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A Novel Mechanism of Intragenic Complementation between Phe to Ala Calmodulin Mutations

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Calmodulin (CaM) performs essential functions in cell proliferation in *Saccharomyces cerevisiae*. Previously, we isolated fourteen temperature-sensitive Phe-to-Ala mutations of the CaM-encoding gene *CMD1*. These mutations were classified into four intragenic complementation groups, suggesting that each group represents a loss of CaM interaction with its specific essential target protein. Nuf1p/Spc110p, one of the essential targets, is a spindle pole body component that is required for proper mitosis. We investigated which intragenic complementation group of CaM represents the malfunction of Nuf1p. Immunoprecipitation analysis showed that two *cmd1* mutations belonging to two distinct intragenic complementation groups had the most severely impaired complex formation with Nuf1p at the restrictive temperature. The temperature-sensitive growth of these *cmd1* mutants was suppressed by a CaM-independent dominant allele of *NUF1*. Additionally, these mutants displayed characteristic mitotic defects: an increased ratio of artificial chromosome loss, which could be suppressed by the CaM-independent dominant allele of *NUF1*, and aberrant microtubule structures. These results indicate that these *cmd1* mutants display the temperature-sensitive growth due to the compromised interaction with Nuf1p. However, the interaction was restored in a heterozygous diploid of the two *cmd1* alleles, suggesting that intragenic complementation between these *cmd1* alleles occurs by a novel mechanism, whereby co-presence of both mutant proteins rescues the interaction with Nuf1p.

Key words: calmodulin, intragenic complementation, *NUF1*/SPC110, *Saccharomyces cerevisiae*, spindle pole body.

Abbreviations: CaM, calmodulin; 5-FOA, 5-fluoroorotic acid; GST, glutathione-S-transferase; PtdIns(4,5)P_2, phosphatidylinositol 4,5-bisphosphate; SD, synthetic medium containing yeast nitrogen base and dextrose; SPB, spindle pole body; YPD, rich medium containing yeast extract, peptone, and dextrose.

Calmodulin (CaM), a highly conserved Ca^{2+}-binding protein, is known to be the major Ca^{2+} signal transducer. CaM conveys the Ca^{2+} signal by binding to its target proteins and regulates their functions in a Ca^{2+}-dependent manner. CaM binds to a wide variety of proteins *in vitro*, and is implicated in diverse cellular functions such as immune response, cell cycle, glycolysis, muscle contraction, motility, secretion, and synaptic plasticity (*1*). In the budding yeast, CaM is encoded by a single structural gene *CMD1*. *CMD1* has been shown to be required for multiple cellular processes such as cell proliferation, endocytosis, survival in pheromone, and regulation of PtdIns(*4,5*)P_2 synthesis (*2–6*). To explore the functions of CaM in cell proliferation, we previously isolated 14 temperature-sensitive CaM mutations by systematic Phe-to-Ala site-directed CaM mutagenesis. Strikingly, we found that the temperature-sensitive CaM mutations were classified into four intragenic complementation groups (*7*).

Intragenic complementation is common to a large number of genes in various organisms. The precise mechanisms underlying the intragenic complementation are yet to be clarified in some cases. Occasionally, mutant alleles are changed by recombination or gene conversion, as observed for *lacZ* mutations in *E. coli* (*8*). It should be noted that this is not authentic intragenic complementation, even though it produces an apparently indistinguishable phenotype from that of authentic intragenic complementation. Our Phe-to-Ala CaM mutations cannot be explained in this way, because it was reported that both complementing *cmd1* alleles could be routinely detected by allele-specific PCR in the diploid (*7*). The currently known mechanisms underlying intragenic complementation can be roughly categorized into the following two cases. In one case, the gene encodes a protein that interacts with itself to form a multimer. The recovery of enzymatic activity by multimer formation was first demonstrated *in vitro* for tryptophan synthetase α subunit of *E. coli* (*9*, *10*), and some intragenic complementation phenotypes, such as *arg-7* mutations in *Chlamydomonas reinhardi* (*11*) and *SUC2* mutations in *S. cerevisiae* (*12*), have been classified in this way. This mechanism is not

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Table 1. **Yeast strains used in this study.** All strains are derived from YPH499 (*MAT***a** *ade2 his3 leu2 lys2 trp1 ura3*) and YPH500 (*MAT*α *ade2 his3 leu2 lys2 trp1 ura3*) (*34*) and hence harbor the identical background (*ade2 his3 leu2 lys2 trp1 ura3*) except for the additional markers listed in the table. "a::b" denotes disruption of gene a with gene b, and "a:b" denotes contiguous array of gene a and gene b.

Strain	Genotype	Source
YOC200	*MAT***a** *cmd1::TRP1 ade3::CMD1:HIS3*	(*6*)
YOC226	*MAT***a** *cmd1::TRP1 ade3::cmd1-226:HIS3*	(*6*)
YOC228	*MAT***a** *cmd1::TRP1 ade3::cmd1-228:HIS3*	(*6*)
YOC231	*MAT***a** *cmd1::TRP1 ade3::cmd1-231:HIS3*	(*6*)
YOC239	*MAT***a** *cmd1::TRP1 ade3::cmd1-239:HIS3*	(*6*)
GHW300	*MAT*α *SUP11:URA3*	(*30*)
YOC2651	*MAT***a** *SUP11:URA3*	This study
YOC2652	*MAT***a** *lys2:LYS2 nuf1^{C911R} SUP11:URA3*	This study
YOC2727	*MAT***a** *cmd1::TRP1 ade3::CMD1:LEU2 SUP11:URA3*	This study
YOC2728	*MAT***a** *cmd1::TRP1 ade3::cmd1-228:LEU2 SUP11:URA3*	This study
YOC2729	*MAT***a** *cmd1::TRP1 ade3::cmd1-239:LEU2 SUP11:URA3*	This study
YOC2730	*MAT***a** *cmd1::TRP1 ade3::CMD1:LEU2 AUR1-C:NUF1-407:AUR1 SUP11:URA3*	This study
YOC2731	*MAT***a** *cmd1::TRP1 ade3::cmd1-228:LEU2 AUR1-C:NUF1-407:AUR1 SUP11:URA3*	This study
YOC2732	*MAT***a** *cmd1::TRP1 ade3::cmd1-239:LEU2 AUR1-C:NUF1-407:AUR1 SUP11:URA3*	This study
YOC3144	*MAT***a**/*MAT*α *leu2/leu2 ura3/ura3 cmd1::TRP1/cmd1::TRP1 ade3::CMD1:LEU2/ade3::CMD1:HIS3 SUP11:URA3*	This study
YOC3145	*MAT***a**/*MAT*α *leu2/leu2 ura3/ura3 cmd1::TRP1/cmd1::TRP1 ade3::cmd1-228:LEU2/ade3::cmd1-228:HIS3 SUP11:URA3*	This study
YOC3146	*MAT***a**/*MAT*α *leu2/leu2 ura3/ura3 cmd1::TRP1/cmd1::TRP1 ade3::cmd1-239:LEU2/ade3::cmd1-239:HIS3 SUP11:URA3*	This study
YOC3147	*MAT***a**/*MAT*α *leu2/leu2 ura3/ura3 cmd1::TRP1/cmd1::TRP1 ade3::cmd1-228:LEU2/ade3::cmd1-239:HIS3 SUP11:URA3*	This study
YOC2721	*MAT***a**/*MAT*α *leu2/leu2:LEU2 ura3/ura3:URA3 cmd1::TRP1/cmd1::TRP1 ade3::CMD1:HIS3/ade3::CMD1:HIS3*	This study
YOC2722	*MAT***a**/*MAT*α *leu2/leu2:LEU2 ura3/ura3:URA3 cmd1::TRP1/cmd1::TRP1 ade3::cmd1-226:HIS3/ade3::cmd1-226:HIS3*	This study
YOC2723	*MAT***a**/*MAT*α *leu2/leu2:LEU2 ura3/ura3:URA3 cmd1::TRP1/cmd1::TRP1 ade3::cmd1-228:HIS3/ade3::cmd1-228:HIS3*	This study
YOC2724	*MAT***a**/*MAT*α *leu2/leu2:LEU2 ura3/ura3:URA3 cmd1::TRP1/cmd1::TRP1 ade3::cmd1-231:HIS3/ade3::cmd1-231:HIS3*	This study
YOC2725	*MAT***a**/*MAT*α *leu2/leu2:LEU2 ura3/ura3:URA3cmd1::TRP1/ cmd1::TRP1 ade3::cmd1-239:HIS3/ade3::cmd1-239:HIS3*	This study
YOC2726	*MAT***a**/*MAT*α *leu2/leu2:LEU2 ura3/ura3:URA3 cmd1::TRP1/cmd1::TRP1 ade3::cmd1-228:HIS3/ade3::cmd1-239:HIS3*	This study

the case for the CaM mutations, since budding yeast CaM, like the mammalian protein, functions as a monomer in solution (*13*). In the other case, the gene encodes a multifunctional protein, as for *arg-6* mutations in *Neurospora crassa* (*14*) and for *HIS4* mutations in *S. cerevisiae* (*15*). *HIS4* protein catalyzes three different enzymatic steps in histidine biosynthesis. Mutations affecting one of these steps complement the defects caused by mutations affecting the others. Although the structural domains in CaM may not be as easily separable as the domains of *HIS4* product, intragenic complementation of the CaM mutations has been interpreted from the viewpoint that each complementation group represents a single functional defect by losing the interaction with its specific target protein (*7*). Biochemical characterization of the mutant CaMs apparently supports this interpretation (*13*).

When we initiated this study, only Myo2p and Nuf1p/Spc110p were known definitely to be essential CaM targets (*16–18*). Myo2p is a class V myosin that is involved in polarized growth, vacuolar inheritance, spindle orientation, and Golgi inheritance (*19–22*). Among the *cmd1* complementing mutations, *cmd1-226* most severely compromised CaM binding to one of the six CaM-binding domains of Myo2p (*23*). Thus, the intragenic complementation group to which *cmd1-226* belongs represents the malfunction of Myo2p. Nuf1p/Spc110p is an essential component of the spindle pole body (SPB), a microtubule-organizing center in yeast (*24*, *25*). The N-terminal region of Nuf1p binds directly to the yeast γ-tublin complex (*26*), whereas the C-terminal region binds to CaM (*17*, *18*). CaM binding to Nuf1p is required for the attachment of the C-terminal region of Nuf1p to the core of the SPB, and thus is essential for proper mitotic functions (*27–29*). The central coiled-coil region acts as a spacer between the spindle microtubule and the core of the SPB (*25*).

In this study, we examined which intragenic complementing CaM mutation specifically compromises Nuf1p function. Surprisingly, we revealed that two of the intragenic complementation groups represent the dysfunction of Nuf1p. Here we show evidence for a novel mechanism that underlies the intragenic complementation between these two CaM mutations.

MATERIALS AND METHODS

Strains and Microbial Techniques—Yeast strains used in this study are listed in Table 1. An artificial chromosome was previously made by inserting the 1.5-kb

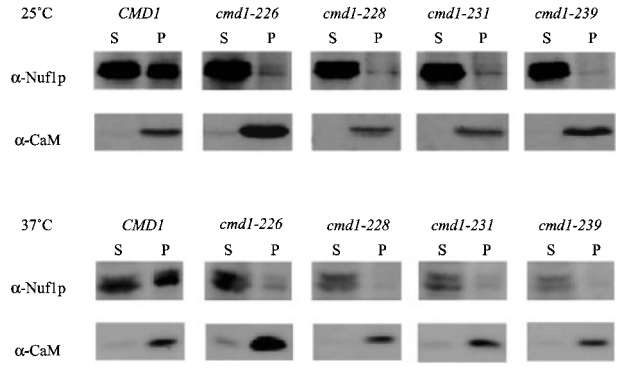

Fig. 1. ***cmd1-228* and *cmd1-239* mutations have the most severely impaired Nuf1p-CaM interaction.** Yeast cells of *CMD1* (YOC200), *cmd1-226* (YOC226), *cmd1-228* (YOC228), *cmd1-231* (YOC231), and *cmd1-239* (YOC239) were grown to early logarithmic phase at 25°C, and then incubated either at 25°C or 37°C for 2 h. Lysates were prepared for immunoprecipitation using the anti-Cmd1p antibody. Immunoprecipitates (P) and supernatants (S) were processed for immunoblot detection of Nuf1p and CaM using anti-Nuf1p and anti-Cmd1p antibodies, respectively.

*Bam*HI fragment of the *CMK1* gene into the *Bam*HI gap of plasmid YCF4 and introduced into YPH500 as a fragment linearized with *Bgl*II and *Eco*RI to produce GHW300 (*30*). Strains carrying an artificial chromosome used in this study, such as YOC2651, were isolated as Ura^+ segregants of diploid strains constructed by crossing GHW300 with the original strain, such as YPH499. Basic yeast methods and growth media were as described previously (*31*). Standard procedures were used for all DNA manipulations and *E. coli* transformation (*32*).

Antibodies—Rabbit polyclonal anti-Nuf1p antibody was generously given by M. Snyder (Yale University, New Haven, Connecticut). Gunea pig polyclonal anti-Nuf1p antibody was raised against GST-Nuf1p as described previously (*24*) and affinity-purified on a HiTrap NHS-activated column with 0.8 mg of GST-Nuf1p bound. Rabbit anti-Cmd1p serum was prepared as described previously (*23*). Rat monoclonal anti-yeast tublin antibody (YOL1/34) was commercially available from Abcam (Cambridge, UK).

Immunoprecipitation—Yeast cells were grown in YPD medium to early logarithmic phase at 25°C, then incubated at 37°C for 2 h. The cells were harvested by centrifugation, washed once with buffer A containing 150 mM NaCl [20 mM Tris-Cl, pH 8.0, 1 mM EDTA, 1 mM EGTA, 0.1 mM dithiothreitol, 5% (v/v) glycerol, and 3 mM NaN_3], and lysed with glassbeads in buffer A containing 150 mM NaCl and protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine-HCl, 5 μg/ml each of leupeptin, pepstatin A, chymostatin, aprotinin, and antipain). After cell lysis, 1 M NaCl and 1% Triton X-100 were added, and the lysate was incubated on ice for 15 min. Cell extracts were separated twice by centrifugation, and the supernatant containing 200 μg of protein was incubated with rabbit anti-CaM antibody in buffer A containing 360 mM NaCl, 1% Triton X-100, and 5 mM $CaCl_2$ at 4°C for 1 h. Protein G-conjugated Sepharose 4B was added to the extract, which was further incubated at 4°C for 1 h. The beads were collected by centrifugation and washed twice with buffer A containing 150 mM NaCl, 1% Triton X-100, and 5 mM $CaCl_2$ at 4°C for 10 min. Bound protein was eluted in sample buffer, and then analyzed by immunoblotting using the rabbit or gunea pig anti-Nuf1p antibody or rabbit anti-Cmd1p antibody.

Chromosome Loss Assay—Analysis of chromosome loss was performed using the chromosome fragment method as described previously (*30*). Strains bearing a marker chromosome were first grown to early logarithmic phase in SD complete medium lacking uracil. These cells were incubated in YPD at 25°C for 3 h, then shifted to 37°C for 1 h. Cells were plated on SD complete medium with or without 1.8 mg/ml 5-FOA. The frequency of chromosome loss was calculated as follows: the number of colonies formed on SD complete medium containing 1.8 mg/ml 5-FOA divided by the number of colonies formed on SD complete medium.

Cytological Techniques—Procedures for immunofluorescence microscopy were as described previously (*33*). For staining of tublin, rat monoclonal anti-yeast tublin antibody (YOL1/34) was used. Cells were observed under the Axioplan2 imaging microscope (Carl Zeiss, Tokyo). Images were captured using a CoolSNAP CCD camera (Nihon Roper, Chiba) and Metamorph Imaging software (Universal Imaging, West Chester, PA). Images were processed using Adobe Photoshop software.

RESULTS

First, we studied the effect of each complementing CaM mutation on the interaction with Nuf1p. CaM mutants belonging to distinct intragenic complementation groups were subjected to immunoprecipitation with anti-Cmd1p serum after growth at the permissive or restrictive temperature. We found that all of the *cmd1* mutations examined significantly impaired CaM/Nuf1 complex formation even at the permissive temperature (Fig. 1). However, *cmd1-228* and *cmd1-239* compromised the complex formation more severely than the other mutations, especially at the restrictive temperature. Next, we examined whether the compromised interaction of CaM with Nuf1p observed in the *cmd1* mutants is a primary cause of their temperature-sensitive proliferation. *NUF1-407* is a dominant allele of *NUF1* that lacks the CaM-binding sequence, and it encodes a protein that can function without CaM binding (*17*). If the temperature-sensitive growth of the *cmd1* mutants is caused by the defective CaM binding to Nuf1p, expression of Nuf1-407p should alleviate the defects of the mutants by circumventing the requirement of CaM binding to Nuf1p. We found that *NUF1-407* restored the growth of *cmd1-228* and *cmd1-239* at the restrictive temperature, but did not suppress the temperature-sensitive growth of *cmd1-226* and *cmd1-231* (Fig. 2). Introduction of the wild-type *NUF1* could not suppress the temperature-sensitivity of *cmd1-228* and *cmd1-239*, suggesting that suppression of the temperature-sensitive growth is not simply due to an increased dosage of Nuf1p. These results indicate that it is the loss of the CaM binding to Nuf1p that causes the temperature-sensitive growth of *cmd1-228* and *cmd1-239*.

Nuf1p has been reported to be essential for the proper mitotic function. To explore the Nuf1p-related phenotype of *cmd1-228* and *cmd1-239*, we examined the accuracy of

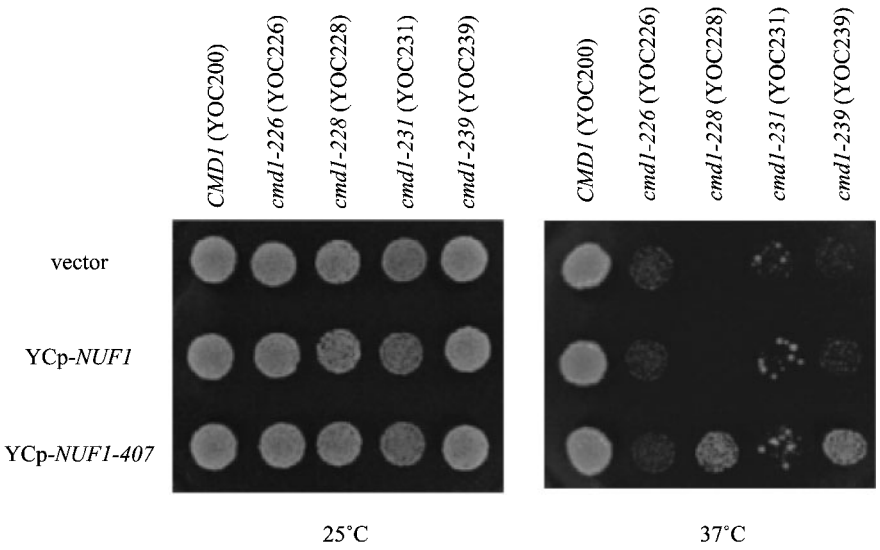

Fig. 2. ***NUF1-407* suppresses the temperature-sensitive growth defects of *cmd1-228* and *cmd1-239* in an allele-specific manner.** Each *cmd1* mutant strain was transformed with *NUF1* or *NUF1-407* on a low-copy plasmid and grown on a YPD plate at 25°C or 37°C. Parental strains used are YOC200, YOC226, YOC228, YOC231, and YOC239.

chromosome segregation. Cells were marked with nonessential artificial chromosomes carrying the *URA3* gene. After incubation at 37°C for 1 h, the number of cells that had lost the marker chromosomes was determined by counterslection on 5-FOA-containing solid medium. Because of the importance of CaM binding for mitotic function of Nuf1p, the *nuf1*^{C911R} mutation, which has been reported to reduce the CaM binding to Nuf1p, elevated the frequency of the marker chromosome loss (Table 2) as expected. The *cmd1-228* and *cmd1-239* mutations also increased the frequencies of chromosome loss both in haploid strains and, to a lesser extent, in homozygous diploid strains. Importantly, the heterozygous *cmd1-228*/*cmd1-239* diploid strain showed the normal segregation of the marker chromosome, indicating intragenic complementation between *cmd1-228* and *cmd1-239* for the defects in chromosome segregation. Notably, the elevated frequencies of chromosome loss in *cmd1-228* and *cmd1-239* were reversed by introducing one copy of *NUF1-407*. Thus, it is also the compromised interaction of CaM to/with Nuf1p in *cmd1-228* and *cmd1-239* that caused the defects in accurate segregation of chromosomes.

Since accurate chromosome segregation requires normal microtubule organization, we investigated the microtubule structures of the *cmd1-228* and *cmd1-239*. Homozygous diploid cells of *cmd1-228* and *cmd1-239* were grown at the restrictive temperature for 4 h, then observed by immunofluorescence microscopy with anti-tublin antibody. We found that both strains displayed the same characteristic phenotypes, which were categorized into four groups (Fig. 3). The first group is composed of large-budded cells with a monopolar spindle (Fig. 3, A and E). The second group consists of large-budded cells with a broken spindle: one of the SPBs is detached from the nucleus (Fig. 3, B and F). The third group contains large-budded cells with a misoriented short spindle (Fig. 3, C and G): cytoplasmic microtubules emanating from the two SPBs were often oriented in the same direction. The fourth group has unbudded cells with two microtubule-organizing foci, one of which is dissociated from the nucleus (Fig. 3, D and H). We quantitated the percentages of these abnormal microtubule structures and found that the homozygous *cmd1-228* and *cmd1-239* diploid strains displayed similar distributions while the heterozygous *cmd1-228*/*cmd1-239* diploid strain barely displayed such aberrant structures (Fig. 4).

Although *cmd1-228* and *cmd1-239* mutations caused loss of interaction with Nuf1p, both mutants exhibited intragenic complementation for temperature-sensitive growth (7), defective segregation of marker chromosome (Table 2), and abnormal microtubule organization (Fig. 4). Hence, we examined whether loss of interaction with Nuf1p is restored in the heterozygous *cmd1-228*/*cmd1-239* diploid strain. Homozygous *cmd1* diploid strains and the heterozygous *cmd1-228*/*cmd1-239* strain were subjected to the immunoprecipitation with anti-Cmd1p

Table 2. **Frequencies of artificial chromosome loss.** Strains bearing a marker chromosome were first grown to early logarithmic phase in SD complete medium lacking uracil. These cells were incubated in YPD at 25°C for 3 h and then shifted to 37°C for 1 h. Cells were plated on SD complete medium with or without 1.8 mg/ml 5-FOA. The frequency of chromosome loss was calculated as follows: the number of colonies formed on SD complete medium containing 1.8 mg/ml 5-FOA divided by the number of colonies formed on SD complete medium.

Strains	Genotype	Relative frequency of chromosome loss
YOC2651	*NUF1*	1[a]
YOC2652	*nuf1*^{C911R}	26[a]
YOC2727	*CMD1*	1[b]
YOC2728	*cmd1-228*	22[b]
YOC2729	*cmd1-239*	59[b]
YOC2730	*CMD1 NUF1-407*	0.87[b]
YOC2731	*cmd1-228 NUF1-407*	3.5[b]
YOC2732	*cmd1-239 NUF1-407*	4.2[b]
YOC3144	*CMD1*/*CMD1*	1[c]
YOC3145	*cmd1-228*/*cmd1-228*	7.5[c]
YOC3146	*cmd1-239*/*cmd1-239*	22[c]
YOC3147	*cmd1-228*/*cmd1-239*	0.84[c]

[a]Each value was normalized to that of YOC2651. [b]Each value was normalized to that of YOC2727. [c]Each value was normalized to that of YOC3144.

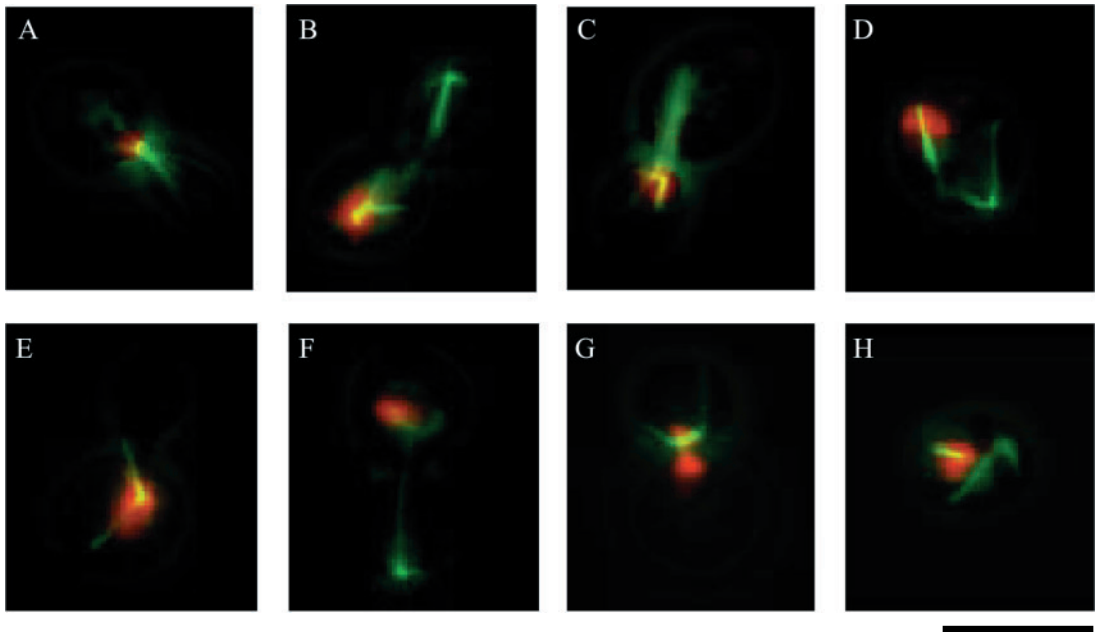

Fig. 3. **The *cmd1-228/cmd1-228* and *cmd1-239/cmd1-239* homozygous diploid strains show four classes of abnormal microtubule organization.** Cells from logarithmic phase cultures of *cmd1-228/cmd1-228* (YOC2723) and *cmd1-239/cmd1-239* (YOC2725) were shifted to 37°C for 4 h, then prepared for microscopy. Microtubules were visualized by anti-tublin immunofluorescence microscopy as shown in green. Nuclei were visualized by DAPI staining as shown in red. Yellow indicates the co-localization of tublin and DNA. (A, B, C, D) *cmd1-228/cmd1-228* cells; (E, F, G, H) *cmd1-239/cmd1-239* cells. (A, E) Large-budded cells with a monopolar spindle; (B, F) large-budded cells with a broken spindle; (C, G) large-budded cells with a misoriented short spindle; (D, H) unbudded cells with two spindle poles. Bar, 5 μm.

serum after grown at 37°C for 2 h. As in the haploid strains, the homozygous *cmd1-228* and *cmd1-239* diploid strains showed the most severe defects in the complex formation with Nuf1p (Fig. 5). However, the impaired complex formation with Nuf1p was restored in the heterozygous *cmd1-228/cmd1-239* strain, indicating that intragenic complementation occurs by a novel mechanism, where the co-presence of both mutant proteins can restore CaM/ Nuf1p binding.

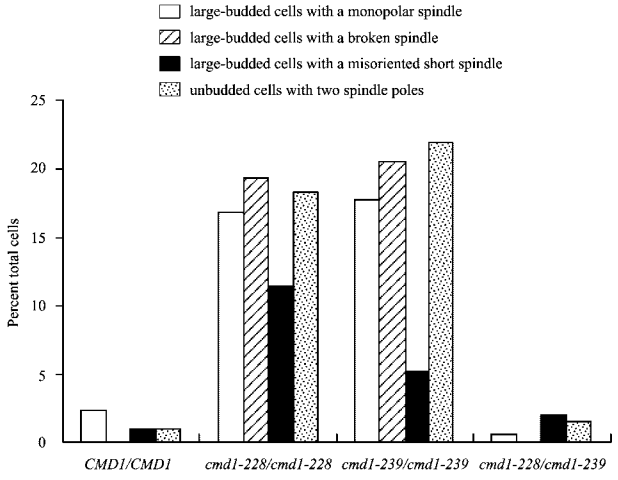

Fig. 4. **The *cmd1-228/cmd1-239* heterozygous diploid cells show minor defects in microtubule organization.** Cells from a logarithmic phase culture of *CMD1/CMD1* (YOC2721), *cmd1-228/cmd1-228* (YOC2723), *cmd1-239/cmd1-239* (YOC2725), and *cmd1-228/cmd1-239* (YOC2726) were shifted to 37°C for 4 h, which were then processed for anti-tublin immunofluorescence and DAPI staining. The staining patterns of microtubules and nuclear DNA were examined under the fluorescence microscope.

DISCUSSION

In this study we demonstrated that two of the CaM intragenic complementation groups show the dysfunctional Nuf1p. Immunoprecipitation study revealed that *cmd1-228* and *cmd1-239,* which belong to different intragenic complementation groups, most severely compromised the complex formation with Nuf1p, notably at the restrictive temperature. Expressing Nuf1-407p could suppress the temperature-sensitive growth of *cmd1-228* and *cmd1-239* in an allele-specific manner by bypassing the requirement of CaM binding to Nuf1p. Furthermore, both *cmd1* mutants share several common phenotypes other than temperature-sensitive cell proliferation and a bilateral mating defect (*6*, *7*, *35*). First, they displayed an increased ratio of artificial chromosome loss, as did the *nuf1*^{C911R} mutation, which is known to impair the Nufp-CaM interaction. It should be noted that the elevated frequency of marker chromosome loss was suppressed by expressing Nuf1-407p. Second, both of the homozygous *cmd1-228* and *cmd1-239* diploid strains exhibited abnormal microtubule structures like those observed in some *nuf1* temperature-sensitive strains. These results suggest that *cmd1-228* and *cmd1-239* cause the temperature-sensitive growth due to the compromised interaction of CaM with Nuf1p.

We previously reported that *cmd1-228*, but not *cmd1-239*, shows two characteristic phenotypes at the restrictive temperature: rapid loss of viability and loss of CaM

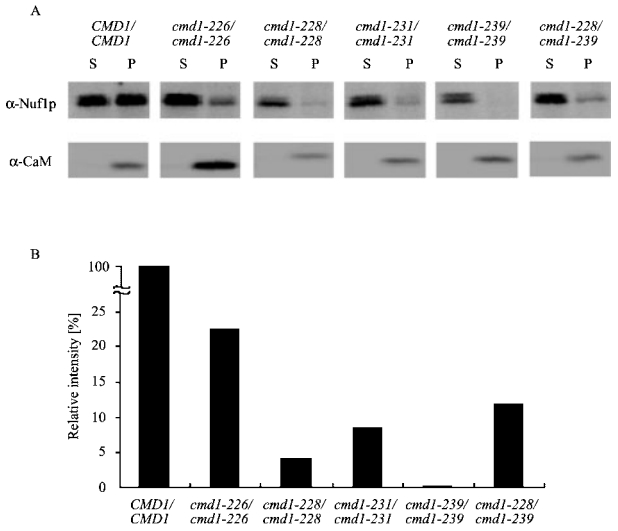

Fig. 5. **Nuf1p-CaM interaction is restored in the *cmd1-228/cmd1-239* heterozygous diploid strain.** (A) Yeast lysates from *CMD1/CMD1* (YOC2721), *cmd1-226/cmd1-226* (YOC2722), *cmd1-228/cmd1-228* (YOC2723), *cmd1-231/cmd1-231* (YOC2724), *cmd1-239/cmd1-239* (YOC2725), and *cmd1-228/cmd1-239* (YOC2726) were immunoprecipitated using the anti-Cmd1p antibody. Immunoprecipitates (P) and supernatants (S) were processed for immunoblot detection of Nuf1p and CaM using anti-Nuf1p and anti-Cmd1p antibodies, respectively. (B) Intensity of each band was measured by densitometry and is represented as percent wild-type intensity.

localization at the bud tip (*7*). The fact that *NUF1-407* restored the temperature-sensitive growth indicates that the former phenotype is caused by the impaired interaction with Nuf1p. Why does the dysfunction of Nuf1p cause the rapid loss of viability in *cmd1-228* but not in *cmd1-239*? It is unlikely that Nuf1p dysfunction is more severe in *cmd1-228* than in *cmd1-239*, because (i) *cmd1-228* and *cmd1-239* compromised the complex formation with Nuf1p to a similar extent, (ii) *cmd1-228* and *cmd1-239* showed similar distributions of the characteristic abnormal microtubule structures, (iii) *cmd1-228* displayed an even milder defect in the artificial chromosome segregation than did *cmd1-239*. One possible explanation is that *cmd1-228* compromises the function of another target protein that itself is not required for cell viability, but is indispensable in the absence of functional Nuf1p. It remains to be clarified whether the loss of CaM localization at the bud tip is caused by the inability of CaM to bind to Nuf1p.

We demonstrated that intragenic complementation between *cmd1-228* and *cmd1-239* occurs by a novel mechanism, whereby the co-presence of both mutations restores the interaction of CaM with Nuf1p. To elucidate the molecular mechanism underlying this phenomenon, we propose the following two possibilities. The first is that the two complexes, Nuf1p/Cmd1-228p and Nuf1p/Cmd1-239p, bind one another to stabilize. This model is consistent with a recent report that Nuf1p forms a dimer *in vitro* and then binds to two CaM molecules (*36*). Recent determination of the three-dimensional structure of a complex between two CaM molecules and a dimer of CaM-binding peptides in the study of a rat Ca^{2+}-dependent K^+ channel revealed that each of the two CaM molecules binds to both submits of the dimer (*37*). Hence, it is necessary to investigate whether Nuf1p also interacts with CaM in a similar. The second possibility is that CaM acts at two steps in the binding process. It is possible to speculate that CaM modifies the CaM-binding region of Nuf1p through another target protein, and that CaM can bind only to this modified region. If Cmd1-228p can perform only one of the CaM functions and Cmd1-239p can only perform the other, the co-presence of Cmd1-228p and Cmd1-239p allows either mutant protein to bind to Nuf1p. Further study should help to clarify the molecular mechanism underlying the intragenic complementation between these *cmd1* alleles.

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