Nnf1p, Dsn1p, Mtw1p, and Nsl1p: a New Group of Proteins Important for Chromosome Segregation in *Saccharomyces cerevisiae*

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Previously, antibodies were raised against a nuclear envelope-enriched fraction of yeast, and the essential gene *NNF1* was cloned by reverse genetics. Here it is shown that the conditional *nnf1-17* mutant has decreased stability of a minichromosome in addition to mitotic spindle defects. I have identified the novel essential genes *DSN1, DSN3,* and *NSL1* through genetic interactions with *nnf1-17.* *Dsn3p* was found to be equivalent to the kinetochore protein Mtw1p. By indirect immunofluorescence, all four proteins, Nnf1p, Mtw1p, Dsn1p, and Nsl1p, colocalize and are found in the region of the spindle poles. Based on the colocalization of these four proteins, the minichromosome instability and the spindle defects seen in *nnf1* mutants, I propose that Nnf1p is part of a new group of proteins necessary for chromosome segregation.

The process of mitotic cell division ensures that chromosomes are faithfully duplicated and equally segregated between mother and daughter cells, as the absence of genes or their presence in irregular numbers is typically lethal. The microtubule (MT) cytoskeleton and its associated structures are responsible for orchestrating chromosome segregation and maintaining genetic continuity. In many eukaryotic cells, chromosomal movements during mitosis are conserved and are mediated by three organelles: the bipolar mitotic spindle, the kinetochores (centromere DNA and associated proteins), and the centrosomes (MT organizing centers). These common features of chromosome segregation can be observed microscopically, as aided by fluorescence in situ hybridization studies of individual chromosomes in fixed cells (20) and green fluorescent protein (GFP)-tagged chromosomes in living cells (23, 46, 49, 66, 67). Separation of sister chromatids is initiated at and progresses from the centromeric regions where the kinetochores mediate the attachment of sister chromatids to the spindle MTs. In *Saccharomyces cerevisiae,* the MTs are nucleated by the spindle pole body (SPB), the yeast equivalent of the centrosome. The SPB is a large laminar structure that is embedded in the nuclear membrane throughout the cell cycle and nucleates both cytoplasmic and nuclear MTs (5, 6). One array of nuclear MTs interdigitate and span from pole to pole, while a second array is captured by the kinetochores. Chromosome segregation requires the shortening of kinetochore MTs (anaphase A) and the elongation of pole-to-pole MTs (anaphase B). In budding yeast, the most dramatic movement of chromatin is concomitant with spindle elongation, although anaphase A has also been observed (21, 49, 67). Sister centromeres that are proximally marked with GFP have been shown to align along a spindle equator, separate, and oscillate between the spindle poles before anaphase B spindle elongation (49). Following the end of mitosis and during much of the cell cycle, the centromeres are clustered near the SPBs (21, 33).

Mutations in components of the mitotic spindle, the SPB, kinetochores, MT-associated motor proteins, and various regulatory enzymes may result in aneuploidy. In one set of mutants, chromosome segregation is asymmetric, with chromosomal DNA segregating to only one pole of a bipolar spindle. Mutants in this group include kinetochore proteins of the Ndc80p complex (76), Ndc10p (16), and the protein kinase Ip1p, which localizes to the mitotic spindle and kinetochores (2, 9, 23, 37). Alternatively, duplication of the SPB can terminate at a particular stage, resulting in a monopolar spindle and diploidization, as seen in the *mps2-1* or *ndc1-1* mutants (70, 78, 79). In other chromosome missegregation mutants, chromosome loss is seemingly random. In certain *tub4* and *spc110* mutants, which are defective in γ-tubulin and the SPB component Spc110p, respectively, the SPBs are assembled but the spindle is compromised during mitosis (36, 61, 64, 68, 69). Failure in chromosome segregation and spindle defects are also seen in *duo1* and *dam1* mutants, as might be expected since Duo1p and Dam1p are found along the mitotic spindle and at the kinetochores in wild-type cells (10, 23, 24, 34). These mutants demonstrate that deficient SPBs, kinetochores, or spindles can lead to disomy of individual chromosomes or diploidization of the entire genome within a single nucleus.

In *S. cerevisiae,* nuclear division occurs along the bud axis, and hence positioning of the mitotic spindle through the narrow bud neck is critical to ensure an equal distribution of DNA between the mother and daughter cells. Dynamic contacts between the cytoplasmic MTs and the cell cortex are chiefly responsible for nuclear migration and orientation of the mitotic spindle (8, 57). Mutants that can complete spindle extension but are defective in nuclear positioning are characterized by populations of anucleate daughter cells and binucleate mother cells. These mutants are typically defective in cytoplasmic MT proteins or have perturbations in the SPB. For example, detachment of the cytoplasmic MTs from the SPBs through loss of either of the outer plaque proteins Cnm67p and Spc72p leads to multinucleated cells (4, 60). Therefore, the observation of binucleate cells may indicate a SPB defect.

In an earlier study, we raised antibodies against a nuclear envelope-enriched fraction of yeast and subsequently cloned...
the essential gene NNF1 (necessary for nuclear function 1) from a yeast expression library (56). Nnf1p is a small coiled-coil protein of 201 amino acids with no homology to any known proteins. Cells carrying mutations in NNF1 exhibit primarily short mitotic spindles and, to a lesser extent, aberrant cytoplasmic MTs and defects in nuclear migration. Nnf1p is a protein of low abundance and cannot be detected by Western blotting unless it is overexpressed. When cells overexpressing NNF1 are fractionated, Nnf1p is found predominantly in the nuclear fraction and can be extracted only in the presence of 8 M urea or guanidine hydrochloride. These studies have verified that Nnf1p is a nuclear protein but have not elucidated the nature of its function. In this work, I present the results of two extensive genetic screens that were initiated with the conditional nff1-17 allele. I show that Nnf1p is important for chromosome segregation and identify three functionally related proteins.

**MATERIALS AND METHODS**

Strains, media, and culture conditions. Yeast cell culture and genetic manipulations, including mating, diploid isolation, sporulation, and tetrad analysis, were performed as described previously (52, 58). Yeast strains (Table 1) are derivatives of W303, except for CH1305 (42) and ABY112 (4). Media were prepared according to standard recipes (5, 58). Selecting assays were performed on low-adenine (5 mg/liter) synthetic medium.

Plasmid constructions. All DNA and bacterial manipulations were by standard protocols (55). Plasmids are listed in Table 2. The CEN vectors pRS313 (23, HIS3 marker), pRS314 (TRP1 marker), and pRS316 (URA3 marker) (59) were primarily used for cloning. The CEN plasmid pCT3 contains the URA3 marker and was a gift from C. Thompson. The 2μm vector YEp352 contains the URA3 marker and was a gift from A. Tzagoloff. Three plasmids (pGE98 to pGE100) were constructed for the synthetic lethal screen. A 1.4-kb NheI/PvuII fragment containing the NSL1-GFP marker was band isolated on a 3.6-kb XmnI/PvuII fragment from pCH1023 and cloned into the EcoRI and SpeI sites of pRS314. Second, the 1.4-kb XmnI/PvuII fragment containing NNF1 and its promoter was inserted in the Smal site of the multiple cloning site to complete pGE100.

**Generation of Nnf1p mutants.** Temperature-sensitive mutations of NNF1 were generated by methods described previously (43, 47). Briefly, NNF1 was amplified by mutagenic PCR, and cotransformed with gapped vector into a nff1::URA3 strain carrying PGAL1-NNF1 in a HIS3 plasmid. Transformants were selected at 28°C on SC-His-Leu medium (containing 2% galactose as the sole carbon source) to maintain expression of NNF1. The transformation plates were then replica plated onto two sets of SC-Leu plates and incubated at 25 or 37°C. Plasmid DNA from potential temperature-sensitive nff1 mutations was rescued into Excerichia coli, restated in the nff1::URA3 yeast strain, and sequenced. The nff1-17 allele analyzed here had three amino acid changes: C53S, V78D, and M187N.

**Selection for dosage suppressors.** High- and low-copy suppressors of the temperature-sensitive growth defect of nff1-17 cells were selected at 35°C following transformation of strain GEY138 with S. cerevisiae genomic libraries in either 2μm (70) or CEN (71) plasmids. Cells were plated on SC-Ura medium for plasmid selection and allowed an initial recovery for 12 h at 25°C before the plates were shifted to the restrictive temperature (35°C) for 4 days. A total of 24 colonies were isolated at 35°C, corresponding to over 150,000 transformants at the permissive temperature of 25°C. Twenty of the 24 plasmids recovered from these colonies allowed growth at 35°C when retransformed into strain GEY138. Restriction digests showed that these 20 plasmids contained nine different inserts. Subclones were made in the 2μm vector YEp352 to identify the genes suppressing the nff1-17 growth defect. Plasmids pGE184, pGE190, and pGE151

| Strain(s) | Genotype |
|-----------|----------|
| W961-5A | MATA leu2-3,112 trp1-1 ura3-1 ade2-1 HIS3+ can1-100 |
| GEY145 | MATA/mao leu2-3,112/trp1-1/NNF1-17 his3-11,15 can1-100/100 |
| GEY146 | MATA/mao leu2-3,112/trp1-1/NNF1-17 his3-11,15 can1-100/100 |
| CH1305 | MATA his2 leu3 ade2 can1-100 |
| ABY112 | MATA/mao leu2-3,112/trp1-1/NNF1-17 his3-11,15 can1-100/100 |

* Strains with a GEY prefix are in a W303 background.
TABLE 2. Plasmids used in this study

| Plasmid* | Relevant markers |
|----------|------------------|
| pCH122* | CEN, URA3, ADE3 |
| pCH1023* | CEN, LEU2, ADE3 |
| pDK243* | CEN, LEU2, ade-3p |
| pW35* | 2μm, URA3, SSD1/SRK1 |
| pGE36 | CEN, TRP1, DSN1-GFP |
| pGE55 | CEN, TRP1, NSL1-GFP |
| pGE74 | CEN, TRP1, DSN3-GFP |
| pGE81 | CEN, URA3, NSL1; pGE180 gap-repaired Snb1-EcoNI in yeast strain YSLP1 |
| pGE82 | CEN, URA3, nsl1-6; pGE180 gap-repaired Snb1-EcoNI in yeast strain YSL6 |
| pGE83 | CEN, URA3, nsl1-8; pGE180 gap-repaired Snb1-EcoNI in yeast strain YSL8 |
| pGE84 | CEN, URA3, nsl1-68; pGE180 gap-repaired Snb1-EcoNI in yeast strain YSL68 |
| pGE89 | CEN, URA3, DSN3/NSL2; pGE170 gap-repaired MluI-Spel in yeast strain YSLP1 |
| pGE90 | CEN, URA3, dsn3-29/DSL2-29; pGE170 gap-repaired MluI-Spel in yeast strain YSL29 |
| pGE99 | CEN, TRP1; NNFI on a 1.4-kb XmnI-PvuII insert and ADE3 on a 3.6-kb Eagl-Nhel insert |
| pGE100 | CEN, TRP1; NNFI on a 1.4-kb XmnI-PvuII insert and ADE3 |
| pGE101 | CEN, URA3; NNFI on a 1.4-kb XmnI-PvuII insert and ADE3 |
| pGE102 | CEN, ade-3p |
| pGE151 | 2μm, URA3, 1.4-kb HindIII subclone from YEp24-based library; contains DSN3/NSL2 |
| pGE164 | 2μm, URA3, 2.7-kb BsaAI subclone from YEp24-based library; contains DSN1 |
| pGE165 | CEN, URA3, 2.7-kb BsaAI subclone from YEp24-based library; contains DSN1 |
| pGE170 | CEN, URA3, 3.1-kb KpnI subclone from YEp24-based library; contains DSN3/NSL2 |
| pGE171 | CEN, URA3, 1.4-kb HindIII subclone from YEp24-based library; contains DSN3/NSL2 |
| pGE180 | CEN, URA3, 2.0-kb PvuII-NruI subclone from pCT3-based library; contains NSL1 |
| pGE190 | 2μm, URA3, 3.3-kb HindIII subclone from YEp24-based library; contains SSL1 |

*Plasmids are from this study unless otherwise indicated.

b From C. Holm.

c From D. Koshland.

d From K. Tatchell.

The screening plasmid pGE98 (CEN/NNFI/ADE3/TRP1) was grown in SC-Ura at 25°C, sonicated, plated on SC low-Ade plates, and UV irradiated to ~10% survival. The mutagenized cells were incubated at 30°C for 7 days, when the colony color had fully developed. From 102,000 colonies that survived the UV mutagenesis, 17 nonsectoring derivatives were identified. As integration of the plasmid or mutations at the chromosomal ade3 locus could also result in a nonsectoring phenotype, the stably red strains were transformed with pGE99 (CEN/NNFI/TRP1) or empty pRS314 (CEN/TRP1) vector. Six mutants which sectord upon transformation with pGE99, but not with empty vector, were selected for cloning by low-copy suppression and nonsectoring phenotype at 30°C.

Cloning and allelic rescue of NLS1. The screening plasmid pGE98 (CEN/NNFI/ADE3/TRP1) in the six candidate strains from the synthetic lethal screen was replaced with pGE100 (CEN/NNFI/ADE3/TRP1) by plasmid shuffling and counterselection on 5-fluoroorotic acid (5-FOA) so that a URA3/CEN yeast genetic library (71) could be used to clone the gene(s) causing synthetic lethality with snl1-17. Ura+ transformants were selected on SC-Ura plates at 30°C. Two sectoring colonies were identified after transformation of the library DNA; one colony from isolate YSL6 (12,000 transformants) and one colony from isolate YSL8 (14,000 transformants). Plasmid DNA from these sectoring colonies was recovered and checked for the ability to confer sectoring in all six of the synthetic lethal strains. Both plasmids complemented the nonsectoring phenotype of strains YSL6 and YSL8 as well as the nonsectoring phenotype of a third strain, YSL89. The plasmids contained a common yeast genomic insert from chromosome XVI. Subcloning of this insert into pRS316 to create pGE180 reduced the complementing region to a 2.0-kb PvuII/NruI fragment that contained NLS1 (YPL233w) as the only open reading frame (ORF).

Chromosomal NLS1 and its alleles nsl1-6, nsl1-8, and nsl1-68 were recovered by gap repair of pGE180 from the parent strain YSLP1 (NSL1) and the mutant strain YSL6 (nsl1-6), YSL8 (nsl1-8), and YSL68 (nsl1-68). For the gap repair, pGE180 was digested with SpeI and EcoNI and band isolated to remove a 920-bp fragment containing 640 bp of wild-type NLS1 and 280 bp of 5′ noncoding region. Plasmid DNA was isolated from Ura+ transformants, and gap repair was verified with an EcoRV digest to produce a 1.2-kb fragment. The gap-repaired vectors were designated pGE81, pGE62, pGE35, and pGE34 for NSL1, nsl1-6, nsl1-8, and nsl1-68, respectively (Table 2). The four gap-repaired plasmids pGE81, pGE62, pGE35, and pGE34 were transformed into the nsl1-17 nsl1 strains YSL6, YSL8, and YSL68 carrying pGE100 (CEN/NNFI/ADE3/TRP1). Only pGE81 with wild-type NSL1 could be plasmid shuffled with pGE100 in YSL6, YSL8, and YSL68 to restore sectoring.

Cloning and allelic rescue of NSL2. All six of the synthetic lethal strains carrying pGE100 (CEN/NNFI/ADE3/TRP1) were also transformed with the dosage suppressors SDI1, SLG1, DS1, and DS3 shuffled into the CEN/URA3 plasmid pR316 to ascertain whether any of the genes might be both a multicopy suppressor of the nsl1-17 allele and also have a mutation that is synthetically lethal in combination with nsl1-17. Plasmid pGE171 containing DS3 was able to complement the nonsectoring phenotype of the mutant YSL29. To distinguish between the possibilities that DSN3/NSL2 is able to act as both a high- and low-copy suppressor of nsl1-17 and that a dsn3(nsl2) mutation is synthetically lethal with nsl1-17, the plasmid shuffle with pGE100 was repeated in synthetic lethal strain YSL29 with DSN3 (YSL68) and dsn3-29 (YSL29) from strains YSLP1 and YSL29 cloned into pRS316. To isolate DSN3/NSL2 and the dsn3-29 (nsl2-29) allele, genomic DNA was prepared from the parent strain YSLP1 and the mutant strain YSL29, and a 1.7-kb region around the ORFs was PCR amplified with primers YAL560 (5′-CTATCCTGGGTCGTGATTTGA-3′) and YAL220 (5′-CTGTCGAACTATTGTCCGCTCA-3′). The 1.7-kb PCR product was then restricted to 1.3 kb with MluI [which cuts 105 bp before the ATG of DSN3 (YSL2) and SpeI which cuts 350 bp after the DSN3 (YSL2) stop codon]. This 1.3-kb MluI/Spel fragment was used to replace the same 1.3-kb MluI/Spel sequence in pGE170, resulting in pGE89 (DNS3/NSL2) from the parent strain) and pGE90 (dsn3-29 (nsl2-29) from the UV-mutagenized strain). Plasmid pGE89 was able to complement the nonsectoring phenotype of YSL29, whereas plasmid pGE90 was not able to complement the nonsectoring phenotype of this strain. Therefore, YSL2 is identical to DSN3.

Gene disruptions. DSN1, DSN3/NSL2, and NLS1 were disrupted singly in a W303 background by one-step gene replacement (53). Correct integrations at the DSN1, DSN3/NSL2, and NSL1 chromosomal loci were verified by PCR or Southern blotting. For each gene disruption, three independent transformants which had a single integration at the intended locus were chosen for tetrad dissection and analysis. For the donl::HIS3 disruption, the resulting heterozygous diploid strains were GEY170, GEY171, and GEY172. For the donl::UFL2 disruption, the resulting heterozygous diploid strains were GEY210, GEY211, and GEY212. For the nsl1::LEU2 disruption, the resulting heterozygous diploid strains were GEY190, GEY191, and GEY192.

Epitope tagging. Six repeats of the Myc epitope on a 270-bp DnuI/XbaI fragment were inserted at the 3′ end of NFP1. The sequence at the C terminus of NFP1 now translates as YWIKAA(MEK/LISLEDELINE)(S)SCP. For integration and selection at the NFP1 chromosomal locus, the LEU2 selectable marker on a 2.0-kb XbaI fragment was ligated after the NFP1 stop codon and before the 164 bp of 3′ noncoding region. Yeast strain W616-5A was transformed with NFP1-myc::LEU2 on a 3.5-kb HindIII fragment, and genomic DNA from Leu− transformants was analyzed by Southern blotting. Strain GEY110 has NFP1-myc::LEU2 successfully integrated at the NFP1 locus. Strain GEY111 was made from GEY110 and expresses a GFP(N63)-tagged allele of CMN67 from the CMN67 chromosomal locus. The CMN67-GFP(N63)-kanMX6 gene fusion was PCR amplified from ABY112 genomic DNA with primers CMN1940 (5′-GAGCTGAGGCCCATTTCTCAG-3′) and CMN3200 (5′-CTCTCATAAGGGCTCGAATCAG-3′). The PCR product was band isolated and trans-
formed into strain GEY110. Transformants were selected on YPD-G418 plates. Correct integration at the CEN67 locus was verified by PCR analysis. GFP(S65T) was fused to the C terminus of DSN3 in several steps. DSN1 was amplified from pGE164 by using primers YIRSac1 (5′-GCGCTTGAAGAGGT AGTCACGCAAGAG-3′) and YIREcoRV (5′-CTTTATTACATCGATTCTTTTACTGA-3′). Primer YIR Sac1 anneals 500 bp before the ATG of DSN1. Primer YIREcoRV mutates an EcoRV site at the C terminus of DSN3, which after EcoRV digestion removes the final codon (leucine) and the stop codon. The resulting 2.3-kb PCR product was digested with SacI and EcoRV, and this fragment was ligated into the SacI and EcoRV sites of pRS313. GFP(S65T) on a NotI fragment was cut from pSF-GP1 (a gift of J. Hirsch), filled in with Klenow polymerase, and ligated into the EcoRV site to create pGE35.

For GFP(S65T)-tagging of NSL1, NSL1 and its promoter were amplified by PCR from pGE180 with the primers T7 and YPL1640 (5′-GACGCCGAGGAACGATTTACTACAATCCGTCGCCGAGAG-3′). The NSL1 PCR product was digested with Smal and PstI and cloned into the EcoRV and PstI sites of pBS SK−. The resulting plasmid was cut with Smal (in the multiple cloning site region next to PstI), and GFP(S65T) on a 750-bp NotI fragment (filled with Klenow polymerase) was ligated into this Smal site. This cloning changes the last amino acids of NSL1 from EED to QP. The entire NSL1-GFP fusion was removed from the pBS SK− backbone with HindIII-XbaI digest and cloned into the HindIII and XbaI sites of pRS316, to make pGE54.

GFP(S65T) was fused to the C terminus of DSN3 by first PCR amplifying the DSN3 promoter and ORF from pGE170 with primers YAL120EcoRI (5′-CTCCGCAGGGATCCAGATGTCGGTATGTGATTAG-3′) and YAL1860NotI (5′-GTAAGCAGGGCAGGCTAACAATCATCACTAGAATACCAATTGAGG-3′). Primer YAL120EcoRI introduces an EcoRI site 900 bp before the DSN3 start codon, and primer YAL1860NotI anneals before the stop of DSN3, to remove the termination codon and add a Nof restriction site. The 1,740-bp PCR product was digested with EcoRI and NotI and cloned into the EcoRI and NotI sites of pRS316. GFP(S65T) on a 750-bp NotI fragment was then ligated into the NotI site, resulting in plasmid pGE73.

Plasmid stability assay. The plasmid stability assay is based on greater incidence of plasmid loss under nonselective conditions in affected mutants (40, 41). Diploid strains GYE160 and GYE165 were transformed with pCT3ade6-2p (pGE102), and single transformants were grown overnight at 25°C in SC-Ura medium. The next day, the starter cultures were diluted in SC complete medium (pGE102), and single transformants were grown overnight at 25°C and then rinsed with pGE102, and single transformants were grown overnight at 25°C and then rinsed in SC-Ura medium. The next day, the starter cultures were diluted in SC complete medium at 30°C for ~4 doublings before plating on SC-low-ade plates prewarmed to 30°C. Colonies were scored after 5 days at 30°C as follows: (i) half-sectored, colonies that are half pink (with red and white sectors) and half white; (ii) pink, colonies pink that have pink and white sectors, not including half-sectored colonies; (iii) white colonies that are solid white with no sectors; and (iv) red, colonies that are dark red. To confirm that plasmid loss in the nff1-17 mutant was due to the nff1-17 mutation alone, the nff1-17 diploid strain GYE165 was cotransformed with pCT3ade6-2p (pGE102), and single transformants were grown overnight at 25°C in SC-Ura medium. The next day, the starter cultures were diluted in SC complete medium, at 30°C for ~4 doublings before plating on SC-low-ade plates prewarmed to 30°C. After 5 days at 30°C, colonies were scored as described above.

Flow cytometry. Cells were prepared for flow cytometry as described previously (27). For each sample a minimum of 25,000 cells were analyzed using a FACs Vantage flow cytometer (Becton Dickinson, San Jose, Calif.).

Fluorescence microscopy. Indirect immunofluorescence against tubulin was carried out essentially as described previously (50). For localization of SPB antigens, short fixation times were used, as suggested previously (54). Cells carrying GFP and/or Myc fusions were fixed for 5 to 10 min in 3.7% formaldehyde at 25°C. Spheroplasts were mounted on polyethyleneimine-coated slides and then rinsed in −20°C methanol for 6 min followed by −20°C acetone for 30 s. Primary antibody sources and dilutions were rat anti-α-tubulin monoclonal antibody YOL1/34 at 1:200 (Harlan Scra-lat, Indianapolis, Ind.), mouse anti-a-Myc epitope monoclonal 9E10 at 1:50 (BABCO, Richmond, Calif.), and rabbit anti-GFP at 1:30 (Clontech, Palo Alto, Calif.). Secondary antibody sources and dilutions were fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit immunoglobulin G (IgG) at 1:200, Cy3-conjugated goat anti-mouse IgG at 1:500, and FITC-conjugated goat anti-rabbit IgG at 1:200. All secondary antibodies were purchased from Jackson Immunoresearch, West Grove, Pa. Cells were photographed with Kodak X-OMAX p3200 film at ASA 1600. Primary antibodies lacking primary antibody were used to make sure that there was not any crossover fluorescence between the FITC and Cy3 fluorophores. First, only faint GFP fluorescence was seen when cells from strain GEY111 were incubated with both FITC- and Cy3-conjugated secondary antibodies in the absence of primary antibodies. Second, FITC signal alone was detected when GEY111 cells were incubated with the anti-GFP polyclonal antibody as the only primary antibody and with both FITC- and Cy3-conjugated secondary antibodies. The third control experiment with the anti-Myc 9E10 monoclonal antibody as the only primary antibody and both FITC- and Cy3-conjugated secondary antibodies had to be carried out with Nnf1p-Myc, cells from strain GEY101 because GFP fluorescence can still be seen even without amplification from the anti-GFP antibody. In summary, no crossover fluorescence between Cy3 and FITC was detected in the absence of either or both anti-Myc and anti-GFP primary antibodies.

For MT depolymerizations, cultures of haploid yeast strains (GEY110, GEY176, GEY206, and GEY216) expressing either Nnf1p-Myc, Dsn1p-GFP, Dsn3p-GFP, or Nofp-GFP from their endogenous promoters were split and either treated with 17 μg of nocodazole (Sigma Chemical Co.) per ml from a 1.5-mg/ml stock in dimethyl sulfoxide (DMSO) or mock treated with DMSO for 2.5 h at 30°C. Cells were fixed and prepared for indirect immunofluorescence, as described above, using either tubulin antibodies or anti-Myc or anti-GFP antibodies. For the nocodazole experiment, cells were imaged with a Leica fluorescence microscope and a CCD camera (Princeton Instruments, Inc.).

RESULTS

The nff1-17 mutant is defective in nuclear division. NNF1 is essential for viability. To determine whether Nnf1p may have a specific role in the cell division cycle and to analyze the onset of the nff1-17 defect, phenotypes of logarithmically growing nff1-17 cells were examined after a shift to the nonpermissive temperature of 37°C. In wild-type strains, large buds are indicative of cells at the G2/M border (reviewed in reference 51). After 3 h at 37°C, large-budded cells comprised more than 70% of the nff1-17 population, compared to ~30% of the NNF1 population (Fig. 1A). The majority of these nff1-17 cells were unineucleate with the nucleus positioned at the bud neck. In a small percentage of nff1-17 cells, nuclear division was completed in the mother cell, resulting in binucleate cells. To estimate DNA content and look for aneuploidy, flow cytometry was carried out on asynchronous cultures of NNF1 and nff1-17 cells. At time zero (Fig. 1B) homozygous diploid NNF1 and nff1-17 cells generated two peaks, corresponding to 2N and 4N DNA contents. After 3 h at 37°C, these peaks are maintained in the wild-type population, whereas in the nff1-17 population, these two peaks merge and broaden, indicating cells with a wide range of DNA content. Thus, it appears that the nff1-17 mutant fails to arrest at the G2/M border. The nff1-17 mutation leads to a mitotic defect, as DNA does not segregate evenly in the mutant.

MT morphologies in large-budded cells were examined after 3 h at 37°C (Fig. 1C). The mitotic spindle was short in most (~85%) large-budded nff1-17 cells (columns a and f to h in Fig. 1C). These short spindles were sometimes misoriented with respect to the mother-daughter axis (column b in Fig. 1C). In a smaller percentage of the nff1-17 population, the cytoplasmic MTs were also affected, in that they were either elongated (columns d and h) or missing altogether (columns c and g). The nff1-17 mutation does not cause a global defect in MT function, because the mutant is not hypersensitive or resistant to benomyl and displays no karyogamy defect (data not shown). Therefore, Nnf1p is required for mitotic spindle elongation and orientation.

nff1-17 cells display plasmid instability. If mitotic spindle function is the primary nff1-17 defect, then there may be a partial spindle defect in nff1-17 cells under semipermissive growth conditions. A centromere-plasmid stability assay was used to detect mitotic segregation defects (40, 41). NNF1 ade2
ade3 and nnf1-17 ade2 ade3 strains carrying the CEN/ade3-2p plasmid pGE102 were grown under nonselective conditions at 30°C (Fig. 2A and B). The rate of plasmid loss can be determined from the number of colonies that are half pink (one copy of pGE102) and half white (zero copies of pGE102) due to plasmid loss during the first mitotic division upon plating. For NNF1 cells, the CEN plasmid loss rate was 1.8% ± 0.3% per generation; for nnf1-17 cells, the rate was 17.0% ± 1.8% (see Materials and Methods) (Fig. 2C). The presence of an NNF1 plasmid had no effect on wild-type plasmid stability but complemented the nnf1-17 defect. Therefore, the nnf1-17 mutation causes CEN plasmid instability under semipermissive growth conditions. These observations support the hypothesis that Nnf1p is required for mitotic spindle function.

**Isolation and characterization of suppressors of nnf1-17.** Dosage suppressors of nnf1-17 were isolated by selection for growth at the minimum restrictive temperature (35°C). In addition to NNF1 itself, four other genes were identified: SSD1 (SRK1), SLG1 (WSC1), and the newly identified genes DSN1 and DSN3 (dosage suppressor of NNF1). Only NNF1 in either a CEN or 2μ vector was able to restore wild-type growth to the nnf1-17 strain at 37°C. High-copy DSN1, SLG1, and DSN3 allowed growth at 35°C to various degrees (Fig. 3).
The DSN1 protein is 576 amino acids and has a predicted molecular mass of 66 kDa and a predicted pI of 5.2. A match to a consensus bipartite nuclear localization sequence is found at residues 414 to 430; a potential EF-hand calcium binding site is found at residues 398 to 410. Expression of DSN1 peaks during G2 (62). Dsn1p has no significant overall similarity to any known protein in the available databases.

The DSN3 protein is 289 amino acids and has a predicted molecular mass of 33 kDa and a predicted pI of 5.1. DSN3 was found to be equivalent to MTW1, a gene identified in S. cerevisiae through its homology to the Schizosaccharomyces pombe gene mis12 (17, 18). In budding yeast, Mtw1p-GFP is closely situated near the SPBs as seen by fluorescence microscopy. Mtw1p coimmunoprecipitates with centromere DNA, and the temperature sensitive mtw1-1 mutant exhibits unequal chromosome segregation (18). Recently Mtw1p was reported to be present in a highly enriched preparation of spindle poles, as detected by matrix-assisted laser desorption-ionization mass spectrometric analysis (76).

SLG1 and SSD1 are both nonessential genes. Slg1p is a plasma membrane protein that is part of the Pkc1p pathway (19, 31, 44, 73). Overexpression of SLG1 may maintain viability in the nbf1-17 background through the heat shock response and cell integrity signaling or through its effects on the cell cycle and SPB duplication (29, 35, 65). Ssd1p is a cytoplasmic protein which may control RNA metabolism by affecting RNA stability (72). Various wild-type yeast laboratory strains have polymorphisms of SSD1. The dominant SSD1-v allele, present in the wild-type strain S288C, has been found in single or multiple copies to partially suppress growth defects associated with diverse mutations (13, 14, 38, 63). In this study nbf1-17 was present in a W303 background, and the wild-type strain W303 has the recessive ssd1-d allele.

Identification of mutations that are lethal in a nbf1-17 strain. A synthetic lethal screen was also used to identify gene products that may act in conjunction with Nnf1p. These clones were called NSL genes (NNF1 synthetic lethal). An ade2 ade3 colony sectoring assay (1, 42) was carried out at 30°C. A total of 102,000 mutagenized colonies of strain YSL1 (ade2 ade3 nbf1-17) carrying an ADE3/NNF1 plasmid were screened to find isolates that had a stable red, nonsectoring phenotype. Four isolates (YSL6, YSL8, YSL29, and YSL68) were chosen for cloning by complementation of the nonsectoring phenotype.

Cloning of NSL1. Plasmids carrying YPL233w and small flanking regions complemented the nbf1 mutations YSL6, YSL8, and YSL68. To verify that mutants YSL6, YSL8, YSL68 carry nbf1 mutations, the alleles nbf1-6, nbf1-8, nbf1-68, and NSL1 were isolated by gap repair of YPL233w from strains YSL6, YSL8, YSL68, and YSL1, respectively. Colony sectoring assays indicated that the retrieved NSL1 allele complemented mutants YSL6, -8, and -68, whereas the retrieved nbf1-6, -8, and -68 did not (Fig. 4). Therefore, YPL233w is NSL1, and it encodes a novel protein of 216 amino acids with a predicted molecular mass of 25 kDa and a predicted pI of 4.7. No significant sequence similarity was found between Nsl1p and proteins in current databases.

![Figure 3](image3.png)

**FIG. 3.** Growth of an nbf1-17 mutant with various suppressors at 30, 35, and 37°C. The nbf1-17 strain GEY138 was transformed to Ura" at 25°C with pGE101 (CEN/NNF1), pRS316 (empty vector), pGE164 (2μ/DSN1), pGE190 (2μ/SLG1), or pGE151 (2μ/DSN3). The transformed strains were streaked on the same minimal SC-Ura plates and incubated for 2 days at the indicated temperatures.

![Figure 4](image4.png)

**FIG. 4.** Complementation of strain YSL68 by NSL1 and of strain YSL29 by DSN3(MTW1). The synthetic lethal mutant YSL68 carrying plasmid pGE100 (CEN/TRP1/ADE3/NNF1) was transformed with either pGE84 (CEN/URA3/nsl1-68) or pGE81 (CEN/URA3/NSL1). The synthetic lethal mutant YSL29 carrying pGE100 was transformed with either pGE90 (CEN/URA3/dsn3-29) or pGE89 (CEN/URA3/DSN3). Transformants were selected on SC-Ura low-Ade plates at 30°C for 5 days. Wild-type NSL1 and DSN3(MTW1) restore the sectoring phenotype to strains YSL68 and YSL29, respectively. Comparable results were obtained with plasmid pGE82 in strain YSL6 and plasmid pGE83 in strain YSL8.
The UV mutagenesis might have produced nsl1 mutations conferring a distinct growth defect. However, no growth defects were observed when the nsl1-6, nsl1-8, and nsl1-68 alleles were placed in a nsl1::LEU2 mutant strain. Hence, nsl1-6, nsl1-8, and nsl1-68 probably represent only a partial loss of Nsl1p function.

Mutations in DSN3 (MTWI) are synthetically lethal in combination with nsl1-17. I reasoned that the same genes might be isolated through nsl1-17 synthetic lethality and dosage suppression. Therefore low-copy plasmids carrying each dosage suppressor were tested for complementation of all YSL mutants. DSN3 (MTWI) complemented the nonsectoring phenotype of mutant YSL29.

To establish that a mutation in DSN3 was synthetically lethal in combination with nsl1-17 and that DSN3 was not just a low-copy suppressor of the mutations in YSL29, chromosomal dsn3-29 and DSN3 were recovered from the mutant strain YSL29 and the parent strain YSLP1, respectively, and a plasmid shuffle experiment similar to the one performed with the nsl1 isolates was carried out (see also Materials and Methods). DSN3 (in plasmid pGE89) fully complemented the nonsectoring phenotype of the YSL29 mutant, but dsn3-29 (in plasmid pGE90) did not (Fig. 4). Therefore, YAL034w-a displays both synthetic lethality and dosage suppression with nsl1-17. The dsn3-29 allele does not confer a growth defect, since no temperature sensitivity was observed in a dsn1::LEU2 strain carrying dsn3-29 in a CEN plasmid as the only source of Dsn3p.

**DSN1, DSN3 (MTWI), and NSL1 are essential genes.** Wild-type diploid strains (CY6 or GEY160) were transformed with dsn1::HIS3, dsn3::LEU2, or nsl1::LEU2 constructs, and for each disruption, three independent transformants that showed a single integration at the DSN1, DSN3, or NSL1 locus were chosen for tetrad analysis. Dissection of the dsn1::HIS3, dsn3::LEU2, or nsl1::LEU2 heterozygotes resulted in poor spore viability (28 to 40%) and a number of His⁺ or Leu⁺ progeny (Tables 3 to 5). Southern analysis showed that all three His⁺ spores from the dsn1::HIS3 heterozygote still maintained a wild-type copy of DSN1, and these spores were not able to mate with MATa or MATα tester strains (Table 3). Therefore, these His⁺ spores may be diploids or disomes. Similar results were obtained for the DSN3 and NSL1 disruptions (Tables 4 and 5). Low spore viability and disome or diploid progeny appear to be part of the mutant phenotypes, as sporulation and dissection of the CY6 and GEY160 parent strains resulted in 95 and 93% viability, respectively. None of these spores was a nonmater. By inference, the His⁺ or Leu⁺ spores recovered from the dsn1::HIS3, dsn3::LEU2, or nsl1::LEU2 heterozygotes are the result of nuclear or chromosome missegregation.

The failure to recover haploid dsn1::HIS3, dsn3::LEU2, or nsl1::LEU2 spores suggested that DSN1, DSN3, and NSL1 are essential for spore germination and perhaps viability. To verify this conclusion, these dsn1::HIS3, dsn3::LEU2, or nsl1::LEU2 heterozygous diploids carrying CEN/URA3 plasmids with DSN1, DSN3, and NSL1, respectively, were tested for the ability to produce meiotic segregants lacking the plasmid (Tables 3 to 5). All of the His⁺ or Leu⁺ spores either were unable to grow on 5-FOA medium (which selects against Ura⁻ and thus against plasmid loss) or still maintained a wild-type copy of the gene targeted for disruption, as detected by PCR analysis. To conclude, DSN1, DSN3, and NSL1 are essential genes.

### TABLE 3. Tetrad data from DSN1 disruption

| Strain(s) (genotype) | % Spore viability | DSN1 (His⁺ spores) | DSN1/dsn1::HIS3 (His⁺ spores)³ | dsn1::HIS3 + pCEN/URA3/DSN1 (His⁺ Ura⁺ spores)³ |
|----------------------|-------------------|---------------------|-------------------------------|-----------------------------------------------|
| CY6 (DSN1/DSN1)      | 95                | 19                  | NA²                          | NA                                            |
| GEY170, GEY171, GEY172 (dsn1::HIS3/DSN1) | 28                | 19                  | 3                             | NA                                            |
| GEY170, GEY171, GEY172 + pGE165 (dsn1::HIS3/DSN1 + pCEN/URA3/DSN1) | 43                | 17                  | 6                             | 6                                             |

¹ All nine of the His⁺ spores were nonmating and still had a wild-type copy of DSN1 which was detected by Southern analysis, as described in Materials and Methods.
² All six of the His⁺ Ura⁺ spores were not able to lose pCEN/URA3/DSN1 on 5-FOA plates.
³ NA, not applicable.

### TABLE 4. Tetrad data from DSN3 disruption

| Strain(s) (genotype) | % Spore viability | DSN3 (Leu⁺ spores) | DSN3/dsn3::LEU2 (Leu⁺ spores)² | dsn3::LEU2 + pCEN/URA3/DSN3 (Leu⁺ Ura⁺ spores)² |
|----------------------|-------------------|---------------------|-------------------------------|-----------------------------------------------|
| GEY160 (DSN3/DSN3)   | 93                | 41                  | NA²                          | NA                                            |
| GEY210, GEY211, GEY212 (dsn3::LEU2/DSN3) | 40                | 35                  | 4                             | NA                                            |
| GEY210, GEY211, GEY212 + pGE171 (dsn3::LEU2/DSN3 + pCEN/URA3/DSN3) | 60                | 43                  | 5                             | 19                                            |

² Six out of the nine Leu⁺ spores were nonmating. All nine of the Leu⁺ spores still had a wild-type copy of DSN3 which was detected by PCR, as described in Materials and Methods.
³ All 19 of the Leu⁺ Ura⁺ spores were not able to lose pCEN/URA3/DSN3 on 5-FOA plates.
⁴ NA, not applicable.
Nnf1p, Mtw1p, Dsn1p, and Nsl1p colocalize and are found near the spindle poles. Epitope-tagged Nnf1p-Myc6 expressed from the NNF1 locus (strain GEY110) was used for subcellular localization studies. Indirect immunofluorescence showed that Nnf1p-Myc6 is present at the nuclear periphery as a single dot in unbudded cells and as side-by-side dots in small-budded cells. In large-budded cells, Nnf1p appears at opposite ends of the dividing nucleus. This localization pattern is typical of spindle pole body proteins (see, e.g., reference 75) and certain kinetochore proteins that transiently cluster at the spindle poles during various stages of mitosis (10, 16, 32, 33, 45, 75, 76).

Double-label immunofluorescence with Nnf1p-Myc6 and an SPB marker, GFP-tagged Cnm67p (4, 75), was undertaken to verify spindle pole proximity of Nnf1p (strain GEY111) (Fig. 5). Several controls indicated an absence of crossover fluorescence between the GFP and Myc detection systems (see Materials and Methods). In cells where both Nnf1p-Myc6 and Cnm67p-GFP were visible, the fluorescent dots were closely positioned (in 54 of 54 cells scored), implying that Nnf1p resides near the spindle poles. This Nnf1p-Myc staining pattern was visible throughout the cell cycle, and no MT staining was observed.

Fusions between GFP(S65T) and the C-terminal ends of DSN1, MTW1, and NSL1 were constructed to allow visualization of Dsn1p, Mtw1p, or Nsl1p in both living and fixed cells. All three fusions were expressed under control of endogenous promoters from CEN plasmids and were able to rescue the respective disruptions in plasmid shuffle experiments. The same localization patterns that are characteristic of SPBs or some kinetochore proteins were seen for Dsn1p-GFP, Mtw1p-GFP, and Nsl1p-GFP. Single cells showed one fluorescent dot which was seen to be nuclear when the cells were grown in the presence of DAPI (4’,6’-diamidino-2-phenylindole). Two fluorescent dots were seen in budding cells. The dots were adjacent in small-budded cells but were separated in large-budded cells. For colocalization with Nnf1p, Dsn1p-GFP, Mtw1p-GFP, and Nsl1p-GFP were transformed separately into the strain GEY110. For colocalization of Nnf1p and the SPB component Cnm67p in strain GEY111. For colocalization of Nnf1p with Mtw1p, Dsn1p, and Nsl1p, strain GEY110 was transformed with pGE74, pGE36, or pGE55, respectively. NNF1-myc6 and CNM67-GFP are integrated in place of wild-type NNF1 or CNM67 in a haploid strain and are expressed from the endogenous promoters. The MTW1-GFP, DSN1-GFP, and NSL1-GFP fusions are expressed from their own promoters in CEN plasmids. For the indirect double-label immunofluorescence, cells were short fixed first in formaldehyde and then in methanol and acetone. The Myc epitope was detected with the monoclonal antibody 9E10 and a Cy3-conjugated goat antimouse secondary antibody. GFP fluorescence was enhanced with a rabbit anti-GFP antibody and an FITC-labeled goat anti-rabbit secondary antibody. Bar, 5 μm.

![Image](Image.png)

**FIG. 5.** Localization of Nnf1p, Mtw1p, Dsn1p, and Nsl1p to the region of the spindle poles. First row, localization of Nnf1p and the SPB component Cnm67p in strain GEY111. For colocalization of Nnf1p with Mtw1p, Dsn1p, and Nsl1p, strain GEY110 was transformed with pGE74, pGE36, or pGE55, respectively. NNF1-myc6 and CNM67-GFP are integrated in place of wild-type NNF1 or CNM67 in a haploid strain and are expressed from the endogenous promoters. The MTW1-GFP, DSN1-GFP, and NSL1-GFP fusions are expressed from their own promoters in CEN plasmids. For the indirect double-label immunofluorescence, cells were short fixed first in formaldehyde and then in methanol and acetone. The Myc epitope was detected with the monoclonal antibody 9E10 and a Cy3-conjugated goat antimouse secondary antibody. GFP fluorescence was enhanced with a rabbit anti-GFP antibody and an FITC-labeled goat anti-rabbit secondary antibody. Bar, 5 μm.
To determine whether the Nnf1p-Myc<sub>6</sub>, Dsn1p-GFP, Mtw1p-GFP, and Nsl1p-GFP localizations could be maintained in the absence of MTs, cultures of haploid yeast strains expressing these fusion proteins were treated with nocodazole to depolymerize MTs or were mock treated with DMSO. After depolymerization, antitubulin immunofluorescence revealed that MT arrays were lost in more than 93% of the cells (data not shown), indicative of effective nocodazole treatment (30). As shown (Fig. 6), the characteristic dot staining patterns were still present for the Nnf1p-Myc<sub>6</sub>, Dsn1p-GFP, Mtw1p-GFP, and Nsl1p-GFP proteins. Nocodazole treatment did not vary the number of cells showing Nnf1p-Myc<sub>6</sub>, Dsn1p-GFP, Mtw1p-GFP, and Nsl1p-GFP staining (>85% for both nocodazole and DMSO treatments), and staining intensities were comparable in treated and untreated cultures. In large-budded cells with a single nucleus, the percentage of cells with only one visible dot was higher in nocodazole-treated Nnf1p-Myc<sub>6</sub>, Dsn1p-GFP, Mtw1p-GFP, and Nsl1p-GFP cultures (~40 to 50%; n > 90) than in the untreated cultures (~35%; n > 100). This increase in the single-dot staining pattern may reflect either duplicated but unseparated SPBs or sister chromatids (15, 30, 66). In short, Nnf1p, Dsn1p, Mtw1p, and Nsl1p staining did not diminish with nocodazole treatment and spindle collapse.

**FIG. 6.** The localization of Nnf1p, Dsn1p, Mtw1p, and Nsl1p does not depend on MTs. Cultures of haploid yeast expressing either NNF1-myc<sub>6</sub> (strain GEY110), DSN1-GFP (strain GEY176), MTW1-GFP (strain GEY216), or NSL1-GFP (strain GEY206) were split and with treated either 17 μg of nocodazole per ml or mock treated with DMSO for 2.5 h. Cells were fixed and prepared for indirect immunofluorescence using either antitubulin antibodies (not shown) or anti-Myc or anti-GFP antibodies. Since MT arrays were lost in nocodazole-treated cells, Nnf1p, Dsn1p, Mtw1p, and Nsl1p localizations are independent of MTs (n > 90 for each strain). Each fusion was expressed from its endogenous promoter, and localizations were not dependent on untagged versions of these proteins as only tagged copies were present in each strain. Bar, 5 μm.

**DISCUSSION**

We previously identified Nnf1p as a protein associated with the nuclear envelope in *S. cerevisiae*. In this study, I show that Nnf1p is needed for genetic stability. I performed two genetic screens and identified novel proteins that have genetic interactions with NNF1. MTW1, a gene encoding a kinesin-like protein, was isolated from both screens. Mutations in MTW1 enhance the temperature-sensitive *nnf1-17* allele, and overexpression of *MTW1* suppresses the *nnf1-17* phenotype. Use of the temperature-sensitive *nnf1-17* mutant has enabled me to propose that Nnf1p is necessary for chromosome segregation. Loss of Nnf1p function leads to abnormal MTs, an accumulation of large-budded cells, altered DNA content, and decreased plasmid stability. Moreover, the genetically related proteins Mtw1p, Dsn1p, and Nsl1p share the same subcellular localization as Nnf1p. Although Nnf1p, Mtw1p, and Nsl1p are similar in size, no significant sequence similarities were found between any two of these proteins. These four proteins must have unique functions, because each is essential for cell viability.

Previously (56), we described two additional phenotypes observed in the *nnf1-17* mutant. First, a small percentage of *nnf1-17* cells (~15%) had a slight nuclear accumulation of poly(A)<sup>+</sup> RNA after 3 h at 35°C. This nuclear accumulation of poly(A)<sup>+</sup> RNA is of uncertain significance and may simply be part of the terminal phenotype due to its late onset and low penetrance. Second, in some *nnf1-17* cells there were changes in the nucleolus as detected by immunofluorescence against a nucleolar antigen. The changes in nucleolar morphology may be related to the phenomenon that the nucleolus in wild-type cells is often found opposite to the SPBs (80). Conceivably, the loss of a crescent-shaped nucleolus may be due to chromosome loss, since *nnf1-17* cells have a wide range of DNA content and nucleolar structure is determined at least in part by ribosomal RNA. The genetic screens reported here did not uncover any links to the nucleolus or mRNA export.

MT defects are the most prominent cytological defect seen in *nnf1-17* cells, and pleiotropic MT defects are observed in large-budded *nnf1-17* cells at the nonpermissive temperature. The range of MT defects observed in the *nnf1-17* mutant are in line with those morphologies seen in mutants lacking spindle function. In the *nnf1-17* mutant, both cytoplasmic and spindle MTs are aberrant, although not always in the same cell. However, a particular MT pattern is not always diagnostic for the role of a protein in a specific cellular substructure, perhaps due to the delicate stoichiometry of many components of the cytoskeleton. For example, mutations in the SPB outer plaque protein Spc72p result mainly in loss of cytoplasmic MTs and binucleate cells, but some *spc72* mutants also exhibit spindle defects (11, 39, 60). After shifting a synchronized *nnf1-17* population to the restrictive temperature (56), short, thick mitotic spindles in nuclei at the bud necks were observed at early time points (1 h after the shift). Binucleate cells and cells with elongated cytoplasmic MTs were not observed until later time points (3 h after the shift). Therefore, one possibility is that spindle elongation is the primary MT defect in *nnf1-17* cells, with a nuclear migration defect occurring subsequently due to perturbations in the nuclear envelope or in tubulin pools.

MTs are essential for mitotic chromosome segregation, and
so mutants with MT defects may also have higher frequencies of chromosome or plasmid loss. To test this, nff1-17 cells were assayed for plasmid stability and were found to lose a marked plasmid at a 10-fold-higher frequency than the wild-type strain. Simple loss could be due to either MT defects or, possibly, errors in DNA replication or repair (26). If the plasmid loss phenotype is accompanied by an increase in mitotic recombination, this could imply that the mutant has DNA lesions and that the wild-type protein has a role in DNA metabolism. Plasmid loss accompanied by nondisjunction only might support a role in the MT cytoskeleton, although DNA replication defects can also prevent sister chromatid separation (25, 48). Neither recombination nor nondisjunction was tested in nff1-17 cells in an attempt to distinguish between these possibilities. However, flow cytometry analysis of DNA content in nff1-17 cells at the restrictive temperature and colocalization of Nnf1p and Mtw1p strongly suggest that the defect in the nff1-17 mutant is in MT or spindle function.

Mtw1p has recently been characterized in a separate study, and the cytological behavior of an mtw1-1 mutant is consistent with defects observed in the nff1-17 cells. The mtw1-1 mutant exhibits unstable transmission of a CEN plasmid at a semi-restrictive temperature, as shown by the colony color assay (18). Synchronous cultures of mtw1-1 cells accumulate as large-budded cells with short spindles at early time points, while at later time points, some binucleate cells are seen (18), as was similarly observed in a synchronous population of nff1-17 cells (56). Goshima and Yanagida (18) also found that in unbudded cells, Mtw1p-GFP was seen as a single dot at the nuclear periphery, whereas two Mtw1p-GFP dots were seen near the SPBs in budding cells. Mtw1p-GFP fluorescence was lost in an ndc10-1 mutant, and from Ndc10p and Mtw1p colocalization studies, these two proteins share the same kinetochore localization but Ndc10p is found additionally along the mitotic spindle (16, 18). The combined use of Mtw1p-GFP, Tub4p-GFP (\(\gamma\)-tubulin), and GFP-tagged chromosomes has facilitated the observations that yeast sister chromatids separate early in the cell cycle and are situated near the SPBs and that sister arms remain connected until spindle elongation (18). Further support of this phenomenon has come from He et al. (22), who have used Mtw1p-GFP as a kinetochore marker to show that sister chromatids undergo transient separations during metaphase and that centromeric chromatin has an elastic quality.

A variety of different relationships among Nnf1p, Dsn1p, Mtw1p, and Nsl1p can be envisioned. In a straightforward model, Nnf1p, Dsn1p, Mtw1p, and Nsl1p exist in a subcomplex. In this model, DSN1 and MTW1 are interaction suppressors of nff1-17. An excess of Dsn1p and Mtw1p at the non-permissive temperature may prevent degradation of nff1-17 protein by sequestering the unstable monomer. Similarly, increases in the concentration of Dsn1p or Mtw1p may stabilize a structure already formed with nff1-17 protein by maintaining an assembled complex through mass action. At lower temperatures, the Nnf1p subcomplex would be functional with the nff1-17 protein, but partial loss of function in either Mtw1p or Nsl1p would destabilize the Nnf1p assembly and the cells would be inviable.

Thus, a future question to be addressed is whether Nnf1p, Dsn1p, Mtw1p, and Nsl1p are present in a subcomplex. I have also found that Dsn1p and Nsl1p interact in the two-hybrid system (G. Euskirchen, unpublished results). Although the mechanism of the Dsn1p and Nsl1p interaction remains to be determined, this two-hybrid result establishes another link between the genes cloned from the suppressor and synthetic lethal analysis starting with the nff1-17 mutant (Fig. 7). The disomy or diploidization that occurs when either DSN1, MTW1, or NSL1 is disrupted implies that these proteins are required in a precise stoichiometry for chromosome segregation and suggests that these loci might be haploinsufficient. Interestingly, haploinsufficient phenotypes are not often observed. In yeast, loss of diploid dosage of the SPB duplication gene, NDC1, results in aneuploidy (12). Chromosome disorders in heterozygous dsn1, mtw1, and nsl1 diploids are consistent with the plasmid loss phenotype observed in the nff1-17 mutant and with the strong spindle defects observed in nff1-17 cells. The most direct explanation is that these four proteins are needed to maintain chromosome number and act together in spindle function.

An extension of this model is that an Nnf1p complex is peripherally associated with the SPB. This model derives from the biochemical identification of Nnf1p from a nuclear envelope preparation (56); the localization patterns of Nnf1p, Cnm67p, Dsn1p, Mtw1p, and Nsl1p; the copurification of Mtw1p with a highly enriched spindle pole preparation (76); and the genetic interactions among NNF1, MTW1, DSN1, and NSL1 (Fig. 7). In S. cerevisiae, comparable patterns of proximal SPB localization are seen with the kinetochore proteins Ndc10p (16), Cse4p (45), Ctf19p (28), Nde80p (75, 76), and Slk19p (81). Cytological observations that centromeres transiently cluster near the SPBs further support this hypothesis (21, 22, 33, 67). A kinetochore association has been found for Spc24p and Spc25p (32, 75), proteins that were originally identified as SPB components, both biochemically and by immu-
noollectron microscopy (76). The essential proteins identified in this study are well positioned to link data from various chromosome segregation studies.

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