# An evolutionarily conserved fission yeast protein, Ned1, implicated in normal nuclear morphology and chromosome stability, interacts with Dis3, Pim1/RCC1 and an essential nucleoporin

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## Summary

**We identified a novel fission yeast gene, *ned1*$^+$, with pleiotropic mutations that have a high incidence of chromosome missegregation, aberrantly shaped nuclei, overdeveloped endoplasmic reticulum-like membranes, and increased sensitivity to a microtubule destabilizing agent. Ned1 protein, which was phosphorylated in a growth-related manner, interacted in a yeast two-hybrid system with Dis3 as well as with Pim1/RCC1 (nucleotide exchange factor for Ran). Ned1 also interacted with an essential nucleoporin, a probable homologue of mammalian Nup98/96. The *ned1* gene displayed a variety of genetic interactions with factors involved in nuclear transport and chromosome segregation, including the *crm1* (exportin), *spi1* (small GTPase Ran), *pim1*, and *dis* genes. A substitution mutation that affected the two-hybrid interaction with Dis3 increased chromosome instability, suggesting the functional importance of the interaction. Overproduction of Ned1 protein induced formation of an abnormal microtubule bundle within the nucleus, apparently independently of the spindle pole body, but dependent on *pim1*$^+$ activity. The *ned1*$^+$ gene belongs to an evolutionarily conserved gene family, which includes the mouse *Lpin* genes, one of whose mutations is responsible for lipodystrophy.**

Key words: Ran, Lipin, Nuclear membrane

## Introduction

A variety of genes and their concerted expression are required for chromosome stability and normal cell proliferation. Model organisms amenable to both genetic and cytologic analyses, like yeast, have been successfully used to identify many such genes and to study their molecular functions. To reveal more of these genes, we screened fission yeast mutants that were unable to form stable diploid cells. One of these mutants displayed a high incidence of chromosome missegregation in diploid mitosis and increased sensitivity to a microtubule destabilizing agent, and also contained highly deformed nuclei. This pleiotropic phenotype was interesting because it suggested a close relation between nuclear structure and chromosome stability. Investigation of the affected gene in this mutant indicated that it was intimately related to the Ran-GTPase system. Ran is an evolutionarily conserved small GTPase that is required for the regulation of several important cellular processes, including cell cycle progression, nuclear envelope formation, microtubule and spindle dynamics, and directional nuclear transport (Sazer and Dasso, 2000; Dasso, 2001; Vasu and Forbes, 2001). The involvement of Ran in the molecular mechanisms of nuclear transport is well understood (Kuersten et al., 2001). Although it is possible that Ran indirectly affects a variety of cellular processes because of its crucial role in nuclear transport, it is more probable that Ran functions in each of the processes independently from nuclear transport (Sazer and Dasso, 2000). In vitro studies using Xenopus egg extracts revealed that the GTP-bound form of Ran actively promotes the assembly of microtubules and subsequent formation of a bipolar spindle in a centrosome-independent manner (Gruss et al., 2001; Nachury et al., 2001; Wiese et al., 2001). Ran-GTP is also required for the formation of a centrosome-dependent bipolar spindle (Carazo-Salas et al., 2001), and appears to have an important role in the regulation of mitosis in yeast. Fleig et al. used fission yeast to demonstrate that an *spi1* mutant that encoded an altered form of Ran was defective in spindle formation and chromosome segregation, but not in nuclear transport (Fleig et al., 2000). Ran/Spi1 binds directly to Dis3 and stimulates the guanine nucleotide exchange activity of RCC1/Pim1 (Noguchi et al., 1996). The *dis3*$^+$ gene is implicated in the regulation of mitosis, and a cold-sensitive mutant of this gene is impaired in chromosome segregation (Ohkura et al., 1988; Kinoshita et al., 1991). The *dis3* gene genetically interacts with other *dis* mutants impaired in chromosome disjunction (Kinoshita et al., 1991). The dosage of Ran is important for chromosome stability both in budding and fission yeast (Ouspenski et al., 1995; Matsumoto and Beach, 1991). In budding yeast, a β-type importin that binds to Ran-GTP, Cse1, is required for accurate chromosome segregation (Kunzler and Hurt, 1998). These findings are

consistent with the notion that Ran has an important role in the regulation of mitosis in yeast. Moreover, the Ran-GTPase system is crucial for nuclear envelope formation (Hetzer et al., 2000; Zhang and Clarke, 2000) and for the integrity of yeast nuclear membranes (Demeter et al., 1995; Matynia et al., 1996). It is also required for the processing of rRNA through interactions with Dis3, a component of the exosome complex (Suzuki et al., 2001).

The nuclear pore complex (NPC) is implicated in chromosome stability. In addition to an essential role in nuclear transport that is of obvious importance for the general chromosome function, NPC appears to have more direct roles in chromosome transmission. A component of NPC, Nup170, is required for kinetochore function in yeast (Kerscher et al., 2001). In mammalian cells, an NPC subcomplex redistributes to mitotic centromeres (Belgareh et al., 2001). Nuclear pores are involved in chromosome arrangement within the nucleus, which might be important for chromosome stability. In yeast, telomeres are tethered to nuclear pores through specific interactions of a telomere component, Ku70, with nuclear pore-associated filamentous proteins Mlp1/2, which appear to interact with a GLFG repeat-containing nucleoporin, Nup145 (Strambio-de-Castillia et al., 1999; Galy et al., 2000).

In the present study, we demonstrated that the affected gene in the mutant belongs to a gene family that is conserved among a wide range of species. A gene named *Lpin1* in mouse is reportedly among this gene family, and its deficiency is responsible for impaired adipose tissue development and other associated phenotypes, including fatty liver, hyperglyceridemia, and insulin resistance. The molecular function of Lipin (the product of *Lpin1*), however, is not known (Peterfy et al., 2001). Loss-of-function mutations of *Saccharomyces cerevisiae SMP2*, also a member of this gene family, results in stabilization of an unstable plasmid and respiration deficiency (Irie et al., 1993). It is not known why these apparently unrelated defective phenotypes are displayed by mutations in the conserved genes in different organisms. We investigated the molecular function of the fission yeast gene in an attempt to determine the molecular basis for the chromosome instability phenotype as well as other associated defective phenotypes, which might contribute to an understanding of the divergence in phenotypes among species. We demonstrated that the gene product interacts with a conserved nuclear pore component as well as with Dis3 and Pim1/RCC1 in a two-hybrid system. Together with other genetic and cytologic results, the findings of the present study suggest that the gene represents a new class of genes that are intimately related to the Ran GTPase system.

## Materials and Methods

### Strains, plasmids and media

The general genetic procedures were described previously (Rose et al., 1990; Alfa et al., 1993). The *ned1* mutant was isolated from a mutant library previously described (Tange and Niwa, 1995). *Schizosaccharomyces pombe* mutants, *crm1-809*, *dis1*-null, *dis2-11*, *dis3-54*, and *nda3-KM311*, were obtained from M. Yanagida and *pim1-46* from T. Matsumoto. *S. cerevisiae* strains used were W303D (a/α *leu2/leu2 his3/his3 ura3/ura3 trp1/trp1 ade2/ade2 can1/can1*; from A. Toh-e) and SH4904 (a *ura3 leu2 trp1 his4 can1*; from S. Harashima). Plasmids pSP17H (*pim1*$^+$) and pSP5HBam (*spi1*$^+$) were from T. Matsumoto, pSMB2 containing the *SMP2* gene was obtained from H. Araki. An *Xba*I fragment of pSMB2 containing the gene was cloned into the pKD10 vector (Shimanuki et al., 1997) to create pKD-SMP2. This plasmid was used for complementation of *S. cerevisiae* mutants. YCp50 (Rose et al., 1987) was obtained from A. Toh-e for the minichromosome stability test. In the plasmid pR1FPap1 (Kudo et al., 1999), Pap1 is fused with green fluorescent protein (GFP) and placed under the control of the inducible *nmt1* promoter in pREP1 plasmid (Maundrell, 1993). pSec61-GFP was constructed by inserting a genomic DNA sequence of the *sec61*$^+$ gene, but lacking a coding sequence for the N-terminal three amino acids, into the *Bgl*II-*Sma*I site in the pREP41EGFPC (Craven et al., 1998). pREP82tubGFP was made by inserting the GFP-fused α-tubulin gene excised from pDQ105 (Ding et al., 1998) into the appropriate site in pREP82 (Basi et al., 1993). Rich media, YE and YPD, and a synthetic medium, EMM2, were used for *S. pombe* (Alfa et al., 1993), and YPD and SD (synthetic medium) were used for *S. cerevisiae* (Rose et al., 1990). MR-plates contained phloxin B in YEA medium, and were used for discerning haploid and aneuploid from diploid colonies. MR-plates were incubated at 26°C for 3 to 4 days. Aneuploid colonies were restreaked on MR-plates to verify the presence of haploid cells, which was expected due to the instability of the aneuploid cells (Niwa and Yanagida, 1985). In some experiments, aneuploidy was verified using differently marked chromosomes I and III (data not shown). Flow cytometric analysis was performed using FACScan (Becton Dickinson, Franklin Lakes, NJ) (Tange and Niwa, 1995). Unless indicated, the incubation temperature was 26°C for *S. pombe* and 30°C for *S. cerevisiae*. The minichromosome stability test was performed as described (Allshire et al., 1995).

### Cloning of *ned1*$^+$ and *nup189*$^+$ genes

The *ned1* mutant was hypersensitive to thiabendazole (TBZ), so colonies did not form on YEA plates containing 20 μg/ml of TBZ at 30°C. A clone derived from the *ned1*$^+$ locus (verified by linkage analysis of a cloned fragment integrated in the chromosome) that complemented the drug sensitivity was isolated from a cosmid library. A 3.1-kb fragment containing the *ned1*$^+$ gene was subcloned into the pKD10 vector to create a pYT205 plasmid. This plasmid complemented the nuclear morphology defect as well as the drug sensitivity. Colony hybridization was used to clone the *nup189*$^+$ gene into the pKD10 vector.

### Gene disruption and site-directed mutagenesis

To disrupt the *ned1*$^+$ gene, parts of the gene corresponding to amino acids 21 through 121 or 121 through 580 were replaced by the *ura4*$^+$ gene. The QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) was used to make new *ned1* mutants. The 3.1-kb DNA fragment was transformed into the *ned1*-disrupted mutant in which the Ned1 segment 21 through 121 was replaced with the *ura4*$^+$ gene. Ura$^-$ transformants were selected and correct replacement was verified by sequencing genomic DNA. The *ura4*$^-$ mutation was removed by genetic crossing before use. For disruption of the *nup189*$^+$ gene, almost the entire open reading frame was replaced by the *ura4*$^+$ gene. Tetrad analysis of diploid cells that were heterozygous for the *nup189* alleles indicated that only two spores with the *ura4*$^-$ marker were viable, suggesting that the *nup189*$^+$ gene is essential for viability. To examine the phenotype of the *nup189*Δ cells, spores were germinated in the absence of supplemented uracil and subsequently examined cytologically. The *S. cerevisiae SMP2* gene was disrupted with the *HIS3* gene from *Candida grablata* (Kitada et al., 1995).

### Preparation and analysis of proteins

Protein samples for sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis were prepared by the alkaline method (Tange et al., 1998). Calf intestine alkaline phosphatase (Roche Diagnostics,

Germany) was used for phosphatase digestion of the cell extract. Phosphatase inhibitor cocktail II (Sigma) was used at the concentration recommended by the manufacturer. Proteins resolved in 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1 mM $MgCl_2$, 0.1 mM $ZnCl_2$, and 0.4% SDS were digested with 20 U of alkaline phosphatase at 30°C for 45 minutes. Western immunoblotting was performed as described (Tange et al., 1998). Cell cultures were synchronized using the *cdc25* arrest-release method (Alfa et al., 1993).

### Two-hybrid interaction

A yeast two-hybrid system (Matchmaker two-hybrid system 3, Clontech, Palo Alto, CA) was used according to the manufacturer's protocol. Two Ned1segments, amino acids 1 through 121 or 314 through 581, were used as bait to screen a cDNA library (Clontech). Segments of Nup189 (460-890 and 511 to 900; 900 is a rough estimate from the DNA segment length), Dis3 (927-970), and Pim1 (332-489) were obtained using the Ned1(1-121) bait. A fragment of Ppa1 (1-214) was also obtained, but because the activity was weak it was not further studied. The Nup189 fragment itself weakly activated the indicator gene, therefore we exchanged the cloning vectors and verified the interaction. There were no positive clones obtained with the other Ned1 segment.

### Nuclear transport assay

Wild-type or *ned1-1* mutant cells were transformed with pR1FPap1 and incubated at 30°C for 13 hours in the absence of thiamine to induce the expression of the GFP-Pap1. Diethyl maleate (2 mM) was added and incubated at the same temperature. The localization of GFP signal was observed at 0, 0.5, and 1 hour after the addition of the drug (Kudo et al., 1999).

### Cytologic observations

Procedures for fluorescence in situ hybridization (FISH) and indirect immunostaining were described previously (Tange et al., 1998). The method described in Demeter et al. was used to stain the nuclear pores (Demeter et al., 1995). For FISH, rDNA was used as a probe for chromosome III and cos713 as a probe for chromosome II (Funabiki et al., 1993; Mizukami et al., 1993). Mouse anti-rat nucleoporin monoclonal antibody (MAb414, Eurogentec, Belgium) or rabbit anti-Nup189 polyclonal antibody was used to stain the nuclear pores. The latter antibody was raised using the GST-fused Nup189 segment (9-140) as an antigen. Rabbit anti-Ned1 antibody was raised using a Ned1 segment (124-258). To visualize nuclear membranes (Tange et al., 1998), the pD817 plasmid was transformed into appropriate cells. For electron microscopic observation, diploid *ned1* cells were incubated in YPD at 36°C for 4 hours. Thin sections were prepared either by the freeze-substituted fixation method (Sun et al., 1992) or by the permanganate fixation method (Kawai et al., 2001) and observed with a H-7600 electron microscope (Hitachi High-Technologies, Tokyo). Three-dimensional reconstitution of the cellular structure from serial thin sections was performed as described previously (Sun et al., 1992).

## Results

### Isolation of the *ned1* mutant

We previously described a genetic method to obtain fission yeast mutants that failed to produce stable diploid cells after conjugation (Tange and Niwa, 1995). Because aneuploidy is lethal or unstable in this yeast (Niwa and Yanagida, 1985), it was anticipated that some of the diploid-less mutants would be chromasomally unstable. Diploid cells of one of the mutants were highly unstable at high temperatures and became haploid or died, probably via the aneuploid state (Fig. 1A; see also Materials and Methods). Mutant diploid cells contained lagging or non-disjoined chromosomes in approximately 20% of the mitotic anaphase cells, as revealed by chromosome-specific FISH analysis (Fig. 1B). Such a cytologically defective phenotype was not apparent in haploid cells, indicating that diploid cells were more susceptible to the mutation. A genetic test using a minichromosome, however, indicated that haploid cells had impaired chromosome maintenance (Fig. 6C).

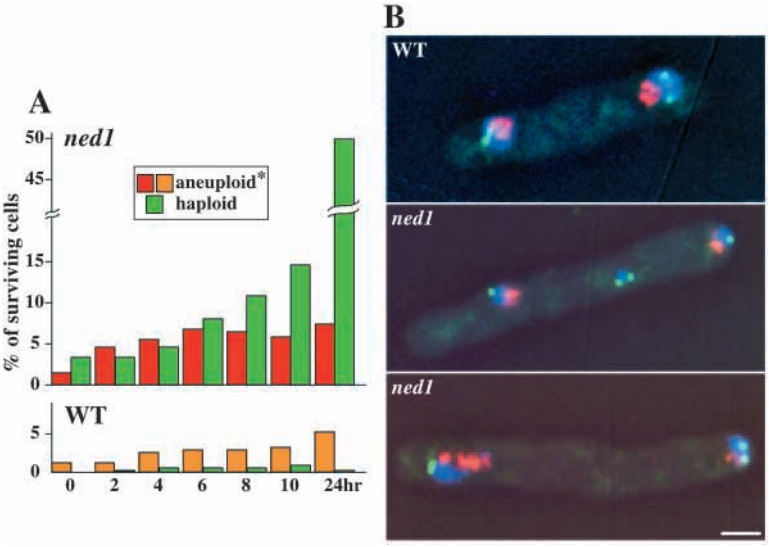



**Fig. 1.** Chromosome missegregation in the *ned1-1* mutant. (A) Diploid cells were incubated in a rich medium YPD at 36°C for indicated periods and plated on MR-plates at 26°C. Haploid (white in color) and aneuploid colonies (deep red, sectored) were scored. *For wild-type (WT), deep red colonies are categorized as aneuploid in the figure, however those with sectors were very rare, with undetermined genetic constituents. (B) FISH analysis of segregating chromosomes. Diploid cells were incubated in YPD medium at 36°C for 4 hours. Red, chromosome III (rDNA); green, chromosome II (cos713); blue, chromatin regions (DAPI). In the second panel, non-disjoined chromosome II is left in the mid-zone. In the bottom panel, one of the two chromosome III shows nondisjunction. Bar, 2 μm.

Another remarkable feature of the mutant was that it contained a highly deformed nucleus as revealed both by DAPI staining and immunostaining with anti-nucleoporin antibodies (Fig. 2A,B). With anti-nucleoporin antibodies, stained interphase nuclei in wild-type cells were usually round, but mutant nuclei were elongated and occasionally constricted. In extreme cases, mutant nuclei were stained in a figure-eight shape. Double staining with DAPI and ethidium bromide indicated that one of the spheres was rich in RNA (Fig. 2A), probably representing the nucleolus (Toda et al., 1981). Consistently, electron microscopic observation indicated that the mutant contained a deformed nucleus (Fig. 2D). In addition, it revealed the presence of enormously overdeveloped endoplasmic reticulum (ER)-like membranes (Fig. 2D). The overdeveloped ER-like membranes often produced mesh-like or multi-layered structures (arrows in Fig. 2D). We also examined the localization of an ER marker, Sec61 (Broughton

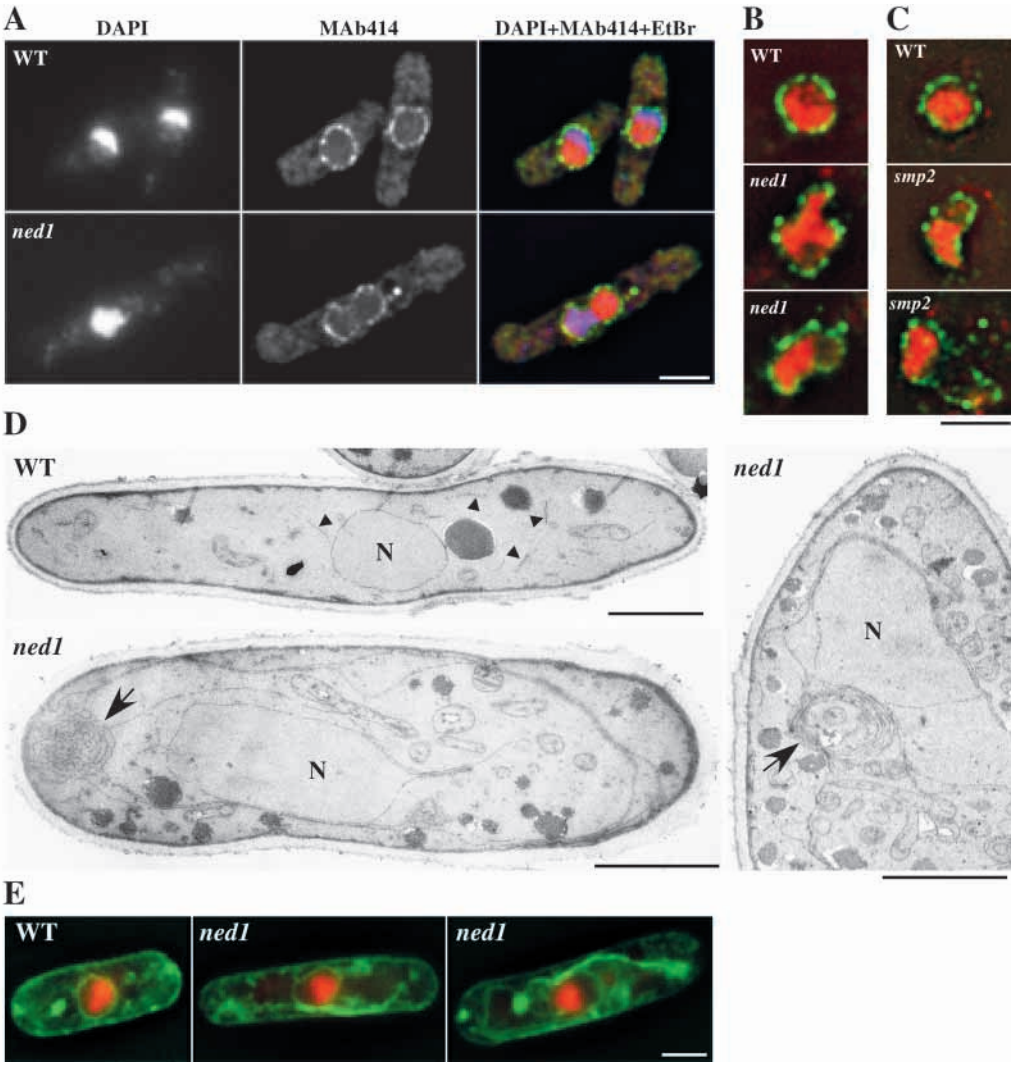



**Fig. 2.** Deformed nuclei and abnormal ER-like membranes in the *ned1-1* mutant. (A) Haploid cells were incubated in YPD medium at 30°C. Merged images: blue, DAPI; red, ethidium bromide; green, MAb414. (B) Haploid cells are incubated in EMM2 medium at 30°C. The anti-Nup189 antibody was used instead of MAb414 (green), chromosomes were in red. (C) *S. cerevisiae* diploid nuclei stained with anti-Nup189. (D) Electron micrographs of diploid cells cultured at 36°C for 3 hours. N, nucleus; arrowheads, ER in wild-type; arrows, mesh-like (left bottom) and multi-layered structure (right) probably formed from enormously overdeveloped ER-like membranes in *ned1-1* cells. (E) Haploid fission yeast cells transformed with pSec61-GFP. Cells are incubated in thiamine-free EMM at 30°C for 20 hours. Green, GFP; Red, DAPI. Bars, 2 μm.

et al., 1997). The results, shown in Fig. 2E, demonstrate that the distribution of GFP-tagged Sec61 was abnormal in the mutant. The affected gene in the mutant was named *ned1*$^+$ (nuclear elongation and deformation).

## Cloning of the *ned1*$^+$ gene

In addition to the phenotype described above, the *ned1* mutant was hypersensitive to TBZ, a microtubule destabilizer. The mutant was unable to form colonies on a YE plate containing 20 μg/ml TBZ at 30°C. We cloned the *ned1*$^+$ gene, which complemented the drug hypersensitivity. The nucleotide sequence of the gene was deposited in public databases (SPAC1952.13) and predicted to encode a protein with 656 amino acid sequences (estimated molecular weight, 73.4 kDa). We sequenced a cDNA clone to verify the predicted introns. In the genomic nucleotide sequence, there were two in-frame ATG codons upstream of the predicted start codon. We therefore verified the prediction by creating termination codons at appropriate positions in the cloned gene (data not shown). The predicted amino acid sequence of the Ned1 protein contains two distinct segments [LNS1 and LNS2 (Lipin/Ned1/Smp2) homology, corresponding to NLIP and CLIP in mouse/human Lipin (Peterfy et al., 2001)], which are highly conserved in a wide range of species, including human, mouse, nematode, Arabidopsis, *S. cerevisiae* (*SMP2*), and fission yeast. The mutation in the original *ned1* mutant (*ned1-1*) was mapped to a completely conserved glycine residue (G402 in wild-type Ned1 is changed to D in the mutant) in the LNS2 segment, consistent with the functional homology.

Using the cloned *ned1*$^+$ sequence, we disrupted the gene (Materials and Methods). Resultant gene disruptants (*ned1*Δ) were viable, indicating that the gene is not essential for cell viability, although *ned1*Δ spores failed to form colonies on rich media, the reason for which is not known.

## Aberrant nuclear morphology in the *smp2* mutant of budding yeast

The *S. cerevisiae smp2* mutant was originally isolated from mutants that stabilized an unstable plasmid, and the gene disruption conferred a respiration-negative and a weak temperature-sensitive phenotype (Irie et al., 1993). To compare the function of the *SMP2* gene with that of the *ned1*$^+$ gene, we examined the nuclear morphology and the sensitivity to a

microtubule agent as well as diploid stability of an *smp2*Δ mutant. Diploid cells of the mutant were more sensitive to benomyl (a microtubule destabilizer) and the nucleus was deformed similarly as in fission yeast (Fig. 2C). Both of these defects were rescued by a plasmid carrying the *SMP2* gene. The stability of chromosomes in the mutant diploid, however, was not appreciably reduced, as judged by the stability of the heterozygosity at the *ADE2* locus (data not shown). The stability of a minichromosome, YCp50, (Rose et al., 1987) was also not impaired by the *smp2* mutation in either haploid or diploid cells. The minichromosome was stabilized in the mutant (data not shown), indicating that the plasmid-stabilization effect of the *smp2* mutation is not confined to the non-centromere plasmid. These results suggest that the function of the *ned1*$^+$/*SMP2* genes in nuclear morphology is conserved, but the function in chromosome stability might be different between these yeasts.

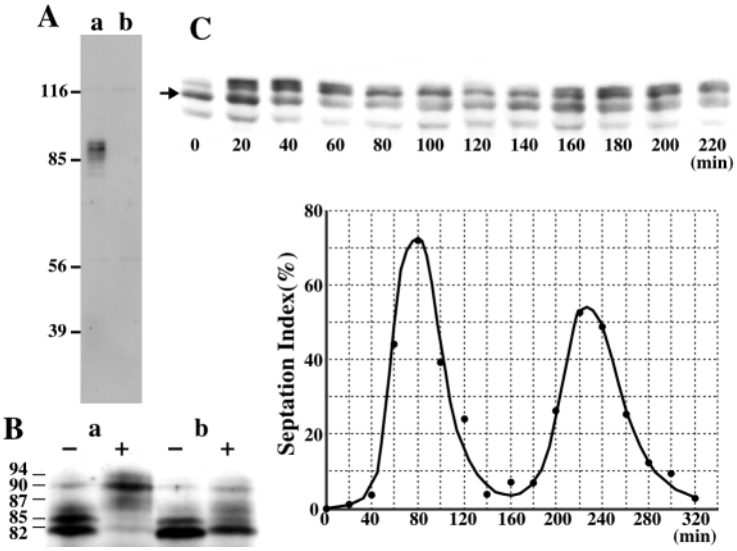



**Fig. 3.** Identification of Ned1 protein. (A) Extracts prepared from a wild-type (a) or a *ned1*Δ strain (b) were examined by western blot analysis. Numbers on the left indicate the molecular weight (kDa) of protein standards. (B) Wild-type fission yeast cell extracts prepared from a logarithmic (a) or from a stationary culture (b) in EMM2 at 30°C. Each of the protein samples was digested with alkaline phosphatase in the absence (–) or presence (+) of phosphatase inhibitors. Numbers on the left indicate the molecular weight (kDa) of each band. (C) Protein samples prepared from a synchronized culture at indicated times are analyzed by western blotting (top). The arrow indicates the position of a 90 kDa band. The synchrony was shown by the profile of the septation index (bottom).

## Identification of Ned1 protein

We raised an anti-Ned1 antibody using an N-terminal portion of the Ned1 protein (see Materials and Methods). Western immunoblotting with this antibody stained several bands; the intensity of each band varied depending on the culture conditions under which the protein extracts were prepared (Fig. 3B). Slower migrating forms were less abundant in stationary phase cell extracts. All of these bands disappeared when we used an extract from *ned1*Δ cells (Fig. 3A), indicating that the bands represented some forms of the *ned1*$^+$ gene product. Digestion of cell extracts with bacterial alkaline phosphatase resulted in the bands shifting to faster migrating forms and these band shifts were inhibited by phosphatase inhibitors, suggesting that Ned1 is phosphorylated at multiple sites (Fig. 3B). Because logarithmic phase cell extracts were rich with slower migrating forms, we examined whether the phosphorylation of Ned1 changed during the cell cycle. In a synchronous culture, the slowest migrating form of Ned1 protein peaked at approximately the M-phase (Fig. 3C).

## Two-hybrid interaction of Ned1 with an essential nucleoporin as well as with Dis3 and Pim1/RCC1

To elucidate the molecular function of Ned1, we performed a two-hybrid search for interacting proteins. As a result, five types of gene segments were isolated using a LNS1-containing part of Ned1 (amino acid sequence 1-121) as bait (see Materials and Methods), while there were no positive clones obtained with the LNS2-containing Ned1 segment (332-563). One of the isolated proteins was a segment of a previously unidentified nucleoporin protein (segment 460-890 of the 1778 amino acid sequence) and others were from two known proteins, Dis3 (927-970) and Pim1 (332-489). Subsequent subcloning indicated that a longer C-terminal segment of Dis3 (440-970) as well as a C-terminal truncated version of this fragment (440-927), were inactive for two-hybrid interaction with Ned1. Why only the shortest fragment was active is not understood. As for Pim1, a segment (412-477) that contained the last RCC1 repeat was more active for two-hybrid interaction than the original longer isolate, but the second to last repeat (346-428) was inactive. A combination of these two repeats as well as longer fragments that contained them gave an intermediate level of the two-hybrid interaction. Subcloning of the nucleoporin gene sequence indicated that a segment ranging from 771 through 863 was responsible for the interaction (Fig. 4A). Further studies are needed, however, to examine whether these proteins are interacting directly as predicted by the two-hybrid analyses.

We cloned the nucleoporin gene (SPAC1486.05), designated *nup189*$^+$ after the calculated molecular weight of its product. An anti-Nup189 antibody was raised using a segment of Nup189 (9-140) as an antigen. Western blot analysis with the antibody indicated one major band migrating at 98 kDa along with a less intense band at 88 kDa and other very faint bands. In cells carrying the *nup189*$^+$ plasmid, the major band became more intense, but other bands were not affected, indicating that the major band represented the *nup189*$^+$ gene product (Fig. 4B, and see Discussion). Indirect immunostaining with the Nup189 antibody was punctate along the nuclear envelope and largely overlapped at sites that were stained by another anti-nucleoporin antibody, MAb414 (Fig. 4C), consistent with Nup189 being a component of the nuclear pore complex. Fission yeast Nup189 was essential for cell viability (see Materials and Methods). Spores with a disrupted *nup189* gene germinated but nuclear division did not follow. The nucleus was prominently enlarged with a vast region devoid of chromatin and often appeared clumpy with DAPI staining (Fig. 4D).

### Localization of Ned1 protein and the mutant effect on nuclear transport

Because Ned1 appeared to interact with a nucleoporin as well as with Dis3 and Pim1, both of which are nuclear or at least enriched in the nucleus (Kinoshita et al., 1991; Matynia et al., 1996), we examined its localization. We failed to determine any particular localization of the protein, although it appeared less abundant in the nucleus. Some accumulation of Ned1 protein was occasionally observed, however, along the periphery of the nucleus in cells with mildly overproduced Ned1 (data not shown). We also examined whether the *ned1* mutation affects nuclear transport. To this end, we used a transcription factor, Pap1, that is largely localized in the cytoplasm dependently on the Crm1/exportin activity, but accumulates in the nucleus following oxidative stress (Kudo et al., 1999). The Pap1 protein fused with the GFP (Kudo et al., 1999) was used to examine the nuclear transport activity in the *ned1* mutant. In both the wild-type and mutant cells, the GFP signals were localized in the cytoplasm before treatment with diethyl maleate and accumulated in the nucleus after treatment. There were only very slight differences, if any, in the accumulation rate, indicating that the effect of the *ned1* mutation on nuclear transport is only marginal, at least as determined by this method.

### Genetic interactions with *crm1*, *pim1* and *dis* genes

We then examined whether the *ned1-1* mutation interacts genetically with factors involved in nuclear transport. A fission yeast mutant with defective Crm1 (exportin) is more resistant to several drugs, including caffeine and staurosporin, probably due to accumulation of the Pap1 transcription factor (Kumada et al., 1996). The resistant phenotype of the *crm1* mutant was suppressed by the *ned1* mutation (Fig. 5A). Consistently, the accumulation of p25*apt1* in the *crm1* mutant (Toda et al., 1992) was virtually abolished in the *ned1 crm1* double mutant (Fig. 5B). The cold-sensitive growth phenotype of the *crm1* mutant was not rescued by the *ned1* mutation, but rather it was enhanced (Fig. 5A). The *ned1*$^+$ gene also interacts genetically with Pim1 [guanine nucleotide exchange factor for Ran-GTPase (Matsumoto and Beach, 1991; Sazer and Nurse, 1994)]. The temperature sensitivity of the *pim1-46* mutation was enhanced by the *ned1* mutation (Fig. 5C). The TBZ hypersensitivity of the *ned1* mutant was suppressed by introducing multiple copies of *pim1*$^+$ or *spi1*$^+$ genes (Fig. 5D). In addition, the *pim1* mutation suppressed the effect of Ned1 overproduction on nuclear shape (see below). We also tested whether the *ned1*$^+$ gene interacts with *dis* genes. The cold-sensitive growth phenotype of all of the *dis* mutants tested [*dis1*-null, *dis2-11* and *dis3-54* (Ohkura et al., 1988; Nabeshima et al., 1995)] was enhanced when combined with the *ned1-1* mutation. The effect appeared to be most prominent for the *dis1* mutation (Fig. 6A).

### Effect of a mutation that affected Dis3-interaction

An N-terminal segment of Ned1 interacts with Dis3 in a two-hybrid system. We substituted a highly conserved glycine residue at position 80 in this segment with arginine (G80R) or with alanine (G80A). G80R is the same point mutation found in a defective *lpin1* locus reported in mouse (Peterfy et al., 2001). Two-hybrid analysis indicated that the G80R mutation affected the interaction with Dis3 (Fig. 6B). This mutation eliminated the residual expression of the indicator genes observed in a control experiment with a blank vector plasmid. The G80A mutant, however, did not affect the interaction. Neither of the mutants, however, altered the activity for the two-hybrid interaction with Pim1 and Nup189 (data not shown).

We then introduced the mutant *ned1* genes using a

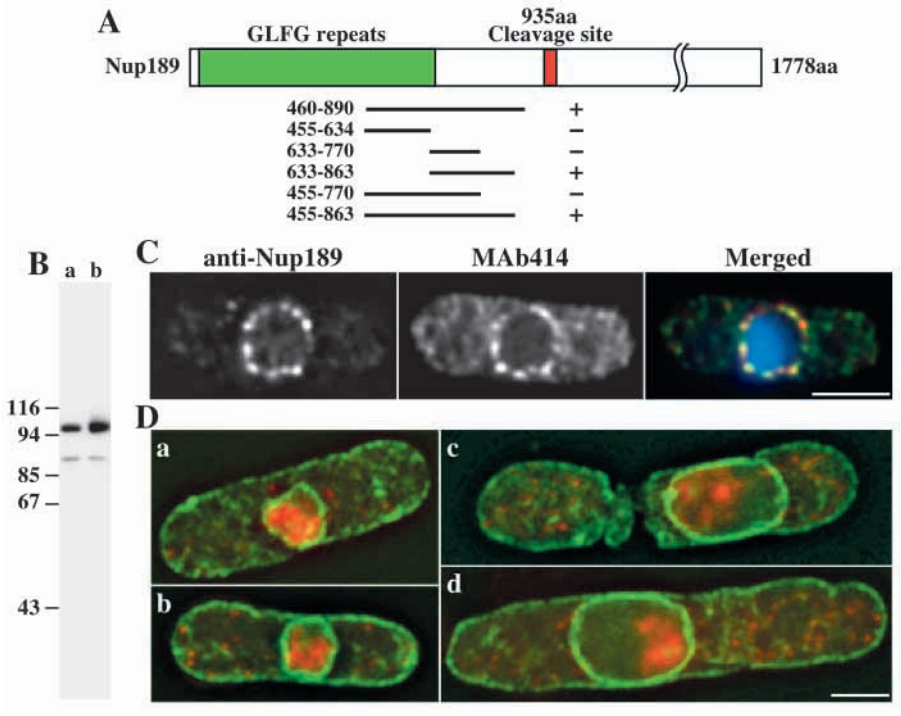



**Fig. 4.** Analysis of Nup189 protein and the defective phenotype of *nup189*-disrupted cells. (A) The two-hybrid interaction activity of indicated protein segments is shown (see text for details). (B) Specificity of the anti-Nup189 antibody. Protein samples prepared from a wild-type strain carrying the vector pKD10 (a) or pKD10-*nup189*$^+$ (b) were electrophoresed in a gel and immunoblotted. The upper band in b is more intense than in a, while the lower bands are of the same intensity. (C) Colocalization of Nup189 and MAb414 antigen. Merged: Nup189, red; MAb414, green; DAPI, blue. (D) Wild-type (a) and mutant spores (b-d) containing pD817 germinated in an appropriate selective medium at 30°C. pD817 carries a segment of cytochrome P450 reductase gene that is fused with the GFP sequence and is used to visualize the nuclear envelope (Tange et al., 1998). (a) 25 hours, (b) 19 hours, and (c,d) 38.5 hours after transfer into the medium. Red, DAPI; green, GFP. Bars, 2 μm.

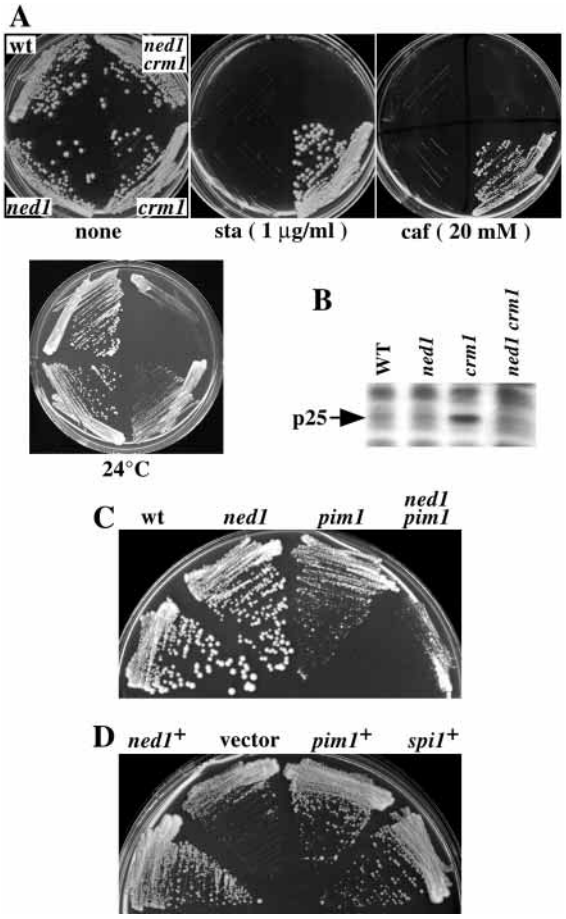
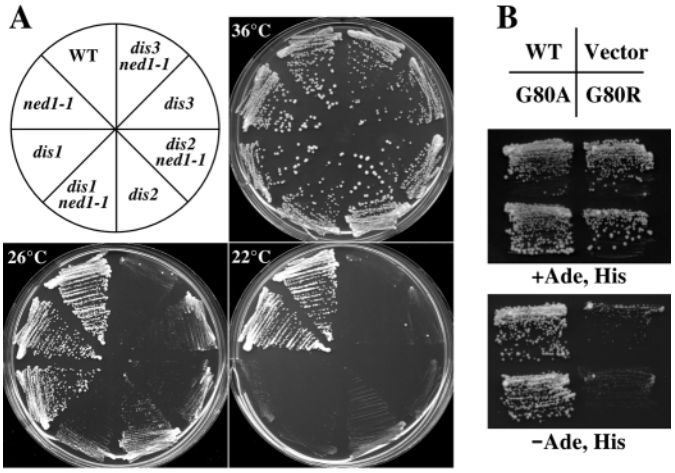



**Fig. 5.** Genetic interactions of *ned1-1* mutant with *crm1*, *pim1* and *spi1*. (A) In the upper panels, indicated strains were incubated at 33°C for 3 days on YEA containing the indicated amounts of staurosporin (sta) or caffeine (caf). In the lower panel, the same strains were incubated on YEA at 24°C for 4 days. (B) Cell extracts prepared from the indicated strains were run on a 10% SDS-polyacrylamide gel and stained with Coomassie Brilliant Blue. The p25 band was only visible in the lane for *crm1* single mutant. (C) Indicated strains were incubated at 33°C for 4 days. (D) The *ned1-1* mutant cells were transformed with multicopy plasmids that carried the indicated gene. Transformants were plated on YEA containing 15 μg/ml TBZ, incubated at 30°C for 3 days.




C

| Minichromosome loss / division | 26°C | 36°C |
|---|---|---|
| wild type | 1.8 (x10$^{-4}$ ) | 3.9 (x10$^{-4}$ ) |
| G402D (*ned1-1*) | 27.0 | 52.9 |
| G80R | 15.7 | 37.3 |
| G80A | 1.9 | 2.6 |

**Fig. 6.** Ned1-Dis3 interactions. (A) Strains indicated are incubated at 36°C or at 26°C for 3 days and at 22°C for 4 days. (B) Two-hybrid interaction between Ned1 (1-121) and Dis3 (927-970). The activity of Ned1 protein segment containing a substitution mutation G80A or G80R as well as blank plasmid is shown on the –Ade, His plate. (C) The rate of minichromosome loss per cell division was determined by the half-sectored colony assay (Allshire et al., 1995). In each experiment, approximately $3\times10^4$ divisions were scored. The values are the average of two experiments.

substitutive transformation (Materials and Methods). The *ned1*-G80R, but not the G80A, mutant was hypersensitive to TBZ (data not shown). A minichromosome assay was performed to examine the effect of *ned1* mutations on chromosome stability. As summarized in Fig. 6C, the rate of minichromosome loss per cell division increased approximately tenfold in the G80R mutant as well as in the original *ned1-1* (G402D) mutant. In both mutants, the loss rate increased another twofold at a high temperature. In contrast, the *ned1*-G80A allele did not affect minichromosome stability. The amount of all of these mutant Ned1 proteins in the cell was not different from that in wild-type cells (data not shown), indicating that the observed phenotypic effect was not likely due to the amount and/or stability of protein. These results suggest that the interaction between Ned1 and Dis3 is important for chromosome stability. Moreover, the *ned1*-G80R mutant produced a severely deformed nucleus in zygotes as in the *ned1-1* mutant (data not shown). The *ned1*-G80A mutant contained a mildly deformed nucleus in zygotes, suggesting that the G80A mutation affects some cellular processes related to nuclear shape.

## Abnormal nuclear events induced by Ned1 overproduction

A full length cDNA sequence of the *ned1*$^+$ gene was placed under the control of a potent inducible promoter in the pREP1 vector (Maundrell, 1993). After the gene was fully induced, cells ceased dividing and became less viable (data not shown). Concomitantly, the nucleus was displaced from the center of the cell and then underwent the characteristic morphologic change, i.e., the nucleus was elongated along the long axis of the cell. Electron microscopic observation revealed a microtubule bundle in the deformed nucleus (Fig. 7A,B). This bundle was not a normal spindle, because, first, there were no spindle pole bodies (SPB) at the ends of the bundle (Fig. 7Bb,c, arrows); rather a single SPB was situated on the nuclear membrane, usually facing the cell wall. The structure of the SPB (Fig. 7Ba, arrowhead) was very similar to that reported in the interphase cell (Ding et al., 1997). Similar to the interphase cell, it was

situated in the cytoplasm and associated with an electron-dense region lining along the inner nuclear membrane near the SPB. Second, cytoplasmic microtubules were present in the affected cell, indicative of an interphase cell (Fig. 7Cd). In addition, the characteristic nuclear elongation was observed in the *cdc25* mutant (data not shown), which blocks the G2/M transition (Millar and Russell., 1992). Fluorescence microscopic observation using a GFP-tagged α-tubulin and an anti-nucleoporin antibody unambiguously showed the coexistence of cytoplasmic microtubules and the intranuclear microtubule bundle (Fig. 7C). The nuclear elongation appeared to be due to the presence of the microtubule bundle, because when cells were placed on ice (to induce microtubule disassembly) the nucleus did not remain elongated. Consistently, nuclear elongation was largely suppressed by the cold-sensitive β-tubulin mutation, *nda3-KM311* [(Hiraoka et al., 1984) and data not shown]. Furthermore, the *pim1-46* mutation significantly reduced the effect of Ned1 overproduction on nuclear elongation (Fig. 7D), raising the possibility that Ran is involved in the formation of the intranuclear microtubule bundle. An attempt to introduce the GFP-tagged α-tubulin into the *pim1* mutant was unsuccessful probably because the mutant was unable to tolerate transformation with the plasmid carrying the GFP-tubulin gene.

Indirect immunostaining with an anti-Sad1 antibody revealed another peculiar nuclear response to the overproduced Ned1. As described above, electron microscopic observation revealed that the elongated nucleus carried only one SPB. Consistently, an SPB component protein, Cut12 (Bridge et al., 1998), was localized to a single site (Fig. 7E). Another SPB component protein, Sad1 (Hagan and Yanagida, 1995), was located at multiple sites along the nuclear periphery (Fig. 7E,F). Chromosomal centromeres, which would form a single cluster beneath the SPB in a wild-type interphase nucleus, also tended to be located at multiple positions that were often close to the scattered Sad1 proteins, as demonstrated using the GFP-tagged Mis6, a centromere protein (Saitoh et al., 1997) (Fig. 7F). Thus, Ned1 overproduction appeared to affect the chromosome arrangement within the nucleus, possibly by affecting the nuclear envelope structure.

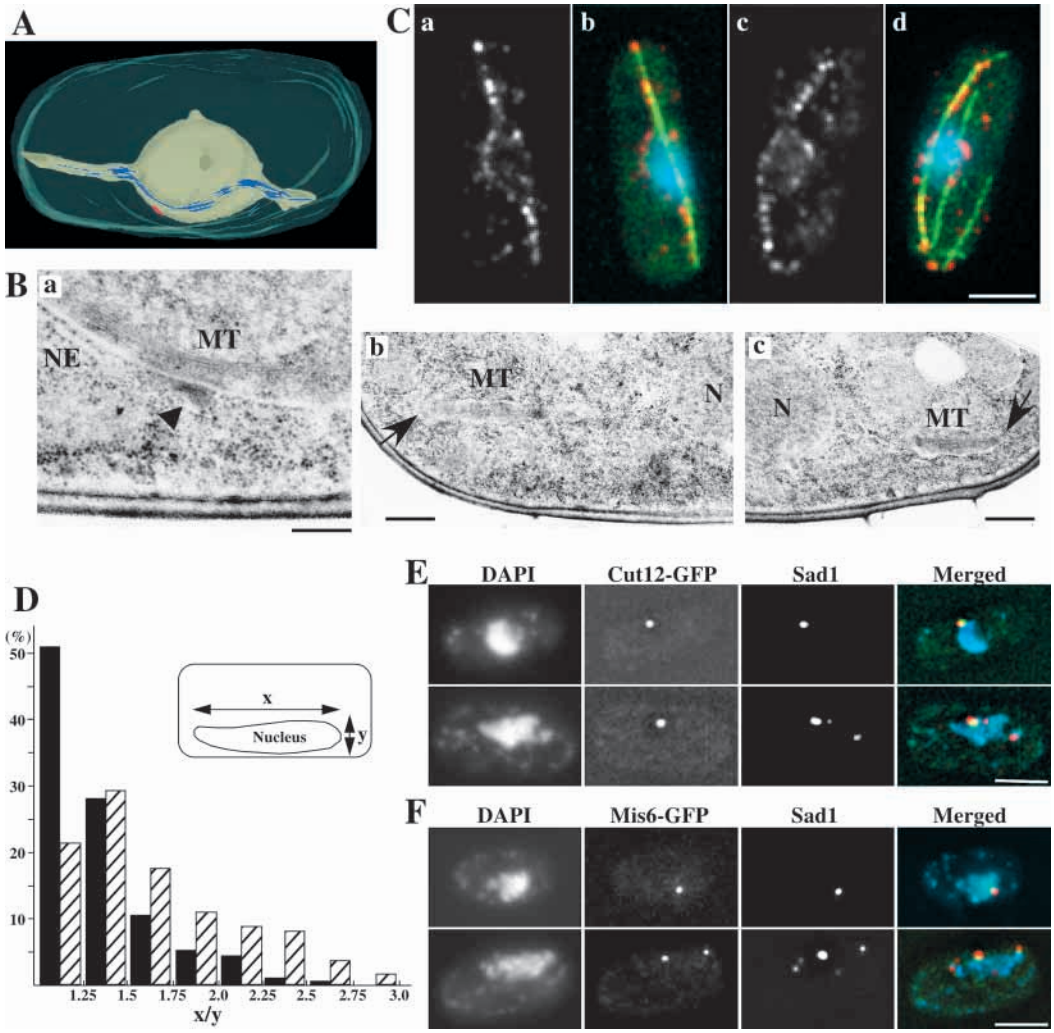



**Fig. 7.** The effect of Ned1-overproduction on the nuclear structure. (A) A 3D image of a cell with overproduced Ned1. Electron micrographs from serial thin sections were used to construct the image (see Materials and Methods). The cell was incubated at 30°C for 24 hours to induce the expression of Ned1 protein. Blue, microtubule bundle in the nucleus; red, the spindle pole body (SPB). (B) Representing parts of electron micrographs used for construction of the 3D image shown in A. N, nucleus; MT, microtubules. (a) Middle part of the cell; arrowhead, the SPB situated in the cytoplasm. (b,c) Left and right parts of the cell, respectively; arrows indicate the ends of extended nuclear envelope. (C) Wild-type cells carrying both pREP82tubGFP and pREP1*ned1*$^{+}$cDNA are incubated in the absence of thiamine at 30°C for 24 hours. (a,c) Localization of Nup189. (b,d) Microtubules (green), Nup189 (red) and DAPI (blue). (D) Suppression of the nuclear elongation by the *pim1-46* mutation. *pim1*$^{+}$ or *pim1-46* cells were induced for full expression of Ned1 (26°C for 28.5 hours) and the nuclei were stained with DAPI. (E,F) Cells carrying either Cut12-GFP (E) or Mis6-GFP (F) were incubated as in A for the Ned1-overproduction (bottom panels; in upper panels cells without Ned1-overproduction) and stained with DAPI and anti-Sad1 antibody. Merged: cyan, DAPI; green, GFP; red, Sad1. Bars in B: 0.2 μm (a), 0.4 μm (b, c); other bars, 2 μm.

The cells with an elongated nucleus were more resistant to cell wall digestion, which made the cell preparation for cytologic staining difficult. Although there was no observed difference in the cell wall structure in electron micrographs, Ned1-overproduction might also affect cell wall structure.

## Discussion

Fission yeast *ned1*$^+$ is an evolutionarily conserved gene. Mutations in homologous genes have been studied in mouse (Peterfy et al., 2001) and in budding yeast (Irie et al., 1993), but common molecular functions of the gene products, if any, are difficult to deduce from their divergent phenotypes. In the present study, apparently unrelated phenotypes that are associated with the mutations in fission yeast were added, although the effect of the mutations on nuclear morphology resembled those in fission and budding yeast. Fission yeast Ned1 interacts with Dis3 and Pim1/RCC1 as well as with a newly identified nucleoporin in a yeast two-hybrid system. The Dis3 segment obtained in the two-hybrid screen consisted of 44 amino acids from the C-terminus. A C-terminal region of Dis3 containing the identified segment is essential for the function of Dis3 (Kinoshita et al., 1991). Pim1 contains seven RCC1 repeats (Matsumoto and Beach, 1991) and the last, but not the second to last repeat, appears to be involved in the interaction between Ned1 and Pim1/RCC1. RCC1-repeats are important for function (Uchida et al., 1990; Amberg et al., 1993), although they are not all functionally equivalent (Lee et al., 1994). The *ned1* gene accordingly displayed genetic interactions with *dis3*, *pim1*, *spi1* and *crm1*. The results generally suggest that *ned1* mutations attenuate the activity of Ran-GTP and that the interaction of Ned1 with Dis3 is important for Ned1 function. Results consistent with this notion are: (1) the *ned1-1* mutation enhanced the temperature sensitivity of a *pim1* mutant; (2) overdosed *pim1*$^+$ or *spi1*$^+$ genes suppressed the TBZ-hypersensitive defective phenotype of the *ned1* mutantp; (3) the cold sensitive growth defect of a *crm1* mutant was enhanced by the *ned1* mutation; (4) the *ned1* mutation interacts with *dis3* as well as other *dis* mutants; and (5) a *ned1* mutant that altered interaction with Dis3 was functionally impaired. In addition, the *ned1* mutation enhanced the cold sensitivity of *dis1*, *dis2* and *dis3* mutants, which is consistent with a previous finding that combinations of a dis3 mutation with *dis1* or *dis2* are lethal (Kinoshita et al., 1991). Dis3 binds to Ran to enhance the guanine nucleotide exchange activity of Pim1/RCC1 (Noguchi et al., 1996). Taken together, these findings suggest that Ned1 enhances the Ran-GTP activity by regulating Dis3/Pim1 activity. Given that Ran has roles in multiple cellular processes, it is conceivable that the pleiotropic defective phenotype of *ned1* mutations arise through a disturbance of the Ran-GTPase system in the mutant. How each of the defective phenotypes is mechanically related with each other remains to be determined.

Overproduction of Ned1 lead to cell death that was accompanied by two peculiar nuclear events. One was the formation of an intranuclear microtubule bundle and the other was the localization of Sad1 protein at multiple sites along the nuclear periphery. The former is similar to the Ran-dependent microtubule assembly reported in other organisms (see Introduction), because the bundle formation is apparently independent of the microtubule organizing center and dependent on active Pim1/RCC1 and therefore on GTP-bound form of Ran. The ectopic formation of the microtubule bundle requires extremely high levels of Ned1 protein and thus it is unlikely to occur under normal physiologic conditions. Nevertheless, investigation of this event might provide useful information regarding the regulation of microtubule dynamics in this yeast. Sad1 protein is usually tightly associated with the SPB (Hagan and Yanagida, 1995). Sad1 protein is a membrane-bound protein (Hagan and Yanagida, 1995) and its mammalian homologue is enriched in an inner nuclear membrane fraction (Dreger et al., 2001). How Sad1 proteins are tethered at the SPB is not known. Ned1-overproduction might impair this tethering mechanism.

Besides Dis3 and Pim1, Ned1 appears to interact with a nucleoporin named Nup189. Nup189 belongs to the family of GLFG repeat-containing nucleoporins and is similar to Nup145 in *S. cerevisiae* and Nup98/96 in mammals (Ryan and Wente, 2000), both of which are required for nuclear transport (Fabre et al., 1994; Wu et al., 2001) and are thought to be involved in nucleoplasmic structure and chromosome positioning (Fontoura et al., 2001; Galy et al., 2000). Nup189 contains an HFS sequence at position 933 through 935, which is conserved among this type of nucleoporin and is the site of autoproteolysis (Fontoura et al., 1999; Rosenblum and Blobel, 1999). The calculated molecular weight of the expected proteolytic product from the N-terminal side is 95 kDa, which is very similar to that estimated from gel mobility. The biologic significance of the presumed in vivo interaction between Ned1 and Nup189 is not clear. Ned1 is exceptional in the homologous proteins, however, as it contains no authentic nuclear localization signal (Peterfy et al., 2001). It is, therefore, possible that Ned1 is translocated into the nucleus, where it might contact Dis3 and Pim1 through interaction with the component of the nuclear pore complex.

The Ned1 homologue in mouse, Lipin, is defective in animals that suffer from a type of lipodystrophy. It was recently reported that lamin A/C deficiency can cause another type of lipodystrophy (Cao and Hegele, 2000; Flier, 2000; Shackleton et al., 2000). Together with our finding that mutations in the *ned1* gene affect nuclear structure and that the overproduction of Ned1 impairs the localization of nuclear membrane-bound protein, we suggest that the primary effect of a Lipin-deficiency might be on the nuclear envelope structure. Finally, although to our knowledge it is not known whether any *lpin* mutations are associated with chromosome instability, this is an important possibility because of the intimate relation between chromosome instability and tumorigenesis (Sen, 2000).

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