kinetochore-microtubule attachments and chromosome segregation. Our data suggests that Ask1 initially binds to microtubules and subsequently binds kinetochores upon microtubule attachment during S phase, when new kinetochores are assembled. We discovered that Ask1 is part of a complex we call DASH that contains multiple essential proteins. The Ipl1 kinase controls the phosphorylation of a component of the DASH complex, Dam1, and may regulate its function. Thus, the DASH complex may alter the property of the kinetochore to allow it to achieve its mature spindle-bound form.

Results

The ask1-1 *mutant is defective in maintaining spindle integrity in HU*

To identify genes involved in the restraint of anaphase entry during S-phase arrest, a genetic screen was conducted to search for mutants that have an elongated spindle phenotype in the presence of hydroxyurea (HU), an inhibitor of DNA replication. Whereas wild-type cells arrested with HU show short spindles, mutants isolated from the screen display elongated spindles in the presence of HU (Alcasabas et al. 2001). Among several mutants isolated from the screen was *ask1-1*. *ask1-1* mutant cells failed to grow on plates containing 150 mM HU (Fig. 1A). Cells lost viability over time when incubated in HU, indicating that they were undergoing a catastrophic event (Fig. 1B). To investigate whether *ASK1* is involved in the replication checkpoint pathway, we examined two well-characterized checkpoint responses in *ask1-1* mutant cells, Rad53 hyperphosphorylation and *RNR3* transcriptional induction (Elledge and Davis 1990; Sanchez et al. 1996). To examine Rad53 phosphorylation, extracts of wild-type or *ask1-1* mutants were prepared from cells treated with 200 mM HU or 0.5% MMS, and Western blotting was carried out using anti-Rad53 antibodies. In both wild-type and *ask1-1* mutant cells, modification of Rad53 was observed upon HU or MMS treatment (Fig. 1C). The transcriptional induction of *RNR3* was also examined under similar conditions and found to be fully intact (data not shown). Thus, we were unable to establish a connection between Ask1 and known components of the replication checkpoint pathway, indicating that Ask1 is either a downstream effector in this pathway or that Ask1 is part of an independent mechanism required for the maintenance of spindle integrity during a prolonged S-phase arrest.

ASK1 *is an essential gene encoding a novel protein*

ASK1 (YKL052C) was cloned by complementing the HU sensitivity of the *ask1-1* mutants. It encodes a protein of 293 amino acids with no obvious conserved motifs. Database analysis identified sequence homologs present in *Schizosaccharomyces pombe* (SPBC27.02c) and *Drosophila melanogaster* (Fig. 1D). Deletion of *ASK1* was performed in diploid cells by gene replacement. Tetrad dissection revealed that the *ask1* null mutant is inviable and is genetically linked to the original *ask1-1* mutation (data not shown).

To investigate the function of *ASK1*, conditional alleles of *ASK1* were generated by PCR mutagenesis. Six temperature-sensitive alleles were isolated and used to replace the endogenous *ASK1* gene. The phenotypes of these mutants were investigated by use of synchronous cultures. Cells were arrested at G_1 with α-factor at 25°C, and then shifted to 37°C for 30 min before release from the block. On the basis of DNA and spindle morphologies, the six temperature-sensitive alleles can be divided into two classes. One class contains a single allele, *ask1-2*, which arrested as large budded cells with a 2N DNA

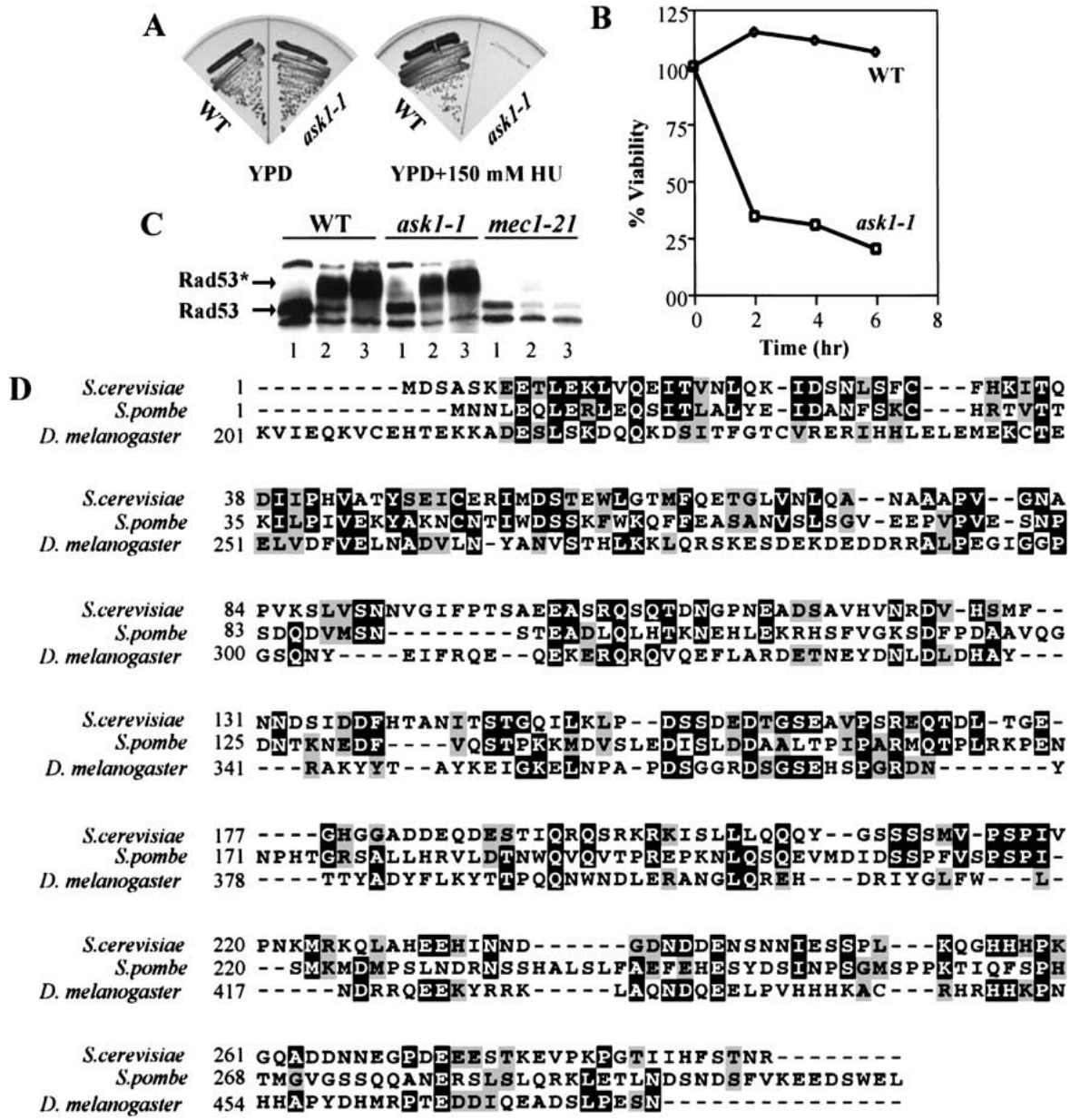

Figure 1. Characterization of *ask1-1* mutant cells. (*A*) *ask1-1* mutants are HU sensitive. Wild-type (Y300) and *ask1-1* (Y928) mutants were streaked on YPD plates containing 150 mM HU at 30°C and incubated for 3 d. (*B*) *ask1-1* loses viability in HU. α-factor arrested wild-type (Y300) and *ask1-1* (Y928) mutant cells were released into YPD containing 200 mM HU for the indicated times and plated on YPD at 30°C to determine survival. (*C*) Proper regulation of Rad53 in *ask1-1* mutants. Log-phase cells of wild-type (Y300), *ask1-1* (Y928), and *mec1-21* (Y604) were treated with 200 mM HU (lane *2*) or 0.5% MMS (lane *3*) for 1.5 h at 30°C. Protein extracts were prepared, separated by SDS/PAGE, and immunoblotted with antibodies against Rad53. (Lane *1*) Untreated control. (*D*) Alignment of *S. cerevisiae* Ask1 protein with its homologs from *S. pombe* and *D. melanogaster*. Identical residues are highlighted in black and conserved residues in gray.

content, undivided nuclei, and short spindles (Fig. 2A). These mutant cells maintained their viability when incubated at 37°C (data not shown). The remaining temperature-sensitive alleles fall into the second class, whose representative mutant is *ask1-3*. When *ask1-3* mutant cells were released from G_1 arrest at the restrictive temperature (35°C), they arrested temporarily with a 2N DNA content for ~120 min before some G_1 cells reappeared (Fig. 2B). Cells analyzed at the 90-min time point showed unusually stretched DNA masses and an abnormal spindle morphology — the microtubule staining between the two SPBs was greatly reduced and the SPBs are further apart relative to normal preanaphase SPBs (Fig. 2B). Meanwhile, the viability of *ask1-3* mutant cells began to decline at 60 min after release (data not shown).

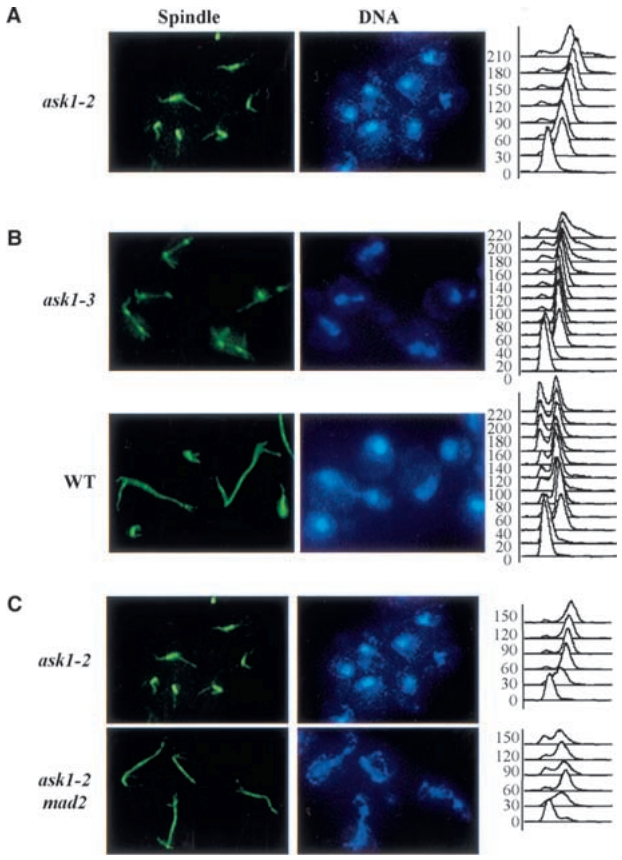

Figure 2. Characterization of temperature-sensitive alleles of *ASK1*. (*A*) *ask1-2* mutant cells arrest before anaphase entry. *ask1-2* mutant cells (Y1104) were synchronized by α-factor and released into YPD at 37°C. (*Left*) The DNA and spindle morphology of *ask1-2* cells at 90 min following the release. (*Right*) The DNA content determined by FACS analysis. (*B*) *ask1-3* mutants display unusual spindle phenotypes at the restrictive temperature. Wild-type (Y300) and *ask1-3* (Y1103) mutant cells were synchronized by α-factor and released at 35°C. At the indicated times after the release, aliquots were withdrawn to examine viability, DNA content, and nuclear spindle morphology. Photomicrographs show wild-type and *ask1-3* mutant cells at 90 min after release. (*C*) *ask1-2* (Y1104) and *ask1-2 mad2* (Y1106) mutant cells were synchronized and released as in *A*. Aliquots were collected for FACS analysis, DNA, and spindle staining at the indicated times. Photomicrographs were taken of cells at 90 min after the release.

The spindle checkpoint is activated in ask1-2 *mutants*

Anaphase entry is tightly regulated by checkpoints. Activation of either the DNA damage checkpoint or the spindle checkpoint leads to preanaphase arrest. To determine the cause of arrest in *ask1-2* mutants, double mutants of *ask1-2* with either *rad9* or *mad2* were constructed. *RAD9* is a key component of DNA damage checkpoint, whereas *MAD2* is required for the spindle checkpoint pathway. In synchronous cultures, *ask1-2 rad9* mutant cells arrested as large budded cells with a 2N DNA content at the restrictive temperature (data not shown), indicating that *ask1-2* mutants do not require the DNA damage checkpoint for arrest. In contrast, *ask1-2 mad2* double mutants failed to arrest and attempted to segregate chromosomes with elongated spindles (Fig. 2C). Thus, *ask1-2* mutants activate the spindle checkpoint to cause a preanaphase arrest.

ask1-3 *mutants undergo massive nondisjuction without sister chromatid separation*

The *ask1-3* class of mutants appear to be more severe than *ask1-2* mutants and would therefore be expected to also activate the spindle checkpoint. Yet, *ask1-3* mutants appear to segregate chromosomes and eventually exit mitosis (Fig. 2B, 220-min timepoint). To explore this hypothesis, we examined several aspects of spindle checkpoint function in *ask1-3* mutants including the phenotypic consequences of *mad2* mutants, the degradation of Pds1, and sister chromatid segregation.

To examine the effects of *mad2* on the phenotype of *ask1-3* mutants, the *ask1-3 mad2* double mutant was constructed and its cell cycle progression was examined in synchronous cultures at 35°C. *ask1-3* mutant cells displayed delayed mitotic progression. Cells with 1N DNA content did not reappear, even at 150 min after G_1 release. However, *ask1-3 mad2* double mutant cells progressed through mitosis without delay (FACS, Fig. 3A) and G_1 cells reappeared around 90 min after release, similar to *mad2* cells. In addition, the abnormal spindle defects evident in *ask1-3* mutants were no longer observed in the double mutants (Fig. 3A), suggesting that the spindle checkpoint is also active in *ask1-3* mutants and has a profound effect on the spindle phenotype of these cells. However, unequal distribution of chromatin was still evident in these *ask1-3 mad2* cells, indicating a defect in anaphase.

Pds1 is an inhibitor of Esp1, the protease that cleaves the Mcd1/Scc1 cohesin subunit. Pds1 is an ubiquitylation substrate of the anaphase promoting complex (APC) and its degradation is required for cells to enter anaphase. Both wild-type and *ask1-3* mutant cells carrying Pds1–18xMyc were analyzed for Pds1 protein levels. Cells were arrested at G_1, shifted to 35°C for 30 min, and then released. Fifty-five minutes after the release, α-factor was added back to prevent cells from entering the next cell cycle. In wild-type cells, Pds1 levels increased 30 min after release, and declined by 75 min. In *ask1-3* mutant cells, Pds1 levels were maintained at high levels throughout the experiment, indicating that Pds1 was stabilized in *ask1-3* mutant cells, presumably due to the activation of spindle checkpoint (Fig. 3B).

If Pds1 levels remain high through activation of the spindle checkpoint, then cohesion should also be maintained unless *ASK1* plays a role in the cohesion process itself. To address this, we followed sister-chromatid separation using GFP-marked chromosomes (Michaelis et al. 1997). α-factor arrested wild-type, *ask1-3*, and *ask1-3 mad2* mutant cells were followed over time after release at 35°C. In both wild-type and *ask1-3 mad2* cells, sister-chromatid separation began between 60 and 90 min after release. However, in *ask1-3* mutants, despite the segregated DNA masses, sister chromatids failed to

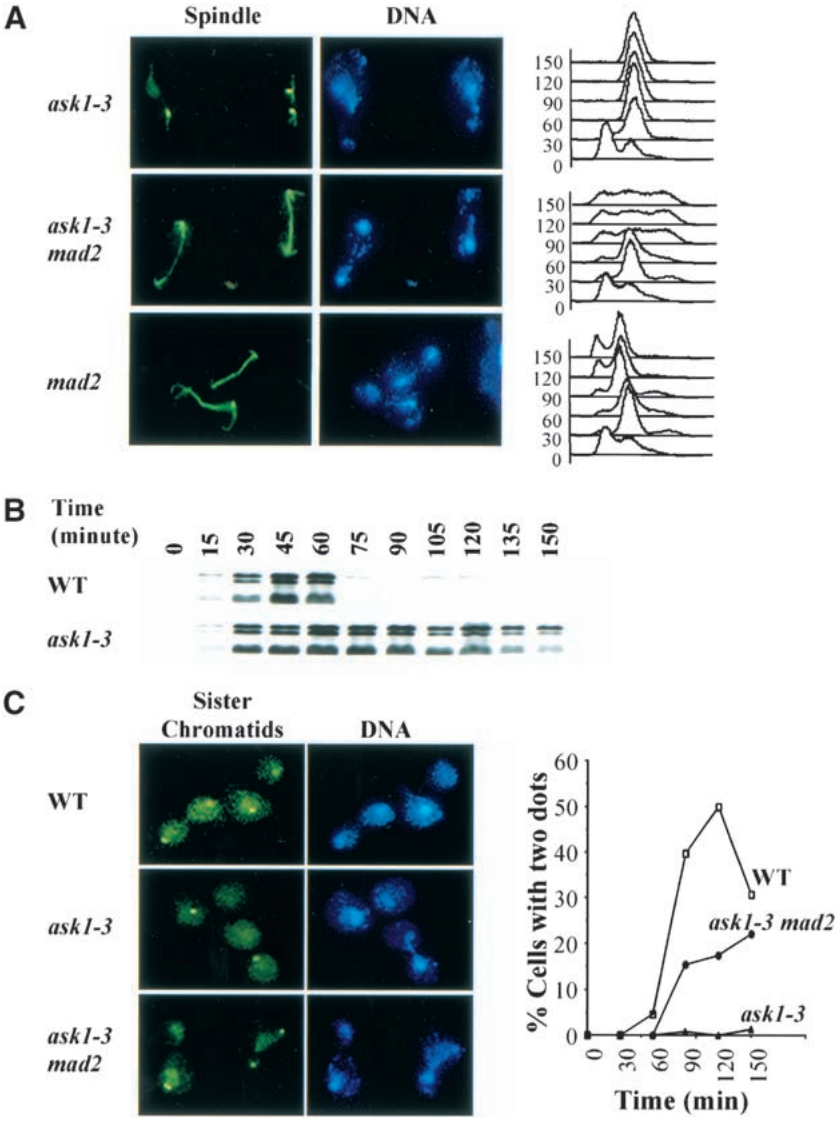

Figure 3. *ask1-3* mutants undergo massive nondisjunction and segregate chromosomes without the separation of sister chromatids. (*A*) The spindle checkpoint is active in *ask1-3* mutants. *ask1-3* (Y1103), *ask1-3 mad2* (Y1107), and *mad2* (Y1101) mutants were arrested in G_1 with α-factor and then released at 35°C. Ninety minutes after the release, samples were collected and stained with DAPI (DNA) and anti-tubulin antibodies (spindles). (*B*) Pds1 is stabilized in *ask1-3* mutants. Wild-type (Y998) and *ask1-3* mutant (Y1108) cells carrying the *PDS1* gene fused to 18 copies of the myc epitope were synchronized with α-factor at 24°C and then released at 35°C. Cells were harvested every 15 min and processed for protein preparation. Pds1 levels were determined by Western blotting using anti-Myc antibodies. (*C*) Analysis of sister-chromatid separation in arrested *ask1-3* mutant cells. Wild-type (Y974), *ask1-3* mutant (Y1109), and *ask1-3 mad2* (Y1110) cells containing a Tet–GFP fusion and tandem repeats of the Tet operator integrated at the *URA3* locus 35 kb away from centromere on the left arm of ChrV were synchronized by α-factor at 24°C and then released at 35°C. Ninety minutes after release, samples were collected to examine sister chromatid separation by immunofluorescence microscopy.

separate even at 150 min after release (Fig. 3C). Thus, *ask1-3* mutant cells undergo premature segregation of chromosome without the separation of sister chromatids, leading to massive nondisjunction. In addition, in *ask1-3 mad2* cells, although sister chromatids separated, 60% of the time both sisters remained in one cell body and failed to segregate. This form of nondisjunction is consistent with a defect in bipolar spindle attachment.

Ask1 associates with centromeres in vivo

Cellular localization is often indicative of protein function. Ask1 was tagged with GFP at its carboxyl terminus and integrated at the *ASK1* locus of wild-type cells. Indirect immunofluorescence microscopy was performed using DAPI, antibodies against GFP, and tubulin to visualize DNA, Ask1p, and spindles, respectively. A dot-like GFP signal was clearly seen in cells of all cell cycle stages. A single dot was seen in G_1 cells. Later, when cells set up and elongated the spindles, two dots were observed in the vicinity of the spindle pole bodies. Ask1 staining was also found along the mitotic spindle in pre-anaphase and during anaphase B (Fig. 4A). The same result was obtained using strains in which Ask1 was tagged with nine copies of the Myc-epitope at its carboxyl terminus (data not shown).

During mitosis, centromeres of sister chromatids transiently separate toward the opposite ends of spindles and localize very close to the SPBs (Goshima and Yanagida 2000; He et al. 2000). To examine whether Ask1 is localized at the SPB or the kinetochore, the localization of Ask1 was examined in a *ndc10-1* mutant background. Ndc10 is an essential kinetochore component. *ndc10-1* mutants elongate spindles without segregating the chromosomes. Cells were grown to mid-log phase at 24°C and synchronized in G_1 using α-factor. After release at 35°C, cells were collected for immunofluorescence staining. In *ndc10-1* mutants, the staining of Ask1 along

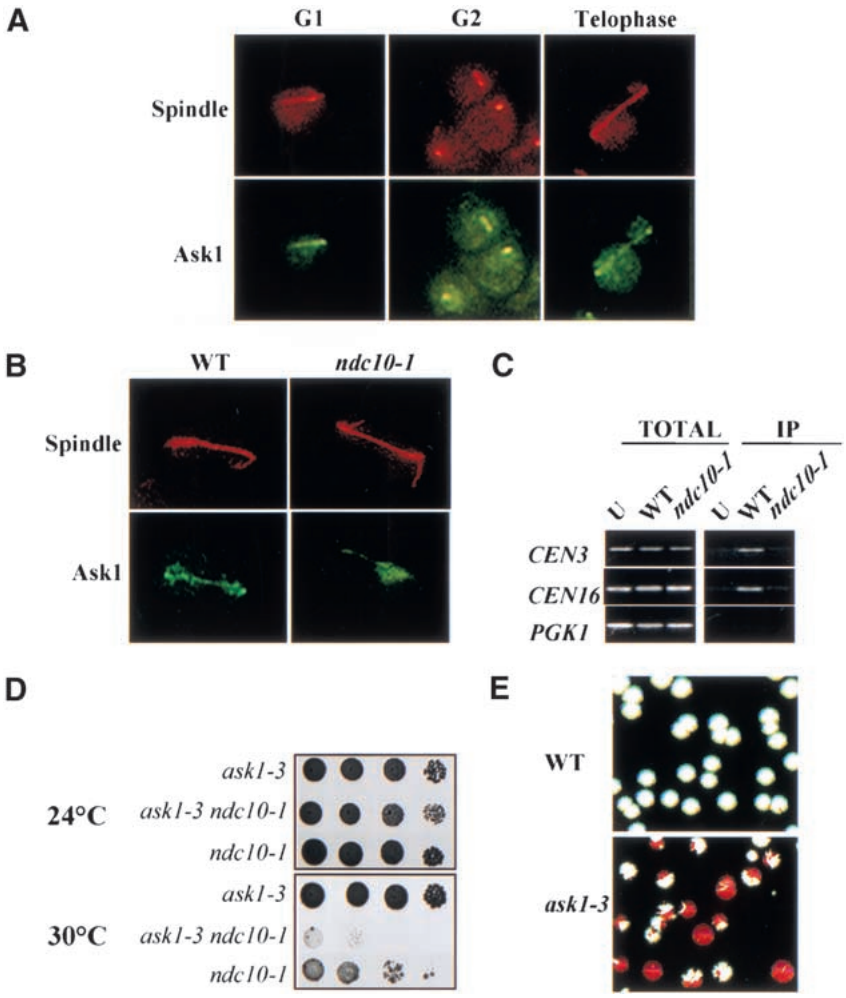

Figure 4. Ask1 is a centromere binding protein. (*A*) Ask1 localizes to the mitotic spindle and spindle-pole bodies. Cells carrying an integrated *ASK1–GFP* gene (Y1111) were synchronized in G_1 with α-factor and released at 30°C. Samples at different stages of the cell cycle were fixed with 5% formaldehyde for 30 min before immunofluorescence staining. (*B*) Localization of Ask1 in wild-type and *ndc10-1* mutants. G_1-arrested wild-type (Y1111) and *ndc10-1* (Y1112) cells containing Ask1–GFP were released into 35°C for immunofluorescence microscopy. Representative telophase cells are shown. (*C*) Chromatin immunoprecipitation analysis (ChIP) of Ask1–Myc in wild-type (Y1113) and *ndc10-1* (Y1115) cells. Untagged wild-type (Y300) cells (U) are included as control. Log-phase cultures were shifted to 35°C for 3 h before collecting samples. (*D*) Synthetic growth defects in *ask1-3 ndc10-1* double mutants (Y1120). Tenfold dilutions of cells were spotted on plates incubated at 24°C and 30°C. (*E*) *ask1-3* mutants exhibit elevated rates of chromosome loss. Wild-type (Y1117) or *ask1-3* mutant (Y1118) strains harboring CFIII were grown in SC-uracil medium at 24°C and then plated out on YPD plates at 32°C and incubated for 4 d. Red sectors represent chromosome loss events.

the spindle was still present, however, the dot-like SPB localization was no longer observed in telophase cells; instead, there was a diffuse nuclear staining (Fig. 4B). Thus, the dot-like localization of Ask1 depended on Ndc10 and raised the possibility that Ask1 associates with the kinetochore.

To test this hypothesis, we used the chromatin immunoprecipitation (ChIP) assay. Formadehyde cross-linked chromatin prepared from *ASK1–MYC* and untagged control strains were sonicated and immunoprecipitated with anti-Myc antibodies. The DNA bound to Ask1 was analyzed by PCR using specific primers. Two *CEN* DNA sequences were tested, *CEN3* and *CEN16*, and both were found enriched in the Ask1–Myc immunoprecipitates, but not in the untagged control strain. The noncentromeric region *PGK1* was analyzed as control and was not found in the Ask1–Myc immunoprecipitates (Fig. 4C). This suggested that Ask1 specifically associates with centromeres. Because the dot-like localization of Ask1 depends on Ndc10, we also examined the centromere binding of Ask1 in the *ndc10-1* mutant background. Untagged control, wild-type *ASK1–MYC* and *ndc10-1 ASK1–MYC* strains were shifted to 35°C for 3 h before the collection of samples. ChIP analysis revealed that the centromere binding of Ask1 is significantly decreased in *ndc10-1* mutants (Fig. 4C). Consistent with the centromere binding of Ask1, *ask1-3* displayed a synthetic, enhanced temperature sensitivity phenotype with *ndc10-1* at 30°C (Fig. 4D).

Given the dramatic nondisjunction phenotypes of *ask1-3* mutants at its nonpermissive temperature, we anticipated that *ask1* mutants would display a significant chromosome loss phenotype. To test this, we examined chromosome segregation in *ask1-3* mutants with a colony color sectoring assay (Spencer et al. 1990). The strains analyzed harbored a mini-chromosome CFIII carrying *SUP11* that suppresses the *ade2-101* mutation present in the cells. In the absence of *SUP11*, the *ade2-101* mutation causes the accumulation of a red pigment. Loss of CFIII therefore results in a red sector. In wild-type cells, the mini-chromosome is stable and colonies appear white. However, in *ask1-3* mutant cells, highly red-sectored colonies appear at semipermissive temperatures 30°C and 32°C (Fig. 4E).

The centromere binding of Ask1 is microtubule dependent

Because Ask1 associated with both the spindles and the kinetochore, it raised the possibility that the kineto-

chore association might be dependent upon the spindle. To examine this, we carried out ChIP analysis in the presence of nocodazole, a microtubule depolymerization reagent. To eliminate cell cycle stage differences between experimental and control samples, a *cdc13-1 ASK1–MYC* strain was used in these experiments because *cdc13-1* arrested at preanaphase. Cells were arrested at G_1 with α-factor and released at 32°C in the presence of nocodazole. Seventy minutes after release, cells reached G_2 and were collected. We found the centromere binding of Ask1 was significantly diminished (10-fold reduction) in cells entering G_2 phase of the cell cycle in the presence of nocodazole (Fig. 5A,F,G). The same result was also observed in wild-type cells releasing from G_1 arrest into nocodazole (data not shown). Titration of the template DNA indicated that the PCR amplification was within the linear range (Fig. 5D).

In addition to depolymerizing microtubules, nocodazole treatment also activates the spindle checkpoint pathway. To distinguish whether the decrease in centromere binding is due to the loss of microtubules or to the activation of the spindle checkpoint, *MAD2* was deleted

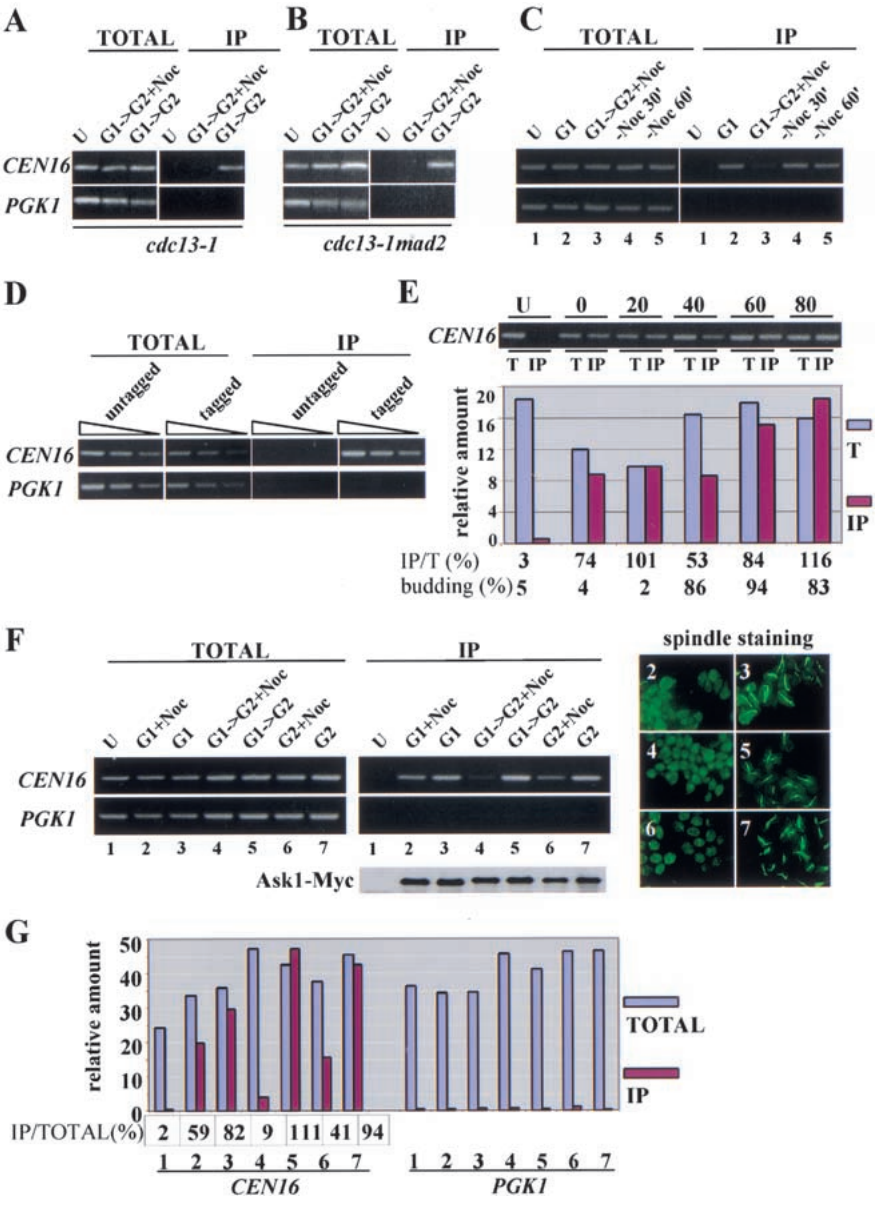

Figure 5. The association of Ask1 to the centromere requires intact microtubules. (*A*) Ask1 centromeric binding is inhibited by nocodazole. α-factor-arrested *cdc13-1 ASK1–MYC* (Y1114) cells were released into prewarmed YPD with (G_1–G_2 + Noc) or without (G_1–G_2) nocodazole at 32°C. Seventy minutes later, samples were collected for ChIP analysis. Untagged wild-type cells are included as a control (U). (*B*) Depolymerization of microtubules, not the activation of the spindle checkpoint, blocks the loading of Ask1 to centromeric DNA. G_1-arrested *cdc13-1 mad2* (Y1116) cells containing Ask1–Myc were released into medium containing nocodazole for 70 min and processed for ChIP analysis as above. Untagged wild-type cells are included as a control (U). (*C*) The nocodazole-dependent centromere binding of Ask1 is reversible. α-factor-arrested *cdc13-1 ASK1–MYC* (Y1114) cells (G_1) were released into prewarmed YPD with nocodazole at 32°C (G_1–G_2 + Noc). Seventy minutes later, cells were spun down and resuspended in fresh YPD at 32°C to remove nocodazole. Samples were collected at 30-min intervals for ChIP analysis (–Noc 30′, –Noc 60′). Untagged wild-type cells are included as a control (U). (*D*) Control showing that the PCR reactions are in the linear range. Twofold serial dilutions of the precipitated and input samples used in lanes *1* and *7* in *F* were subjected to PCR. (*E*) The centromere binding of Ask1 in the cell cycle. *ASK1–MYC* (Y1113) cells were arrested at G_1 with α-factor and then released at 30°C. Samples were collected every 20 min for ChIP analysis. DNAs from total (T) and immunoprecipitaed (IP) samples were analyzed and the PCR products were quantitated using NIH image software. Percent budding for the samples is shown below. (*F*) The centromere binding of Ask1 in G_1 and G_2-arrested cells is less sensitive to nocodazole treatment. *cdc13-1 ASK1–MYC* (Y1114) cells were arrested in G_1 with α-factor or in G_2 by incubating at 32°C before nocodazole was added. Seventy minutes later, samples were collected for ChIP analysis. Cells that were released from G_1 to G_2 were included as a control (lanes *4*,*5*). (*Bottom*) The amounts of Ask1–Myc protein immunoprecipitated as determined by Western analysis. (*Right*) The control showing that nocodazole treatment destabilized microtubules. Shown here are anti-tubulin immunoflourescence images of cells used to prepare the DNA used in lanes *2*–*7*. (*G*) Quantification of the PCR reactions in *F*. PCR products in *F* were run on a 3% agarose gel, visualized with ethidium bromide staining, and scanned for quantitation using NIH Image software.

from the strain above and ChIP analysis was carried out. When *cdc13-1 mad2* cells were released from G_1 to 32°C in the presence of nocodazole, the centromere binding of Ask1 also decreased (Fig. 5B), indicating that the failure in Ask1 binding to centromeric DNA in the presence of nocodazole is not due to the activation of the spindle checkpoint, but due to the loss of microtubules. We conclude that the mitotic spindle is required for Ask1 binding to the centromere.

Nocodazole does not permanently inactivate the Ask1 protein because the nocodazole-dependent interference with Ask1's centromeric association is reversible. When nocodazole was removed from these cells and spindles are allowed to form and be captured by the kinetochore, Ask1 resumed its centromeric association (Fig. 5C). This provides further support for the notion that microtubules are required for the binding of Ask1 to the centromere.

To gather insight into the nature of Ask1's centromeric association, we examined this association through the cell cycle. Using synchronous cultures, we found that Ask1 remained centromere associated throughout the cycle but showed a twofold reduction as cells entered S phase (Fig. 5E). We further examined its association in cells arrested in different cell cycle stages and the effects of nocodazole during these arrests. We found that in G_1 and G_2-arrested cells, Ask1 bound centromeric DNA. However, treatment of these cells with nocodazloe resulted in significantly less interference, 59% and 41% residual binding, respectively (Fig. 5F,G) than observed when cells were traversing S phase in the presence of nodcodazole (Fig. 5F,G). These differences are not due to different amounts of Ask1 protein immunoprecipitated under different conditions (Fig. 5F). These data suggest that either nocodazole is less effective in G_1 or G_2-arrested cells, or that traversing S phase in the presence of nocodazole causes a greater sensitivity. Because nocodazole appears to be equally effective at disrupting microtubule staining under each of these conditions (Fig. 5F), the former explanation is unlikely. A plausible explanation is that kinetochore structures are disassembled as replication forks replicate centromeric DNA. After replication, kinetochores must be reassembled and reattach to the spindle to load Ask1. Thus, S phase might constitute a highly nocodazole-sensitive period and is consistent with the slight reduction in binding in synchronous cultures passing through S phase (Fig. 5E).

The identification of the DASH complex

To understand the pathways in which Ask1 functions, we carried out a large-scale affinity purification using TAP-tagged Ask1 (Rigaut et al. 1999). Approximately 10 bands were specifically present after two sequential affinity purifications from the *ASK1–TAP* strain and were absent from strains lacking the tag. By use of mass spectrometry, several of these bands were identified as Ask1, Dam1, Duo1, Spc34, and ribosomal proteins L17A and S17A (Fig. 6A), and two additional proteins, Spc19 and Hsk1 were identified as described below. We named the complex DASH (for Dam1/Duo1, Ask1, Spc34/Spc19, Hsk1).

Dam1 and Duo1 were identified previously as proteins that localized to both spindles and kinetochores like Ask1, and function to maintain spindle integrity and the fidelity of chromosome segregation (Jones et al. 1999; Cheeseman et al. 2001; He et al. 2001). The interaction between Ask1 and these two proteins was confirmed by coimmunoprecicpitation using Ask1–Myc (Fig. 6B), indicating that the association was not dependent upon the epitope tag used. The reciprocal immunoprecipitation of Duo1 with anti-Duo1 antibodies led to the coprecipitation of Ask1–Myc (Fig. 6B), further supporting the existence of a complex. The interaction was also evident by genetic analysis. We found that *ask1* mutants were synthetically lethal with mutations in either *DAM1* or *DUO1*. Double mutants of *ask1-3 dam1-1* and *ask1-3 duo1-2* can survive only in the presence of wild-type *ASK1* (Fig. 6C). Finally, we observed that Ask1–GFP failed to localize to the spindle or kinetochore in *dam1* or *duo1* mutants by immunofluorescence microscopy (data not shown).

From the mass spectrometry, we also identified Spc34, a protein that copurified biochemically with the spindle-pole bodies (Wigge et al. 1998). Like Ask1, Dam1, and Duo1, it also binds to both kinetochores and spindles, and its kinetochore binding is Ndc10 dependent (Wigge et al. 1998; He et al. 2001). That Spc34 is a component of DASH was confirmed by immunoprecipitation experiments carried out in strains expressing Spc34-HA (Fig. 6D).

Spc34 copurifies and colocalizes with Spc19 (He et al. 2001) and interacts with Spc19 in a two-hybrid assay (Yeast Protein Database). To test whether Spc19 is present in DASH, we precipitated Ask1–TAP from strains expressing Spc19-HA and found that Spc19 is present in the immunoprecipitates (Fig. 6E). Precipitating Spc19-HA led to the precipitation of Duo1, further confirming that Spc19 exists in DASH with Ask1 and Duo1 (Fig. 6E).

Ask1 interacts with a novel ORF (YKR083C) in a two-hybrid assay (Yeast Protein Database). We named the protein Hsk1 (Helper of Ask1) and tagged it with the HA-epitope to test whether it was also in DASH. Immunoprecipitation experiments indicated that Hsk1 is a component of the complex (Fig. 6F).

The integrity of large complexes is often dependent upon several of their components. Therefore, we examined the complex formation in mutants of *ASK1*, *DAM1*, or *DUO1*. Immunoprecipitation experiments were carried out in *ask1-2–MYC*, *dam1-1 ASK1–MYC*, or *duo1-2 ASK1–MYC* strains. Although the total amount of all three proteins was not affected significantly in the mutants, the amount of Dam1 and Duo1 in the immunoprecipitates was greatly reduced in each mutant, whereas the amount of Ask1 immunoprecipitated remained constant (Fig. 6G). This indicates that Ask1, Dam1, and Duo1 are all required for the integrity of the DASH complex and its function in mitosis.

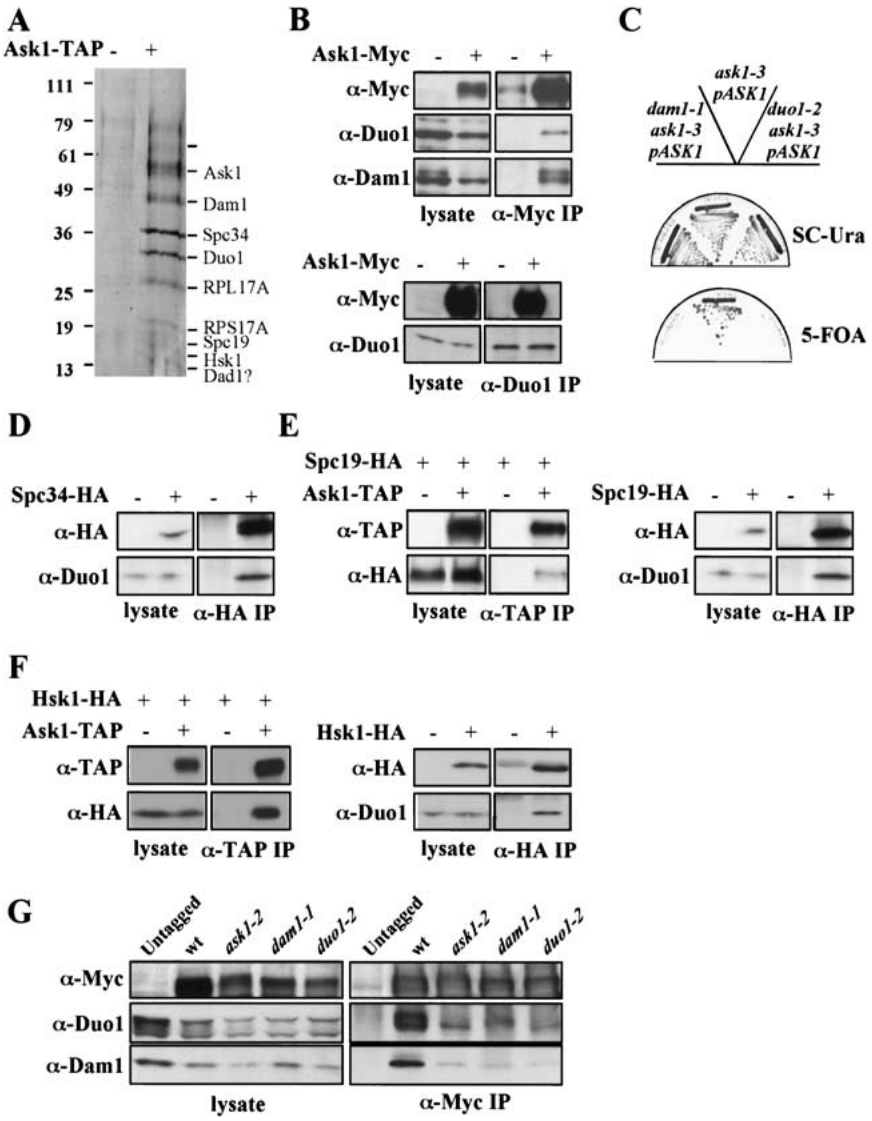

Figure 6. The identification of DASH complex. (*A*) TAP-purified protein extracts from 2L of yeast culture from either *ASK1–TAP* (Y1173) or untagged strains (Y300) were resolved on 4%–20% SDS–polyacrylamide gels and stained with Coomassie blue. Protein bands were cut out and identified by mass spectrometry as described previously (Wang et al. 2000). Question marks indicate proteins whose identities are suspected but not yet determined. (*B*) Coimmunoprecipitation of Dam1 and Duo1 with Ask1–Myc. (*Top*) Ask1–Myc was immunoprecipitated with anti-Myc antibodies. Proteins from total protein extracts (lysate) or from the immunoprecipitated fractions (IP) were analyzed by immunoblotting, using antibodies against Dam1 and Duo1. (*Bottom*) The same extracts as above were immunoprecipitated with anti-Duo1 antibodies and Western blots were probed with anti-Myc antibodies. (*C*) *ask1-3* is synthetically lethal with *dam1-1* and *duo1-2*. Both *ask1-3 dam1-1* (Y1171) and *ask1-3 duo1-2* (Y1172) containing pJBN81 (*ASK1–URA3*) were struck upon SC-Ura plates or SC plates containing 5-FOA. (*D*) Coimmunoprecipitation of Duo1 with Spc34. Immunoprecipitation was carried out with anti-HA antibodies and Western blots were probed with anti-Duo1 antibodies. (*E*) Spc19 is a component of the DASH complex. (*Left*) Extracts from *SPC19-HA* and *SPC19-HA ASK–-TAP* strains were immunoprecipitated with anti-TAP antibodies (PAP: peroxidase-anti-peroxidase) and Western blots were probed with anti-TAP and anti-HA antibodies. (*Right*) Extracts from *SPC19-HA* and nontagged strains were immunoprecipitated with anti-HA antibodies and Western blots were probed with anti-HA and anti-Duo1 antibodies as indicated. (*F*) Hsk1 is a component of the DASH complex. (*Left*) Extracts from *HSK1-HA* and *HSK1-HA ASK1-TAP* strains were immunoprecipitated with anti-TAP antibodies (PAP) and Western blots were probed with anti-TAP and anti-HA antibodies. (*Right*) Extracts from HSK1-HA and nontagged strains were immunoprecipitated with anti-HA antibodies and Western blots were probed with anti-HA and anti-Duo1 antibodies. (*G*) *ASK1*, *DAM1*, and *DUO1* are required for the formation of the DASH complex. Immunoprecipitations were carried out on extracts from *ask1-2–MYC* (Y1174), *dam1-1 ASK1–MYC* (Y1176), or *duo1-2 ASK1–MYC* (Y1175) strains and probed for Dam1, Duo1, and Ask1–Myc.

Ipl1 controls Dam1 phosphorylation

Purification of the DASH complex revealed a number of protein bands, several of which appeared to have a heterogeneous migration pattern characteristic of phosphoproteins. This prompted us to examine the mobility of these complex members in strains mutant for protein kinases that localize to kinetochores. Ipl1 is a protein kinase important for chromosome segregation (Chan et al. 1993; Biggins et al. 1999) and localizes to kinetochores (He et al. 2001). We therefore examined the effect of Ipl1 on DASH. TAP-purified DASH complexes from either wild-type or *ipl1-321* mutant cells were fractionated on SDS-PAGE and silver stained to visualize proteins (Fig. 7A). We observed a difference in mobility of one band in *ipl1* mutants. That band corresponded to the Dam1 protein identified by mass spectrometry, suggesting Dam1 is a potential target of Ipl1.

To determine whether Dam1 was a phosphoprotein, Dam1 was tagged with the Myc epitope and prepared from synchronized cells. The protein was treated with phosphatase alone (AP) or with phosphatase inhibitor (PI). Dam1 migrates as a doublet and phosphatase treatment significantly reduced its mobility to the faster migrating species, indicating that Dam1 is a phosphoprotein (Fig. 7B). To provide further evidence that Ipl1 is

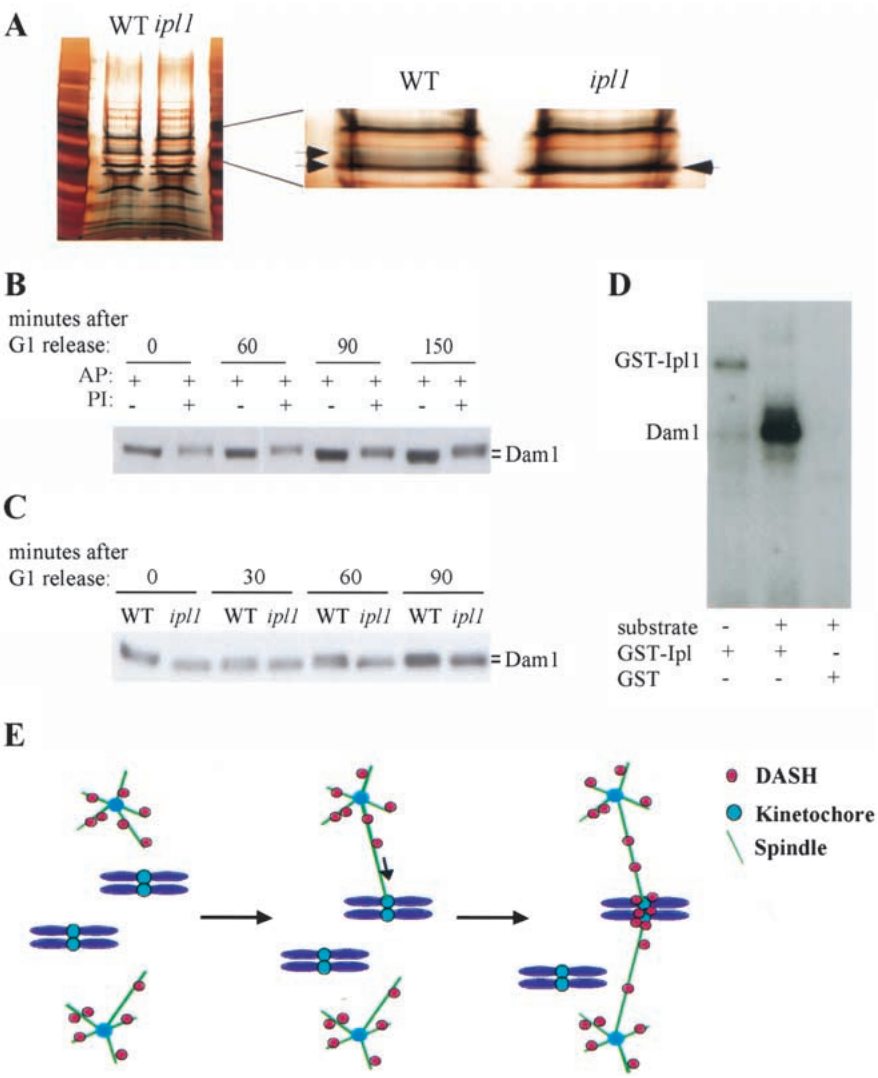

Figure 7. Ipl1 controls Dam1 phosphorylation. (*A*) The mobility of DASH complex proteins is altered in *ipl1* mutants. TAP-purified protein extracts from 2 L of yeast culture from either *ASK1–TAP* (Y1173) or *ASK1–TAP ipl1-321* mutants (Y1182) were resolved on 4%–20% SDS–polyacrylamide gel and silver stained. An expanded section is shown to illustrate the absence of certain bands in an *ilp1* mutant strain. (*B*) Dam1 is a phosphoprotein. Dam1–Myc was prepared from extracts of Y1183 and treated with alkaline phosphatase in the presence or absence of phosphatase inhibitors. Proteins were then immunoblotted and probed with anti-Myc antibodies. (*C*) Ipl1 controls the phosphorylation of Dam1 in vivo. Protein extracts were prepared from Y1183 (*DAM1–MYC*) and Y1184 (*DAM1–MYC ipl1-321*) cells synchronized in G_1 with α-factor and released into the cell cycle for the indicated time, and were then immunoblotted and probed with anti-MYC antibodies. (*D*) Ipl1 directly phosphorylates Dam1. Kinase assays were performed using GST–Ipl1 kinase (lanes *1*,*2*) or GST alone as a control (lane *3*). The substrate tested was the DASH complex purified from *ipl1-321 ASK1–TAP* (Y1182). The autophosphorylation of GST–Ipl1 and the phosphorylation of Dam1 are indicated at *left*. (*E*) Hypothetical model depicting the microtubule-dependent loading of the DASH complex to the kinetochore.

controlling Dam1 phosphorylation in vivo, the mobility of Dam1 in wild-type and *ipl1* mutants was examined in synchronized cells. The presence of the *ipl1* mutation abolished the slower migrating form of Dam1 (Fig. 7C). To test whether Ipl1 phosphorylated Dam1 directly, GST and GST–Ipl1 were purified from bacteria (Biggins et al. 1999) and tested for kinase activity toward TAP-purified DASH complexes. GST–Ipl1 specifically phosphorylated a band corresponding to the position of Dam1 on the gel (Fig. 7D), indicating that Dam1 is a substrate of Ipl1 and that Ipl1 control of Dam1 phosphorylation in vivo is likely to be direct.

Discussion

In this study, we describe the identification of Ask1 and the DASH complex in which it resides. Genetic analysis of *ask1* mutants revealed that it plays an essential role in chromosome segregation. *ASK1* and at least three additional members of the DASH complex are essential genes required for proper execution of anaphase and maintenance of spindle-kinetochore integrity. The similarity in phenotype among components of DASH suggests that the entire complex is required for a common function.

We show that Ask1 is a centromere-binding protein by several lines of evidence. First, *ask1-3* mutants exhibit severe chromosome loss, a phenotype associated with kinetochore defects. Second, a genetic interaction was found between *ASK1* and *NDC10*, a gene encoding the essential component of CBF3 kinetochore protein complex. Third, immunofluorescence staining of Ask1 shows that, in addition to associating with the mitotic spindle, it also localizes in the vicinity of the SPBs. This is consistent with the immunolocalization of many other centromere-binding proteins such as Ndc10 and Ctf19 (Hyland et al. 1999). This localization is abolished in *ndc10-1* mutants. Finally, formaldehyde cross-linking followed by chromatin immunoprecipitation reveals

that Ask1 localizes to *CEN* DNA in vivo in a Ndc10-dependent manner.

Ask1, and presumably the entire DASH complex, plays a critical role in chromosome segregation and maintenance of spindle-kinetochore integrity. *ask1-3* mutants lose chromosomes at a very high rate at semi-permissive temperatures and at their restrictive temperature, undergo massive nondisjunction. Furthermore, in *ask1-3* mutant cells, we also observed an abnormal spindle in checkpoint-arrested cells in which the spindle staining between SPBs disappeared after segregation of the DNA masses. This defect is similar to that observed in *dam1-1* mutants (Jones et al. 1999; Cheeseman et al. 2001), in which electron micrographs show a severe defect in spindle integrity.

The phenotypes of *ask1* mutants can best be explained through a defect in kinetochore–spindle interaction. *ask1-3* mutants attempted to segregate chromosomes to daughter cells despite activation of the spindle checkpoint, suggesting cohesed sisters are pulled randomly to one of the two spindle poles and spindles are functional. Even in the absence of a spindle checkpoint in which a normal elongated spindle is observed, nondisjunction occurs at a high rate. A possible explanation for this is that, on average, one of the two spindle–kinetochore interactions on a pair of sister chromatids is defective. Whether this is a random, stochastic event or that one particular kinetochore is defective or misoriented is unknown. An alternative explanation is that chromosomes remain monoattached to a given spindle-pole body and fail to set up a bipolar attachment because they fail to release. This phenotype would be similar to that of the *ipl1* mutant (T. Tanaka et al., in prep.), which also shows high rates of chromosome non-disjunction (Chan and Botstein 1993).

Ask1 is unique among kinetochore-binding proteins because its centromeric association is dependent upon an intact spindle. Ask1's kinetochore localization is most sensitive to spindle perturbation when cells are traversing S phase, the period in which new kinetochores are assembled and must re-establish their spindle attachment. Because Ask1 is associated with the spindle, the simplest interpretation is that Ask1 is transferred directly from the spindle to the kinetochore once kinetochores are replicated and microtubules are captured (Fig. 7E). Once Ask1 is loaded onto the kinetochore, it only partially requires the presence of microtubules to maintain kinetochore localization, indicating that it is not simply a microtubule-binding protein that is localized near the kinetochore after microtubule capture, but is actually loaded onto the kinetochore in a microtubule-dependent manner. Thus, DASH requires microtubules for loading but not maintenance of its kinetochore binding. It should be noted, however, that our results cannot distinguish between loading of the Ask1 protein directly from the spindle to the kinetochore versus induced binding of Ask1 to the kinetochore upon spindle interaction.

The dynamic microtubule-dependent association of Ask1 with the kinetochore indicates that spindle binding has additional consequences for the kinetochore other than establishing a connection. DASH may be transferring information to the kinetochore in order to prepare it for future activities. How might a kinetochore benefit from DASH loading? One possibility is that DASH stabilizes the microtubule–kinetochore attachment through maturation of the kinetochore, a possibility consistent with its role in preventing nondisjunction and maintaining a short spindle during cell cycle arrest. Alternatively, DASH may act to turn off the spindle checkpoint, causing the dissociation of Mad2 and associated proteins. This would be consistent with the observation that the spindle checkpoint is maintained in an active state in *ask1* mutants. Another possibility is that DASH might help orient sister kinetochores in a back-to-back orientation to facilitate bipolar attachment once one kinetochore has captured a microtubule.

The DASH complex consists of at least ten copurifying proteins. We have identified Ask1, Dam1, Duo1, Spc34, Spc19, Hsk1, RPL17A, and RPS17A as eight of these. We have confirmed that each of these, with the exception of the ribosomal proteins, exist in a complex with at least two other components of DASH, suggesting one large complex. From sizing columns, we have detected Ask1 in a complex of >1 Mda, which is large enough to accommodate all of these proteins (data not shown). Beside the phenotypic similarity between mutants of *ASK1*, *DUO1*, and *DAM1*, all three proteins localize to kinetochore in a Ndc10-dependent manner (He et al. 2001). In addition, Spc34 also binds to both spindles and kinetochores, the latter in a Ndc10-dependent manner (Wigge et al. 1998; He et al. 2001). Another, as yet unidentified, component is likely to be a known protein, YDR016C (Dad1), which interacts with Duo1 in a two-hybrid assay (Yeast Protein Database) and has been shown recently to exist in a complex with Dam1 and Duo1 (Enquist-Newman et al. 2001).

The connection between Ipl1 and the DASH complex ties together two pathways important for proper control of chromosome segregation. Ipl1 has been implicated recently in controlling the sensing of tension on chromosomes. In the absence of DNA replication such as in *cdc6* mutants, Pds1 degradation is delayed in a spindle checkpoint-dependent manner (Stern and Murray 2001). Unlike other aspects of spindle checkpoint function such as cell cycle arrest in response to nocodazole treatment, this aspect of the spindle-assembly checkpoint is specifically dependent on Ipl1 (Biggins and Murray 2001). The fact that Ipl1 controls Dam1 phosphorylation in vivo, most likely by direct phosphorylation, suggests that Ipl1 is controlling DASH function in some capacity. Whether this is part of Ipl1's role in controlling the spindle checkpoint or a distinct role in control of chromosome segregation remains to be determined. However, as both *ipl1* and *ask1* mutants show severe nondisjunction phenotypes, it is likely that they are working together to ensure proper bipolar kinetochore–spindle interactions.

All of the identified members of DASH are essential proteins, indicating that it is likely to perform functions essential for mitosis. The discovery of DASH has established the existence of a new pathway controlling inter-

actions between the spindle and the kinetochore. Furthermore, the analysis of Ask1 and the DASH complex has established a new step in kinetochore maturation and has revealed that the spindle transfers information to the newly captured kinetochore to alter its function in an important manner. The finding that Ask1 and other DASH component homologs exist in other organisms indicates that this protein complex will be conserved throughout eukaryotic evolution and is likely to play an important role in regulation of kinetochores in higher eukaryotes.

Materials and methods

Yeast strains and plasmid construction

All strains used in these experiments are isogenic with the W303-derived Y300 strain and are listed in Table 1. Gene disruptions were introduced into the Y300 background by use of standard methods. Additional strains were subsequently constructed by use of standard genetic crosses. The *ASK1* deletion strain was generated by use of a 1-kb Δ*ask1*::*HIS3* PCR fragment. This fragment replaces amino acids 0–177 of the 293 amino acid *ASK1* ORF with *HIS3*. The deletion strain was covered with *pASK1-CEN/ARS/URA3* (pJBN81) to maintain viability.

The GFP-tagged Ask1 was generated as follows: A 490-bp fragment of Ask1 was amplified by PCR using the following primers: Ask1-23, 5′-CCCGCGGTGCCTTCAAGGGAGCAAACA GACC-3′ and Ask1-27, 5′-CTAATCGATTTCTATTCGTAGA AAAATGAATG-3′. As a result, a *Cla*I site was introduced at the termination codon of the *ASK1* gene. The resulting *Sac*II–*Cla*I fragment was cloned into pRS404 together with a *Cla*I–*Cla*I cassette containing GFP provided by Dr. John Kilmartin (MRC, Cambridge, UK). The resulting plasmid was introduced into a haploid wild-type strain Y300 at the endogenous *ASK1* locus after linearization with *Sac*I.

Isolation of temperature-sensitive mutants of ASK1

Temperature-sensitive alleles of *ASK1* were generated using the PCR random mutagenesis method described previously (Wang and Elledge 1999). pJBN79 (*pASK1-CEN/ARS/TRP1*) was used as a template. Primers used in the PCR were as follows: Ask1-5 containing *Pst*I site, 5′-GGTTGATCAACGTATGGA AAGAGCAACATAAGGGATTGTTGACACAAAAAATGG-3′, and Ask1-6 containing *Kpn*I site, 5′-GAGCCGGTACCAT GCTGGAACTAGACCCGTACTGCTGCTGCAAAGTAGCG AATC-3′. Mutagenic PCR was carried out using 20 ng of plasmid DNA, 1 μM of each primer, 5 mM $MgCl_2$, and 1 mM $MnCl_2$. The PCR products were purified, digested with *Kpn*I and *Pst*I, and cloned into pYL61 (pJBN79 with the *Kpn*I site on vector filled in). Approximately 10,000 *Escherichia coli* transformants were obtained. This library was used to transform Δ*ask1*::*HIS3* covered by pJBN81 (Y1102) and Trp^+ transformants were selected at room temperature. They were then replica plated to plates containing 0.1% 5-fluoroorotic acid (5-FOA) at 24°C and 37°C to identify temperature-sensitive mutants. From 10 temperature-sensitive clones, plasmid DNA were recovered and transformed back to confirm the phenotype. Six temperature-sensitive alleles were integrated into the *ASK1* locus using two-step gene replacement, and the temperature-sensitive phenotypes of each allele could be complemented by the wild-type *ASK1* gene on a *CEN* plasmid.

Table 1. *Strains used in this study*

Y300	*MAT***a** *trp-1-1 ura3-1 his3-11, 3 leu2-3, 112 ade2-1 can1-100*
Y604	as Y300 except *mecl-21*
Y928	as Y300 except *LEU2, askl-1*
Y974	as Y300 except *ura3::tetO-URA3, leu2-3, 112::GFP::TetR-LEU2*
Y998	as Y300 except *PDSI-18MYC-LEU2*
Y1101	as Y300 except Δ*mad2::URA3*
Y1102	as Y300 except Δ*ask1::HIS3* + JBN81*(pRS426-ASK1)*
Y1103	as Y300 except *ask1-3*
Y1104	as Y300 except *ask1-2*
Y1106	as Y300 except *ask1-2* Δ*mad2::URA3*
Y1107	as Y300 except *ask1-3* Δ*mad2::URA3*
Y1108	as Y300 except *PDS1-18MYC-LEU2, ask1-3*
Y1109	as Y300 except *ura3::tetO-URA3, leu2-3,112::GFP::TetR-LEU2, ask1-3*
Y1110	as Y300 except *ura3::tetO-URA3, leu2-3,112::GFP::TetR-LEU2, ask1-3,* Δ*mad2::URA3*
Y1111	as Y300 except *ASK1-GFP-TRP*
Y1112	as Y300 except *ASK1-GFP-TRP, ndc10-1*
Y1113	as Y300 except Δ*ask1::LEU2, ASK1-9MYC-HIS3*
Y1114	as Y300 except Δ*ask1::LEU2, ASK1-9MYC-HIS3, cdc13-1*
Y1115	as Y300 except Δ*ask1::LEU2, ASK1-9MYC-HIS3, ndc10-1*
Y1116	as Y300 except Δ*ask1::LEU2, ASK1-9MYC-HIS3, cdc13-1,* Δ*mad2::URA3*
Y1117	as Y300 except *CFIII, URA3, SUP11*
Y1118	as Y300 except *CFIII, URA3, SUP11, ask1-3*
Y1119	as Y300 except *ndc10-1*
Y1120	as Y300 except *ask1-3 ndc10-1*
Y1171	as Y300 except *ask1-3 dam1-1* pJBN81
Y1172	as Y300 except *ask1-3 duo1-2* pJBN81
Y1173	as Y300 except *ASK1-TAP-TRP*
Y1174	as Y300 except *ask1-2-9Myc*
Y1175	as Y300 except Δ*ask1::LEU2, ASK1-9MYC-HIS3, duol-2*
Y1176	as Y300 except Δ*ask1::LEU2, ASK1-9MYC-HIS3, dam1-1*
Y1177	as Y300 except *SPC34-HA*
Y1178	as Y300 except *SPC19-HA*
Y1179	as Y300 except *SPC19-HA, ASK1-TAP-TRP*
Y1180	as Y300 except *HSK1-HA*
Y1181	as Y300 except *HSK1-HA, ASK1-TAP-TRP*
Y1182	as Y300 except *ipl1-321, ASK1-TAP-TRP*
Y1183	as Y300 except *DAM1-MYC-TRP1*
Y1184	as Y300 except *ipl1-321, DAM1-MYC-TRP1*

Cytological techniques

FACS analysis was performed as described previously (Desany et al. 1998). Immunofluorescence microscopy using both anti-tubulin and anti-GFP antibodies was performed after formaldehyde fixation as described (Sanchez et al. 1996). Cells were fixed by 5% formaldehyde for 0.5 h and then stained by anti-GFP polyclonal (1:2000 dilution, provided by Dr. Silver, Harvard Medical School and Dana Farber Cancer Institute, Boston, MA) and anti-tubulin Yol1/34 (1:50 dilution, Desany et al. 1998) antibodies after methanol/acetone treatment. Other immunofluorescence microscopy using anti-tubulin antibodies was done as described previously (Desany et al. 1998). DAPI was used for

staining of DNA after fixation. Observation of TetR–GFP was performed directly on a Zeiss Axioskop after cells were fixed for 5 min.

Protein purification

Protein extracts from a 2-L yeast culture of OD 1.2 were prepared using RIPA buffer (150 mM NaCl, 50 mM Tris-Cl, 5 mM EDTA, 0.5% Triton X-100), and subsequently, TAP purification was carried out as described (Rigaut et al. 1999). After centrifugation to obtain the supernatant, Tris-Cl (pH 8.0) was added to a final concentration of 10 mM and NP40 was added to a final concentration of 0.1%. The extracts were then subjected to two-step affinity purification. After a 2-h incubation with IgG Sepharose (400 µL) at 4°C, the beads were washed three times with IPP150 (10 mM Tris-Cl at pH 8.0, 150 mM NaCl, and 0.1% NP-40), once with TEV buffer (IPP150 containing 0.5 mM EDTA and 1 mM DTT), and incubated in 1-mL TEV buffer containing 100 U of TEV protease (GIBCO BRL) at room temperature for 2 h in a column. The eluate was diluted to 4.8 mL with calmodulin-binding buffer (IPP150 containing 10 mM β-mercaptoethanol, 1 mM Mg-acetate, 1 mM imidazole, 2 mM $CaCl_2$), 3.6 µL 1M $CaCl_2$ and 200 µL of calmodulin beads were added and incubated at 4°C for 1 hr. After washing, bound proteins were eluted (five times, 200 µL each) with calmodulin elution buffer (IPP150 containing 10 mM β-mercaptoethanol, 1 mM Mg-acetate, 1 mM imidazole, and 2 mM EGTA). Purified proteins were precipitated with TCA and analyzed by 4%–20% SDS-PAGE, followed by Coomassie blue staining and silver staining.

Kinase assay

TAP-purified DASH complex from an *ipl1-321 ASK1–TAP* strain was eluted in 200 µL kinase buffer (50 mM Tris-Cl at pH 8.0, 100 mM NaCl, 2 mM EGTA, 1 mM imidazole, 25 mM β-glycerophosphate, 1 mM DTT, 10 mM ATP, 5 mM $MgCl_2$, 2.5 µCi [γ^{32}-P]ATP) and 1 µg GST or GST–Ipl1 on beads were added to the reaction for 20 min at 30°C as described (Biggins et al. 1999).

Chromatin immunoprecipitations

Chromatin immunoprecipitation assays were conducted essentially as described previously (Aparicio et al. 1997; Zou and Stillman 2000). Ask1–Myc was immunoprecipitated by the monoclonal antibody 9E10. Primers for *CEN3*, *CEN16*, and the AT-rich region near *PGK1* are the same as described (Meluh and Koshland 1997).

The nocodazole treatment of the cells were carried out as follows: cells were arrested with α-factor for 3 h, or 32°C incubation for 2 h before being spun down and resuspended in YPD containing 15 µg/mL nocodazole and 1% DMSO. YPD containing 1% DMSO was used as control. Seventy minutes later, the cells were spun down for ChIP analysis.

Acknowledgments

We thank K. Nasmyth, P. Hieter, J. Kilmartin, and S. Biggins for strains; P. Silver, D.G. Drubin, and G. Barnes for antibodies; and K. Nasmyth and T. Tanaka for sharing unpublished results. We also thank X. He, M. Winey, A. Hyman, and members of the Elledge laboratory for comments and helpful discussions. We thank L. Zou and D. Zhang for sharing their technical expertise. This work was supported in part by a National Institute of Health Grant. S. J. E. is an investigator with the Howard Hughes Medical Institute and a Welch professor of biochemistry.

The publication costs of this article were defrayed in part by payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 USC section 1734 solely to indicate this fact.

References

Alcasabas, A.A., Osborn, A.J., Bachant, J., Hu, F., Werler, P.J.H., Bousset, K., Kanji Furuya, K., Diffley, J.F.X., Carr, A. and Elledge, S.J. 2001. Mrc1 transduces DNA replication stress signals to activate Rad53. *Nat. Cell. Biol.*. **3:** 958–965.

Amon, A. 1999. The spindle checkpoint. *Curr. Opin. Genet. Dev.* **9:** 69–75.

Aparicio, O.M., Weinstein, D.M., and Bell, S.P. 1997. Components and dynamics of DNA replication complexes in *S. cerevisiae*: Redistribution of MCM proteins and Cdc45p during S phase. *Cell* **91:** 59–69.

Biggins, S. and Murray, A.W. 2001. The budding yeast protein kinase Ipl1/Aurora allows the absence of tension to activate the spindle checkpoint. *Genes & Dev.* **15:** 3118–3129.

Biggins, S., Severin, F.F., Bhalla, N., Sassoon, I., Hyman, A.A., and Murray, A.W. 1999. The conserved protein kinase Ipl1 regulates microtubule binding to kinetochores in budding yeast. *Genes & Dev.* **13:** 532–544.

Cai, M. and Davis, R.W. 1990. Yeast centromere binding protein CBF1, of the helix-loop-helix protein family, is required for chromosome stability and methionine prototrophy. *Cell* **61:** 437–446.

Chan, C.S. and Botstein, D. 1993. Isolation and characterization of chromosome-gain and increase-in- ploidy mutants in yeast. *Genetics* **135:** 677–691.

Cheeseman, I.M., Enquist-Newman, M., Muller-Reichert, T., Drubin, D.G., and Barnes, G. 2001. Mitotic spindle integrity and kinetochore gunction linked by the Duo1p/Dam1p complex. *J. Cell Biol.* **152:** 197–212.

Chen, R.H., Waters, J.C., Salmon, E.D., and Murray, A.W. 1996. Association of spindle assembly checkpoint component XMAD2 with unattached kinetochores. *Science* **274:** 242–246.

Ciosk, R., Zachariae, W., Michaelis, C., Shevchenko, A., Mann, M., and Nasmyth, K. 1998. An ESP1/PDS1 complex regulates loss of sister chromatid cohesion at the metaphase to anaphase transition in yeast. *Cell* **93:** 1067–1076.

Cohen-Fix, O., Peters, J.M., Kirschner, M.W., and Koshland, D. 1996. Anaphase initiation in *Saccharomyces cerevisiae* is controlled by the APC-dependent degradation of the anaphase inhibitor Pds1p. *Genes & Dev.* **10:** 3081–3093.

Connelly, C. and Hieter, P. 1996. Budding yeast SKP1 encodes an evolutionarily conserved kinetochore protein required for cell cycle progression. *Cell* **86:** 275–285.

Desany, B.A., Alcasabas, A.A., Bachant, J.B., and Elledge, S.J. 1998. Recovery from DNA replicational stress is the essential function of the S-phase checkpoint pathway. *Genes & Dev.* **12:** 2956–2970.

Doheny, K.F., Sorger, P.K., Hyman, A.A., Tugendreich, S., Spencer, F., and Hieter, P. 1993. Identification of essential components of the *S. cerevisiae* kinetochore. *Cell* **73:** 761–774.

Elledge, S.J. and Davis, R.W. 1990. Two genes differentially regulated in the cell cycle and by DNA- damaging agents encode alternative regulatory subunits of ribonucleotide reductase. *Genes & Dev.* **4:** 740–751.

Enquist-Newman, M., Cheeseman, I.M., Van Goor, D., Drubin,

D.G., Meluh, P.B., and Barnes, G. 2001. Dad1p, third component of the duo1p/dam1p complex involved in kinetochore function and mitotic spindle integrity. *Mol. Biol. Cell* **12:** 2601–2613.

Farr, K.A. and Hoyt, M.A. 1998. Bub1p kinase activates the *Saccharomyces cerevisiae* spindle assembly checkpoint. *Mol. Cell. Biol.* **18:** 2738–2747.

Funabiki, H., Yamano, H., Kumada, K., Nagao, K., Hunt, T., and Yanagida, M. 1996 Cut2 proteolysis required for sister-chromatid seperation in fission yeast. *Nature* **381:** 438–441.

Goh, P.Y. and Kilmartin, J.V. 1993. NDC10: A gene involved in chromosome segregation in *Saccharomyces cerevisiae*. *J. Cell Biol.* **121:** 503–512.

Gorbsky, G.J. and Ricketts, W.A. 1993. Differential expression of a phosphoepitope at the kinetochores of moving chromosomes. *J. Cell Biol.* **122:** 1311–1321.

Goshima, G. and Yanagida, M. 2000. Establishing biorientation occurs with precocious separation of the sister kinetochores, but not the arms, in the early spindle of budding yeast. *Cell* **100:** 619–633.

Hardwick, K.G. and Murray, A.W. 1995. Mad1p, a phosphoprotein component of the spindle assembly checkpoint in budding yeast. *J. Cell Biol.* **131:** 709–720.

Hardwick, K.G., Weiss, E., Luca, F.C., Winey, M., and Murray, A.W. 1996. Activation of the budding yeast spindle assembly checkpoint without mitotic spindle disruption. *Science* **273:** 953–956.

He, X., Asthana, S., and Sorger, P.K. 2000. Transient sister chromatid separation and elastic deformation of chromosomes during mitosis in budding yeast. *Cell* **101:** 763–775.

He, X., Rines, D.R., Espelin, C.W., and Sorger, P.K. 2001. Molecular analysis of kinetochore-microtubule attachment in budding yeast. *Cell* **10:** 195–206.

Hegemann, J.H. and Fleig, U.N. 1993. The centromere of budding yeast *BioEssays* **15:** 451–460.

Hoyt, M.A. and Geiser, J.R. 1996. Genetic analysis of the mitotic spindle. *Annu. Rev. Genet.* **30:** 7–33.

Hoyt, M.A., Totis, L., and Roberts, B.T. 1991. *S. cerevisiae* genes required for cell cycle arrest in response to loss of microtubule function. *Cell* **66:** 507–517.

Hoyt, M.A., He, L., Loo, K.K., and Saunders, W.S. 1992. Two *Saccharomyces cerevisiae* kinesin-related gene products required for mitotic spindle assembly. *J. Cell Biol.* **118:** 109–120.

Hwang, L.H., Lau, L.F., Smith, D.L., Mistrot, C.A., Hardwick, K.G., Hwang, E.S., Amon, A., and Murray, A.W. 1998. Budding yeast Cdc20: A target of the spindle checkpoint. *Science* **279:** 1041–1044.

Hyland, K.M., Kingsbury, J., Koshland, D., and Hieter, P. 1999. Ctf19p: A novel kinetochore protein in *Saccharomyces cerevisiae* and a potential link between the kinetochore and mitotic spindle. *J. Cell Biol.* **145:** 15–28.

Jensen, S., Segal, M., Clarke, D.J., and Reed, S.I. 2001. A novel role of the budding yeast separin Esp1 in anaphase spindle elongation. Evidence that proper spindle association of esp1 is regulated by pds1. *J. Cell Biol.* **152:** 27–40.

Jiang, W., Lechner, J., and Carbon, J. 1993. Isolation and characterization of a gene (CBF2) specifying a protein component of the budding yeast kinetochore. *J. Cell Biol.* **121:** 513–519.

Jones, M.H., Bachant, J.B., Castillo, A.R., Giddings, T.H., and Winey, M. 1999. Yeast Dam1p is required to maintain spindle integrity during mitosis and interacts with the Mps1p kinase. *Mol. Biol. Cell* **10:** 2377–2391.

Kim, S.H., Lin, D.P., Matsumoto, S., Kitazono, A., and Matsumoto, T. 1998 Fission yeast Slp1: An effector of the Mad2-dependent spindle checkpoint. *Science* **279:** 1045–1047.

Lechner, J. 1994. A zinc finger protein, essential for chromosome segregation, constitutes a putative DNA binding subunit of the *Saccharomyces cerevisiae* kinetochore complex, Cbf3. *EMBO J.* **13:** 5203–5211.

Lechner, J. and Ortiz, J. 1996. The *Saccharomyces cerevisiae* kinetochore. *FEBS Lett.* **389:** 70–74.

Li, R. and Murray, A.W. 1991. Feedback control of mitosis in budding yeast. *Cell* **66:** 519–531.

Meluh, P.B. and Koshland, D. 1995. Evidence that the MIF2 gene of *Saccharomyces cerevisiae* encodes a centromere protein with homology to the mammalian centromere protein CENP-C. *Mol. Biol. Cell* **6:** 793–807.

———. 1997. Budding yeast centromere composition and assembly as revealed by in vivo cross-linking. *Genes & Dev.* **11:** 3401–3412.

Meluh, P.B., Yang, P., Glowczewski, L., Koshland, D., and Smith, M.M. 1998. Cse4p is a component of the core centromere of *Saccharomyces cerevisiae*. *Cell* **94:** 607–613.

Michaelis, C., Ciosk, R., and Nasmyth, K. 1997. Cohesins: Chromosomal proteins that prevent premature separation of sister chromatids. *Cell* **91:** 35–45.

Ortiz, J., Stemmann, O., Rank, S., and Lechner, J. 1999. A putative protein complex consisting of Ctf19, Mcm21, and Okp1 represents a missing link in the budding yeast kinetochore. *Genes & Dev.* **13:** 1140–1155.

Pangilinan, F. and Spencer, F. 1996. Abnormal kinetochore structure activates the spindle assembly checkpoint in budding yeast. *Mol. Biol. Cell* **7:** 1195–1208.

Pellman, D., Bagget, M., Tu, Y.H., Fink, G.R., and Tu, H. 1995. Two microtubule-associated proteins required for anaphase spindle movement in *Saccharomyces cerevisiae*. *J. Cell Biol.* **130:** 1373–1385.

Pidoux, A.L. and Allshire, R.C. 2000. Centromeres: Getting a grip of chromosomes. *Curr. Opin. Cell Biol.* **12:** 308–319.

Rigaut, G., Shevchenko, A., Rutz, B., Wilm, M., Mann, M., and Séraphin, B. 1999. A generic protein purification method for protein complex characterization and proteome exploration. *Nat. Biotechnol.* **17:** 1030–1032.

Roof, D.M., Meluh, P.B., and Rose, M.D. 1992. Kinesin-related proteins required for assembly of the mitotic spindle. *J. Cell Biol.* **118:** 95–108.

Sanchez, Y., Desany, B.A., Jones, W.J., Liu, Q., Wang, B., and Elledge, S.J. 1996. Regulation of RAD53 by the ATM-like kinases MEC1 and TEL1 in yeast cell cycle checkpoint pathways. *Science* **271:** 357–360.

Saunders, W.S. and Hoyt, M.A. 1992. Kinesin-related proteins required for structural integrity of the mitotic spindle. *Cell* **70:** 451–458.

Spencer, F., Gerring, S.L., Connelly, C., and Hieter, P. 1990. Mitotic chromosome transmission fidelity mutants in *Saccharomyces cerevisiae*. *Genetics* **124:** 237–249.

Stern, B.M. and Murray, A.W. 2001. Lack of tension at kinetochores activates the spindle checkpoint in budding yeast. *Curr. Biol.* **11:** 1462–1467.

Strunnikov, A.V., Kingsbury, J., and Koshland, D. 1995. CEP3 encodes a centromere protein of *Saccharomyces cerevisiae*. *J. Cell Biol.* **128:** 749–760.

Uhlmann, F., Lottspeich, F., and Nasmyth, K. 1999. Sister-chromatid separation at anaphase onset is promoted by cleavage of the cohesin subunit Scc1. *Nature* **400:** 37–42.

Wang, H. and Elledge, S.J. 1999. DRC1, DNA replication and checkpoint protein 1, functions with DPB11 to control DNA replication and the S-phase checkpoint in *Saccharomyces cerevisiae*. *Proc. Natl. Acad. Sci.* **96:** 3824–3829.

Wang, Y. and Burke, D.J. 1995. Checkpoint genes required to

delay cell division in response to nocodazole respond to impaired kinetochore function in the yeast *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* **15:** 6838–6844.

Wang, Y., Cortez, D., Yazdi, P., Neff, N., Elledge, S.J., and Qin, J. 2000. BASC, a super complex of BRCA1-associated proteins involved in the recognition and repair of aberrant DNA structures. *Genes & Dev.* **14:** 927–939.

Wigge, P.A., Jensen, O.N., Holmes, S., Soues, S., Mann, M., and Kilmartin, J.V. 1998. Analysis of the *Saccharomyces* spindle pole by matrix-assisted laser desorption/ionization (MALDI) mass spectrometry. *J. Cell Biol.* **141:** 967–977.

Winey, M. and O'Toole, E.T. 2001. The spindle cycle in budding yeast. *Nat. Cell Biol.* **3:** 23–27.

Yamamoto, A., Guacci, V., and Koshland, D. 1996 Pds1p, an inhibitor of anaphase in budding yeast, plays a critical role in the APC and checkpoint pathway(s). *J. Cell Biol.* **133:** 99–110.

Zou, L. and Stillman, B. 2000. Assembly of a complex containing Cdc45p, replication protein A, and Mcm2p at replication origins controlled by S-phase cyclin-dependent kinases and Cdc7p-Dbf4p kinase. *Mol. Cell Biol.* **20:** 3086–3096.