

The Ran GTPase cycle is required for yeast nuclear pore complex assembly

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Here, we report the first evidence that the Ran GTPase cycle is required for nuclear pore complex (NPC) assembly. Using a genetic approach, factors required for NPC assembly were identified in *Saccharomyces cerevisiae*. Four mutant complementation groups were characterized that correspond to respective mutations in genes encoding Ran (*gsp1*), and essential Ran regulatory factors Ran GTPase-activating protein (*rna1*), Ran guanine nucleotide exchange factor (*prp20*), and the RanGDP import factor (*ntf2*). All the mutants showed temperature-dependent mislocalization of green fluorescence protein (GFP)-tagged nucleoporins (nups) and the pore-membrane protein Pom152. A decrease

in GFP fluorescence associated with the nuclear envelope was observed along with an increase in the diffuse, cytoplasmic signal with GFP foci. The defects did not affect the stability of existing NPCs, and nup mislocalization was dependent on de novo protein synthesis and continued cell growth. Electron microscopy analysis revealed striking membrane perturbations and the accumulation of vesicles in arrested mutants. Using both biochemical fractionation and immunoelectron microscopy methods, these vesicles were shown to contain nups. We propose a model wherein a Ran-mediated vesicular fusion step is required for NPC assembly into intact nuclear envelopes.

Introduction

Nuclear pore complexes (NPCs)* are required for the exchange of all molecules between the nucleus and cytoplasm. This includes the diffusion of small molecules and the selective, facilitated transport of large proteins and RNAs (Görllich and Kutay, 1999). These large, proteinaceous structures are believed to be architecturally and functionally similar in all eukaryotic organisms (Yang et al., 1998; Stoffler et al., 1999). Many questions concerning biochemical composition, structure, and the mechanism of translocation through the NPC have been answered in recent years. However, the molecular pathway of NPC biogenesis remains largely undefined.

The three-dimensional structure of NPCs has been studied in a wide range of organisms including the budding yeast *Saccharomyces cerevisiae* and *Xenopus* oocytes (Yang et al., 1998; Stoffler et al., 1999). NPCs are embedded in a pore of ~90-nm diameter that joins the inner and outer nuclear

membranes. All show a conserved, eightfold rotational symmetry with a series of spokes and rings forming the core and filaments extending to make both cytoplasmic fibrils and a nuclear basket. Recent proteomic-based studies have reported that multiple copies of ~30 different proteins, termed nucleoporins (nups), comprise the calculated 44-MD yeast or 60-MD mammalian NPC (Rout et al., 2000; Cronshaw et al., 2002). Experimentally, these different nups can be isolated in discrete subcomplexes and localized to specific substructures (Ryan and Wente, 2000; Vasu and Forbes, 2001). Analysis of these subcomplexes has been critical to begin understanding the physical contacts between the 30 different nups in the final NPC structure (Finlay et al., 1991; Grandi et al., 1993, 1997; Kita et al., 1993; Hu et al., 1996; Belgareh et al., 1998; Marelli et al., 1998; Siniossoglou et al., 2000; Vasu et al., 2001; Lutzmann et al., 2002).

In cells undergoing an open mitosis, NPCs are disassembled as the nuclear envelope (NE) breaks down at the onset of mitosis (Burke and Ellenberg, 2002). After the separation of chromatids, the NE and NPCs must be reformed. In addition, NPC assembly occurs continuously throughout the cell cycle. The number of NPCs is lowest just after mitosis or in resting cells, and steadily increases until doubled in preparation for the next cell division (Maul et al., 1971, 1972; Winey et al., 1997). These new NPCs must be assembled de novo into the intact NE double membrane. Despite conceptual differences

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*Abbreviations used in this paper: GAP, GTPase-activating protein; GEF, guanine nucleotide exchange factor; NE, nuclear envelope; *npa*, nuclear pore complex assembly mutant; NPC, nuclear pore complex; nup, nucleoporin; ts, temperature-sensitive.

Key words: Ran; vesicular trafficking; nucleoporin; NPC; nuclear envelope

in NPC reassembly after mitosis and assembly during interphase, *in vitro* studies using *Xenopus* egg extracts have suggested that mitotic NPC reassembly also occurs within closed double membranes (Macaulay and Forbes, 1996). In yeast and other organisms undergoing a closed mitosis in which the NE remains intact, all NPC assembly requires insertion into a preexisting double nuclear membrane.

Working models for both NE and NPC assembly predict stepwise mechanisms with requirements for vesicular and soluble components (Burke and Ellenberg, 2002; Hetzer et al., 2002). A focus on the post-mitotic reassembly pathway in vertebrate cells has revealed distinct assembly intermediates and mechanistic separation of the NE reformation and NPC assembly events (Macaulay and Forbes, 1996; Goldberg et al., 1997; Wiese et al., 1997). Membrane vesicles first bind chromatin and fuse to form the closed inner and outer nuclear membranes. The fusion process is inhibited by GTP γ S (Pfaller et al., 1991; Boman et al., 1992; Newport and Dunphy, 1992; Macaulay and Forbes, 1996), and subsequent studies have demonstrated that the small GTPase Ran is required for fusion and NE reformation (Hetzer et al., 2000; Zhang and Clarke, 2000). After NE reformation, there is a second, distinct NPC assembly step that is also inhibited by GTP γ S. Blocking this step results in intact inner and outer nuclear membranes that lack any detectable NPC structures or pores (Macaulay and Forbes, 1996). To date, the mechanism by which GTP γ S specifically inhibits NPC formation has not been elucidated. Finally, after the two GTP γ S-sensitive steps, NPC assembly can be inhibited by the Ca²⁺ chelator BAPTA (Sullivan et al., 1993; Macaulay and Forbes, 1996). The specific NPC assembly steps inhibited by GTP γ S and BAPTA may involve the fusion machinery required for joining the inner and outer nuclear membrane during nuclear pore formation (Macaulay and Forbes, 1996). It is not known how pore formation is coordinated with the other stages of NPC assembly.

In addition to mediating NE reformation, Ran GTPase plays at least two other essential roles in nuclear physiology (Dasso, 2002; Hetzer et al., 2002). Ran was originally identified as a regulator of nucleocytoplasmic transport (Melchior et al., 1993; Moore and Blobel, 1993), and has more recently been shown to mediate spindle assembly during mitosis (Carazo-Salas et al., 1999; Ohba et al., 1999; Wilde and Zheng, 1999; Bamba et al., 2002). RanGTP interacts with a family of shuttling transport factors (termed karyopherins, importins, exportins, and transportins) that recognize nuclear localization or export sequences in their respective protein cargos (Pemberton et al., 1998; Görlich and Kutay, 1999). The GTPase activity of Ran is regulated by a specific GTPase-activating protein (GAP), and Ran requires a guanine nucleotide exchange factor (GEF). RanGAP (Rna1 in *S. cerevisiae*) is localized in the cytoplasm, whereas the RanGEF (RCC1 in vertebrates; Prp20 in *S. cerevisiae*) is nuclear. The spatial segregation of the regulatory factors is predicted to maintain Ran in a predominately GDP-bound state in the cytoplasm and in a GTP-bound state in the nucleus (Moore, 1998; Görlich and Kutay, 1999). Ntf2 acts as the specific nuclear import factor for RanGDP (Görlich and Kutay, 1999). Extensive studies have firmly established that Ran acts as a molecular switch for regulating karyopherin as-

sociation/disassociation with cargo. For nuclear import, RanGTP triggers cargo release, whereas RanGTP stabilizes the karyopherin–cargo interaction during export (Nakielnny and Dreyfuss, 1999). The mechanism for Ran-mediated spindle formation may follow the same paradigm wherein localized RanGTP controls the release of spindle assembly factors that are inhibited by interactions with karyopherins (Gruss et al., 2001; Nachury et al., 2001; Wiese et al., 2001). It is possible that the Ran step in NE biogenesis involves the control of karyopherin–cargo or karyopherin–nup interactions (Zhang et al., 2002).

In this paper, we report the first evidence that the Ran GTPase cycle is required for NPC assembly. Using a genetic approach in the budding yeast *S. cerevisiae*, we have identified nuclear pore complex assembly mutants (*npa*) with temperature-sensitive (ts) perturbations in the direct fluorescence properties of GFP-tagged nups (Ryan and Wentte, 2002). Four of the complementation groups correspond to mutations in the genes encoding Ran (*Gsp1*), the RanGAP (*Rna1*), the RanGEF (*Prp20*), and the Ran import factor, *Ntf2*. Strikingly, the mutants accumulate nup-containing vesicles coincident with defects in new NPC assembly at the NE. We speculate that the Ran GTPase is required for vesicle-mediated NPC assembly into intact NEs.

Results

Isolation of Ran GTPase cycle mutants in a screen for defective NPC assembly

Previously, we conducted a GFP-based screen in the yeast *S. cerevisiae* for *npa* mutants (Ryan and Wentte, 2002). Mutants were identified by direct fluorescence analysis of members of a ts bank expressing both chromosomally integrated GFP-Nic96 and Nup170-GFP. In total, 121 individual mutant strains failed to correctly localize the two GFP-Nups when shifted to the nonpermissive temperature of 34°C. In this work, we focused on three *npa* complementation groups, each represented by a single allele, and identified mutations in *ntf2*, *rna1*, and *prp20* in the respective mutants (*npa11*, *npa13*, and *npa14*). Transformation of each mutant strain with a wild-type copy of its mutated gene rescued both the ts growth defect and GFP-Nup mislocalization phenotype (unpublished data). Because the three proteins encoded by the *NTF2*, *RNA1*, and *PRP20* regulate the small GTPase Ran (encoded by *GSP1* and *GSP2* in yeast; Belhumeur et al., 1993), direct complementation analysis was performed by crossing a strain harboring a ts allele of *gsp1* with the remaining *npa* mutants. A single *gsp1* mutant strain was identified among the *npa* groups (*npa15*) and rescued with a wild-type copy of *GSP1*. *GSP1* is essential and accounts for the majority of Ran in the cell, whereas *GSP2* is not essential and is expressed at low levels under most growth conditions (Belhumeur et al., 1993). Thus, we did not directly test for *gsp2* mutants in the *npa* collection, as they would not have been isolated in our screen for ts alleles.

To determine the exact nature of each mutation, mutant alleles were cloned and sequenced. Each contained a point mutation that resulted in a single amino acid substitution. The *ntf2* allele resulted in a His to Tyr substitution at amino acid 104 (*ntf2-H104Y*). The *rna1* mutation led to a

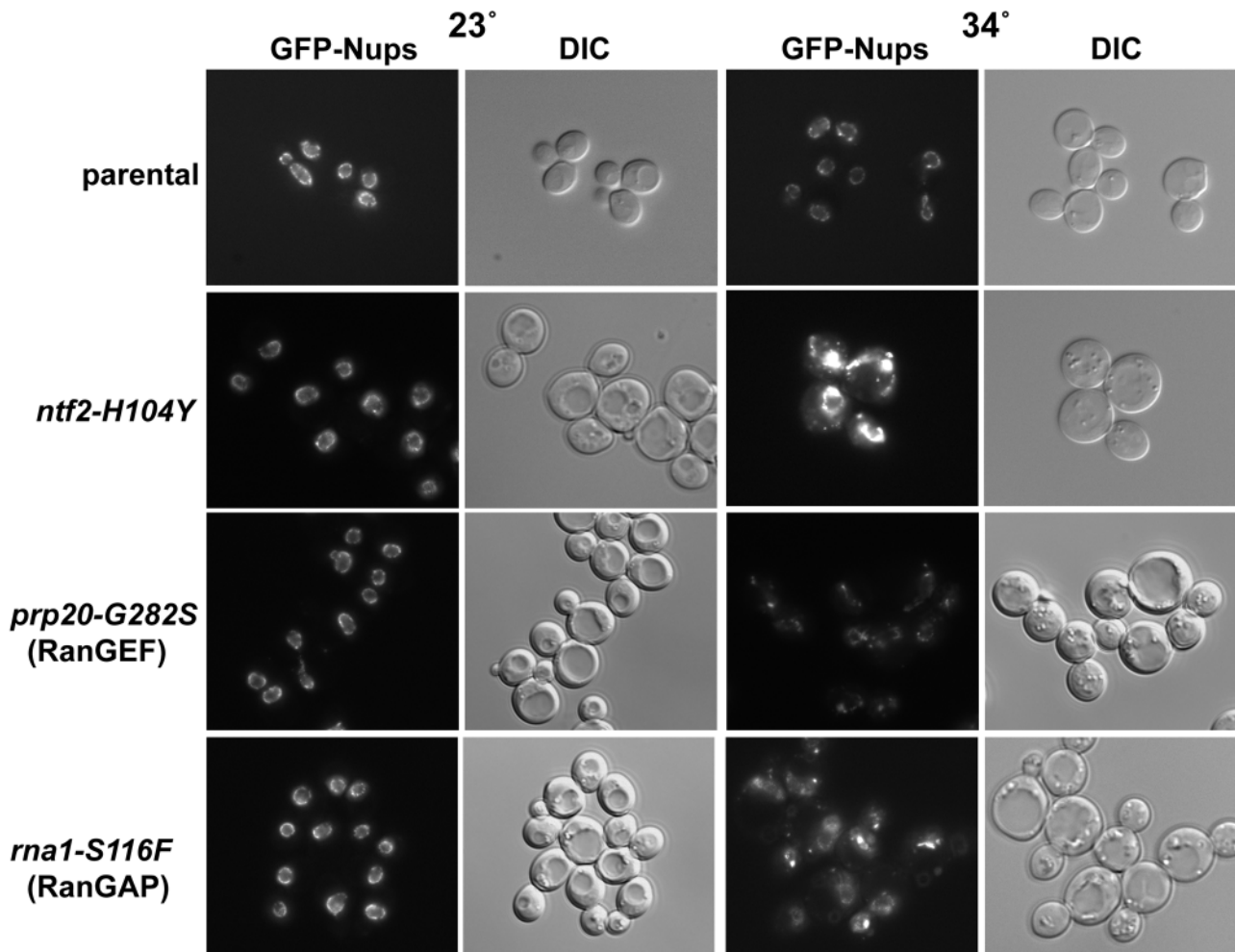


Figure 1. GFP-Nups are mislocalized in Ran GTPase cycle mutants. Ran GTPase cycle mutant and parental strains were grown to early log phase at 23°C, and were then shifted to 34°C for 5 h. Localization of GFP-Nic96 and Nup170-GFP was visualized by direct fluorescence microscopy. Differential interference contrast images (DIC; columns 2 and 4) show cell morphology. Parental, SWY2090; *ntf2-H104Y*, SWY2514; *prp20-G282S*, SWY2515; *rna1-S116F*, SWY2516.

Ser to Phe change at position 116 (*rna1-S116F*), and a Gly to Ser change at amino acid 282 in Prp20 was generated from the mutation in *prp20* (*prp20-G282S*). The *gsp1* allele resulted in a Pro to Leu change at amino acid 162 (*gsp1-P162L*). Additionally, we found that the W303 strain differs from the S288C strain used in the *S. cerevisiae* Genome Sequencing Project for *PRP20*, having sequence coding for Ser instead of Phe at residue 431 in Prp20. The mutations in *rna1*, *prp20*, and *gsp1* occur in sequence for identical residues in the *S. cerevisiae*, *Schizosaccharomyces pombe*, mouse, and human proteins. Although not invariant, the mutation in *ntf2* is linked to a conserved residue. Because *gsp1-P162L* could not be backcrossed, it was not characterized further.

Together, it was striking that a single genetic screen for NPC structure and assembly defects resulted in the independent isolation of multiple mutants that have different defects in maintaining the Ran GTPase cycle. Recent studies have defined a role for Ran in the reestablishment of an intact NE after an open mitosis (Hetzer et al., 2000; Zhang and Clarke, 2000, 2001). However, as yeast undergo a closed mitosis and thereby always maintain an intact NE, our results provide the

first evidence of a role for the Ran GTPase cycle in assembling NPCs into a preexisting, double-lipid bilayer.

To further investigate the defect in these mutants, the localization of GFP-Nic96 and Nup170-GFP was analyzed in cells shifted to 34°C for 5 h (Fig. 1). In the parental strain, GFP-Nic96 and Nup170-GFP localized in a punctate nuclear rim pattern, typical for NPC localization. The localization was maintained when the parental cells were grown at 34°C (Fig. 1, parental). In contrast, after growth at 34°C, all three Ran GTPase cycle mutants showed dramatic perturbations in GFP-Nup localization. Although nuclear rim localization was still detected in most cells, there was a marked decrease in the relative GFP fluorescence level associated with the NE along with a coincident increase in the level of diffuse, cytoplasmic GFP signal. Mutant cells also had foci or clusters of GFP-Nups that were not observed in the parental strain (Fig. 1). Mutant cells were also processed for indirect immunofluorescence microscopy to localize the integral membrane pore-associated protein, Pom152 (Fig. 2). Although previous studies have localized Pom152 exclusively to NPCs (Wozniak et al., 1994; Strambio-de-Castillia et al., 1995), our parental and mutant strains showed a diffuse non-NE pool of Pom152 at

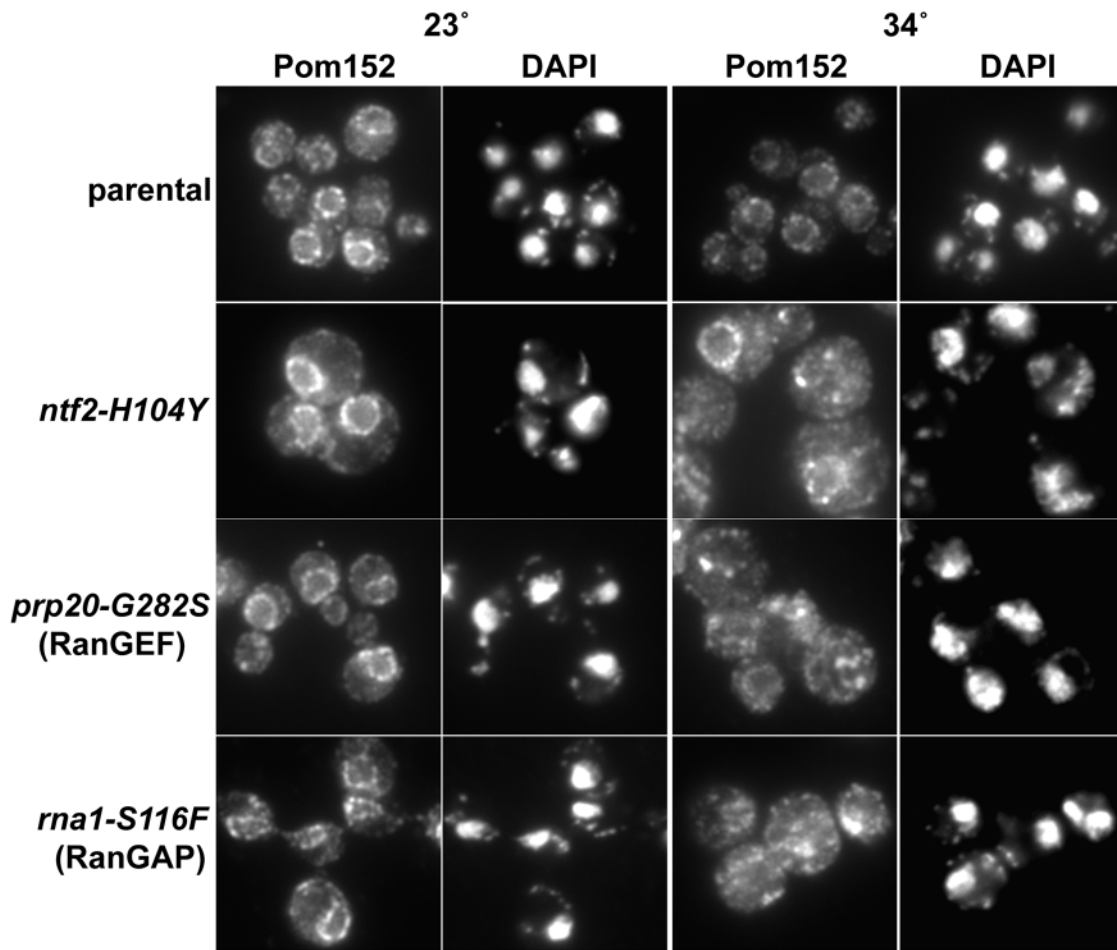


Figure 2. **Ran GTPase cycle mutants result in mislocalization of Pom152.** Cells in early log phase were incubated at 23°C or 34°C for 5 h and processed for indirect immunofluorescence microscopy. Pom152 was visualized using the antibody mAb118C3. Nuclei and mitochondria were detected with the DNA dye DAPI. Strains as in Fig. 1.

the permissive growth temperature (Fig. 2, left column). This could be due to the epitope tagging of both Nic96 and Nup170 in these strains (see section on genetic interactions), as both *NIC96* and *NUP170* are genetically and functionally linked to *POM152* (Aitchison et al., 1995; Tcheperegine et al., 1999). Despite the slight mislocalization of Pom152 in our parental strain, the phenotype was not exacerbated when cells were shifted to 34°C (Fig. 2, top row). In contrast, the localization of Pom152 in the mutants was altered at 34°C with levels at the NE rim diminished, cytoplasmic/reticular levels increased, and concentrated foci or clusters present. Thus, the GFP-Nic96, Nup170-GFP, and Pom152 localizations were coincidentally altered in similar manners. This suggests that multiple (if not all) components of the NPC were mislocalized in the Ran GTPase cycle mutants.

Nup mislocalization in Ran GTPase cycle mutants reflects defects in new NPC assembly

The mislocalization of nups in the Ran cycle mutants could reflect a defect in either new NPC assembly or the stability of existing NPCs. To distinguish between these two possibilities, cycloheximide treatment was used to inhibit protein synthesis (Fig. 3). In cells treated with cycloheximide, the GFP-Nups remained associated with the NE after 6 h at 34°C (Fig. 3,

right columns), whereas untreated cells showed characteristic GFP-Nup mislocalization (Fig. 3, left columns). Thus, preexisting NPCs in these mutants were stable at the nonpermissive temperature and mislocalization was dependent on new protein synthesis. This suggests that the Ran GTPase cycle mutants are defective in the assembly of new NPCs.

To further analyze whether NPC assembly was altered, cell division was monitored after shifting the *ntf2-H104Y*, RanGEF (*prp20-G282S*), and RanGAP (*rna1-S116F*) strains to 34°C. The cells underwent roughly three doublings before ceasing to divide (Fig. 4 A; unpublished data). These results directly parallel the number of cell divisions observed before the arrest of a *nic96* mutant strain that is blocked for new NPC assembly (Zabel et al., 1996; Gomez-Ospina et al., 2000). In the absence of new NPC assembly, the number of NPCs per nucleus would be diluted in half at every mitosis, and after three rounds of cell division, each resulting nucleus would harbor only one eighth of the original NPCs and GFP-Nup fluorescence at the NE. Thus, the time course and relative fluorescence level decrease of GFP-Nups at the NE (Fig. 1) for the Ran GTPase cycle mutant phenotypes correlate with a defect in new NPC assembly.

Additional *ts* alleles of RanGEF (*prp20*) and RanGAP (*rna1*) have been isolated and characterized in previous ge-

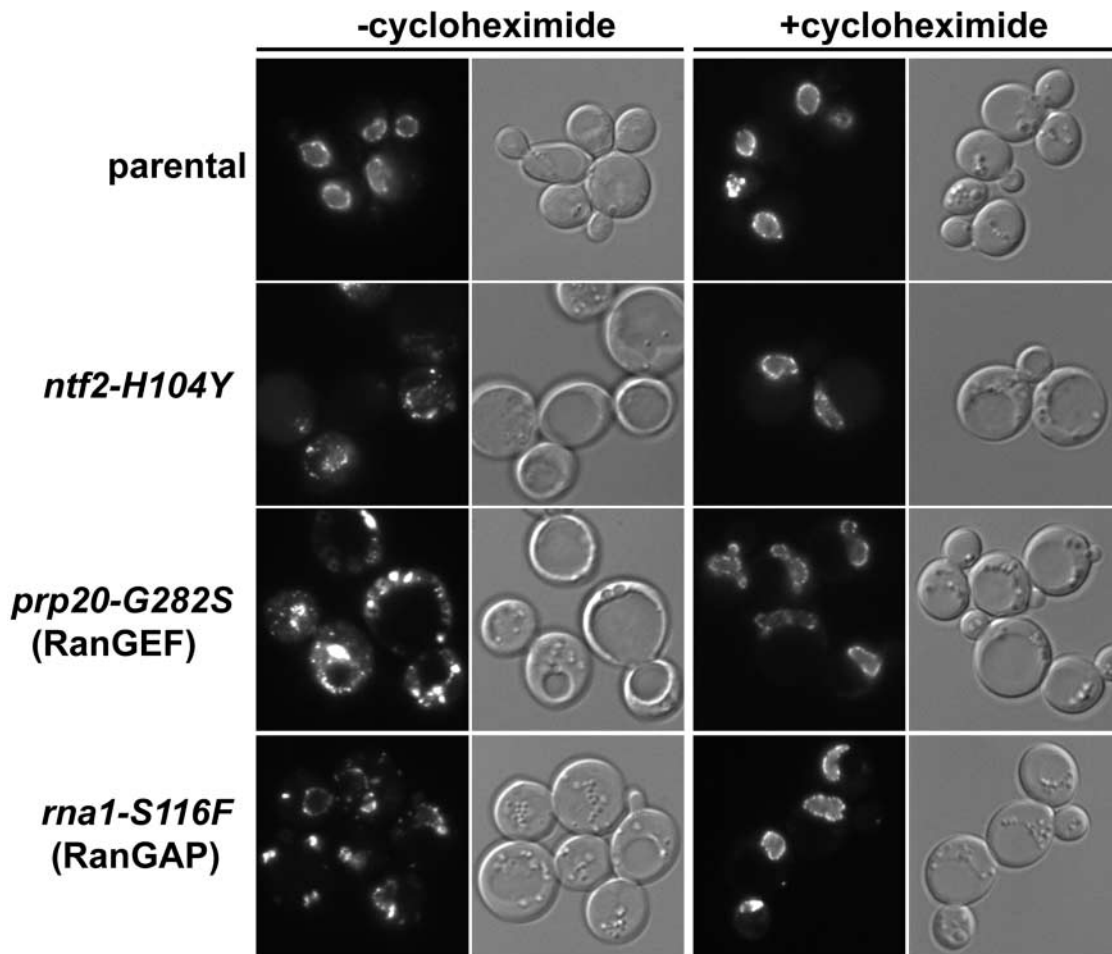


Figure 3. **Cycloheximide treatment prevents GFP-Nup mislocalization.** Cells in early log phase were shifted to 34°C for 6 h in the absence or presence of 10 μ g/ml cycloheximide. GFP-Nic96 and Nup170-GFP localization was visualized by direct fluorescence microscopy. Strains as in Fig. 1.

netic screens. The point mutations in *prp20-1* (Amberg et al., 1993) and *rna1-1* (Traglia et al., 1989) result in amino acid substitutions distinct from our *prp20-G282S* and *rna1-S116F*. To determine if these independent mutant alleles result in a similar NPC assembly defect, the *prp20-1* and *rna1-1* mutations were crossed into the *GFP-nic96 nup170-GFP* background. Interestingly, neither *prp20-1* or *rna1-1* cells showed GFP-Nup mislocalization after 5 h at 34°C (Fig. 4 B). Moreover, the *prp20-1* and *rna1-1* cells only underwent a single round of cell division before arresting at the nonpermissive temperature (Fig. 4 A). The allele specificity observed supports the idea that nup mislocalization in Ran GTPase cycle mutants results from the failure to incorporate nups into new NPCs during multiple rounds of cell growth and division.

Genetic interactions between Ran GTPase cycle mutants and the *GFP-NIC96* and *NUP170-GFP* alleles

Extensive studies suggest that Nic96 and Nup170 play structural roles in the mature NPC and serve as scaffolds for nups that interact with shuttling transport factors (Grandi et al., 1993; Aitchison et al., 1995; Zabel et al., 1996; Marelli et al., 1998). There is also direct evidence that Nic96 is required for

NPC assembly in yeast (Zabel et al., 1996; Gomez-Ospina et al., 2000). Our strains expressing either GFP-Nic96 or Nup170-GFP showed GFP-Nup localization exclusively at the NPC/NE and had no growth defects compared with a wild-type strain at any temperature (unpublished data); however, combined expression of GFP-Nic96 and Nup170-GFP in the same haploid strain resulted in a ts lethal phenotype at 37°C (Fig. 5, parental+). This indicated that the GFP fusion proteins were not fully functional, although they were exclusively NPC localized. Originally, we predicted that using such a sensitized genetic background would bias our screen toward the isolation of NPC assembly mutants. To determine if expression of GFP-Nic96 and Nup170-GFP contributed to the mutant phenotypes, the *ntf2-H104Y*, *prp20-G282S*, and *rna1-S116F* alleles were crossed into a wild-type *NIC96 NUP170* background. The absence of GFP-Nups had no effect on the growth of the RanGEF (*prp20-G282S*) mutant cells, and this strain remained ts at 34°C (unpublished data). In contrast, both *ntf2-H104Y* and RanGAP (*rna1-S116F*) mutants showed increased viability at 34°C in the absence of the GFP-Nups (Fig. 5, middle). However, these strains remained ts at 37°C (Fig. 5, right). The enhanced lethality was not due to either GFP-Nic96 or Nup170-GFP alone, but re-

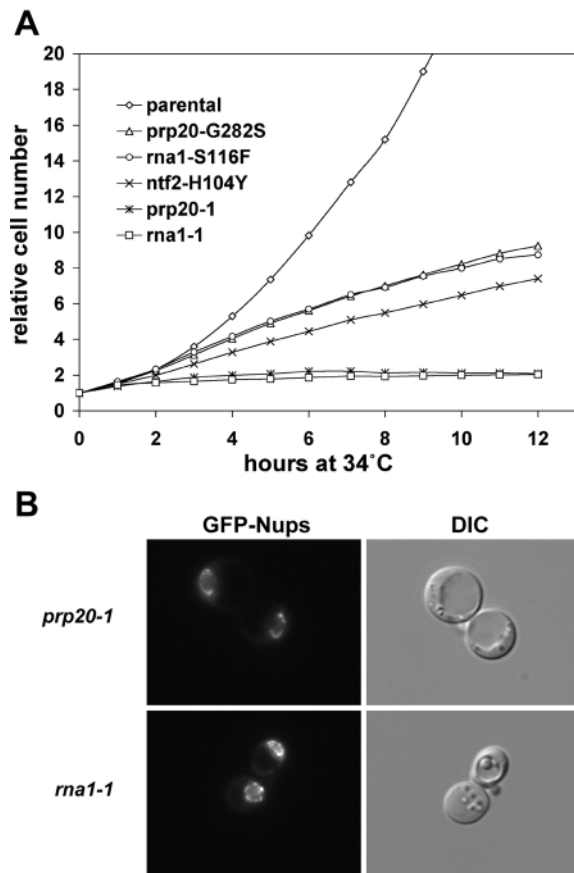


Figure 4. Allele specificity of Ran GTPase cycle mutants. (A) Logarithmically growing parental, *npa* mutants (see Fig. 1 for strains) and previously isolated *prp20-1* (SWY2541) and *rna1-1* (SWY2542) cells in a GFP-*nic96 nup170-GFP* background were shifted to 34°C and monitored for growth over a period of 12 h by measuring OD₆₀₀ nm. Starting densities were normalized to 1. (B) GFP-Nups were localized in *prp20-1* and *rna1-1* cells after 5 h at 34°C as in Fig. 1.

quired expression of both GFP-Nups (unpublished data). These genetic interactions suggest that Nic96, Nup170, and the Ran GTPase cycle are required in a common pathway and provide a genetic link for Ran GTPase regulators to the NPC biogenesis mechanism.

Ran GTPase cycle mutants display NE abnormalities and accumulate small vesicles

Conventional thin-section transmission electron microscopy was used to investigate possible ultrastructural changes associated with the Ran GTPase cycle mutants and their inability to assemble new NPCs. Wild-type NPCs appear as electron-dense structures spanning both the inner and outer nuclear membranes (Fig. 6 I, arrowheads). Mutant cells grown at the permissive temperature had only subtle alterations in cell and nuclear morphology (unpublished data). In contrast, the mutant strains showed massive membrane-based defects after growth at 34°C. Most striking was the accumulation of vesicles of 80–100 nm in diameter. Vesicle accumulation was most prevalent in the RanGEF (*prp20-G282S*) cells (Fig. 6 A). Often, the vesicles formed large aggregates that localized near the plasma membrane (Fig. 6, B

and F, asterisk). In addition to vesicles, these mutants also showed increased amounts of extended cytoplasmic membrane structures. In RanGEF (*prp20-G282S*) and RanGAP (*rna1-S116F*) arrested mutant cells, the expanded membranes appeared as long ribbons stretching throughout the cell (Fig. 6, B and E, arrows). The *ntf2-H104Y* mutant cells had shorter stretches of membranes that were primarily localized near the cytoplasmic face of the NE and often arranged in loose stacks (Fig. 6, F–H, arrows). The general nuclear morphology was also dramatically changed in the temperature-arrested RanGAP (*rna1-S116F*) and RanGEF (*prp20-G282S*) mutant cells. The nuclear region was lobulated and the NE was invaginated (Fig. 6, B and C). In *ntf2-H104Y* mutant cells, the nuclear region was more typical of a wild-type sphere (Fig. 6, F and G). Finally, in RanGAP (*rna1-S116F*) arrested cells, the NE was discontinuous in many sections (Fig. 6 E). Overall, the Ran GTPase cycle mutants showed remarkable changes in NE, cytoplasmic, and vesicular membrane structures.

Despite the membrane perturbations at 34°C, the Ran GTPase cycle mutant cells still had identifiable NPC-like structures embedded in the NE (Fig. 6, B, C, F, and G, arrowheads). As preexisting NPCs are stable for at least 6 h (Fig. 3), these NPCs were likely assembled before the shift to 34°C. Previous studies have analyzed the effect of depleting Ntf2 from yeast cells and reported nuclear pores lacking NPC-like structures (Paschal et al., 1997). Although we did not observe such “empty pores” in our *ntf2-H104Y* strain, some of the NPCs did not appear to span both the inner and outer nuclear membranes and, to a lesser extent, were associated with inner nuclear membrane herniations (unpublished data). The differences between the *ntf2-H104Y* and Ntf2-depletion phenotypes are likely related to allele-specific differences between expressing a ts point mutant versus an effective null mutant.

Nups are associated with the vesicles accumulating in mutant cells

The accumulation of small vesicles is often observed in cells that are blocked in protein secretion or have a defect in pro-

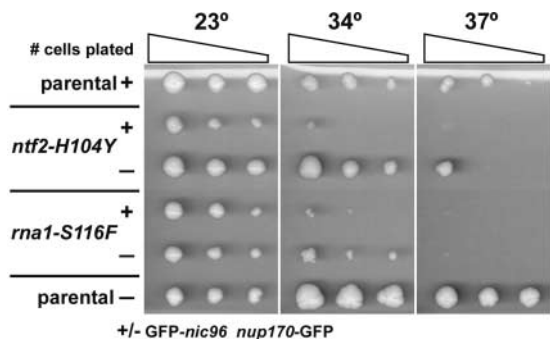


Figure 5. GFP-*nic96* and *nup170-GFP* genetically interact with *ntf2-H104Y* and *rna1-S116F*. Serial dilutions of the parental strain and Ran cycle mutants with integrated GFP-*nic96 nup170-GFP* (+) or lacking GFP-Nups (–) were pinned to plates and incubated for growth at 23, 34, or 37°C. GFP-*nic96 nup170-GFP* strains as in Fig. 1; SWY2517 (*ntf2-H104Y*) and SWY2518 (*rna1-S116F*) are NIC96 NUP170.

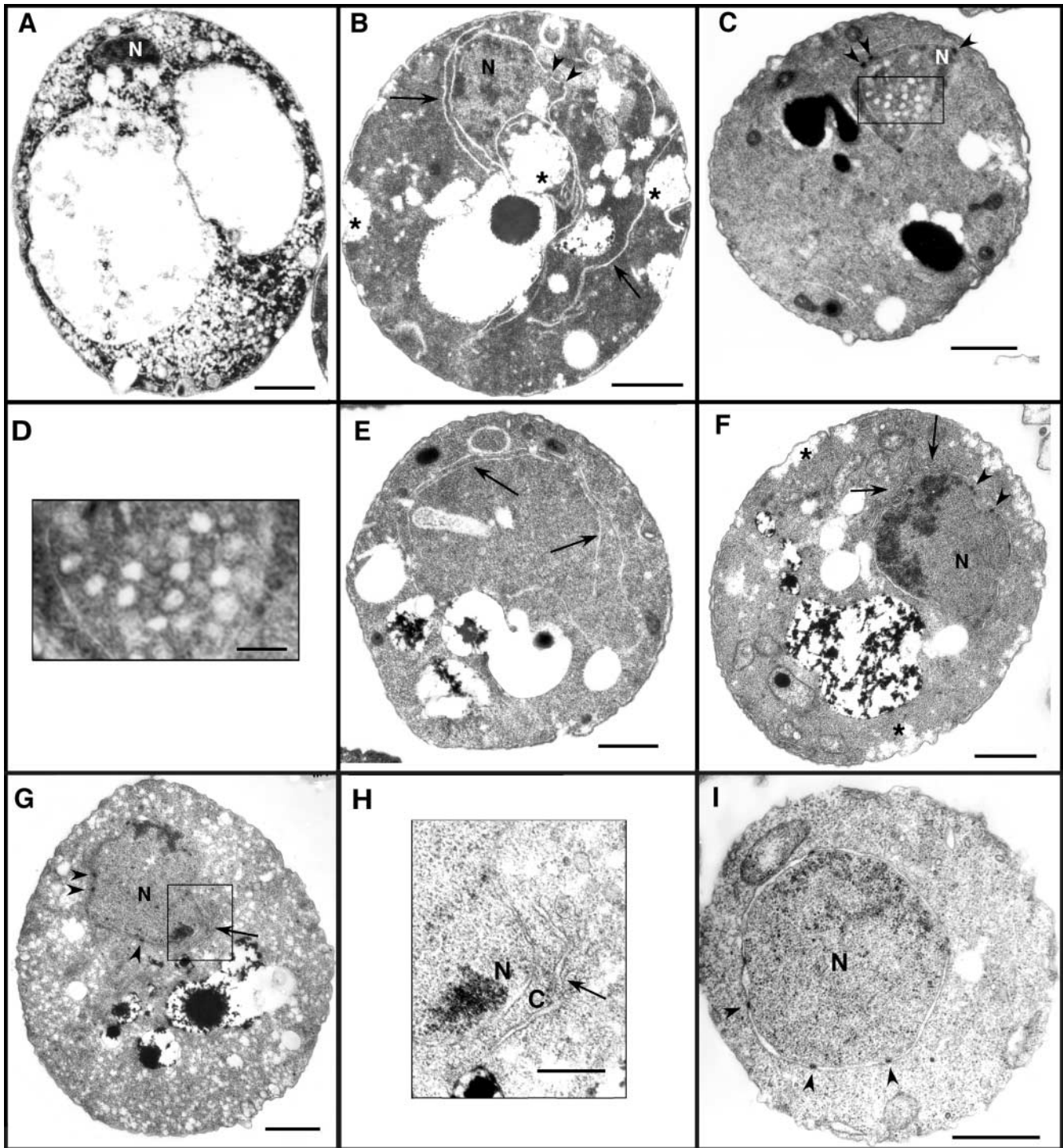


Figure 6. Ran GTPase cycle mutants have massive membrane-based defects at the nonpermissive temperature. Cells in early log phase were shifted to 34°C for 5 h and processed for thin-section transmission electron microscopy. (A and B) RanGEF (*prp20-G282S*, SWY2515) mutant cells; (C–E) RanGAP (*rna1-S116F*, SWY2516) mutant cells; (F–H) *ntf2-H104Y* (SWY2514) mutant cells. Although NPCs (arrowheads) were present, mutants accumulated 80–100 nm vesicles (asterisk, vesicle clusters) or more extended, flattened membranes (arrows). The boxes in C and G correspond to the areas showing individual vesicles (D) and flattened membranes (H), respectively. (I) A cell from the parental strain showing wild-type morphology. N, nucleus; C, cytoplasm. Bars, 0.25 μm in D and H. All other bars, 1 μm .

tein trafficking (Schekman, 1992). Although Ran has not been linked to secretion, many of the other characterized *npa* strains have mutations in genes encoding components of the secretory machinery (Ryan and Wentz, 2002). To determine if the vesicles generated in the Ran GTPase cycle mutants were intermediates in the secretory process, the ability of cells

to secrete the enzyme invertase in response to low glucose was monitored at 34°C. When normalized to the activity of the parental strain, *ntf2-H104Y* cells had a somewhat reduced relative activity of 54%. The RanGEF (*prp20-G282S*) and RanGAP (*rna1-S116F*) strains had essentially wild-type invertase activities of 76 and 130%, respectively. In contrast, invertase

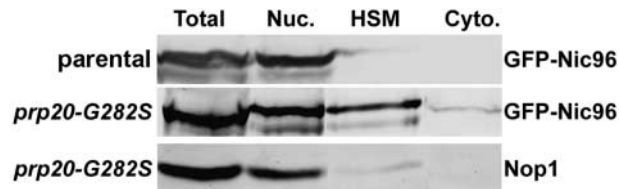


Figure 7. Nups are associated with vesicles in mutant cell lysates. Parental (SWY2090) or RanGEF mutant (*prp20-G282S*, SWY2515) strains were incubated at 34°C for 5 h, and were then fractionated into a nuclear and large organelle fraction (Nuc.), a high speed membrane fraction (HSM), and a cytoplasmic fraction (Cyto.) by differential centrifugation. An equal number of cell equivalents from each fraction were separated by SDS-PAGE and analyzed for GFP-Nic96 by immunoblotting with an antibody against GFP. As a control, the distribution of the nuclear protein Nop1 was also analyzed. Strains are indicated on the left, and antibodies on the right.

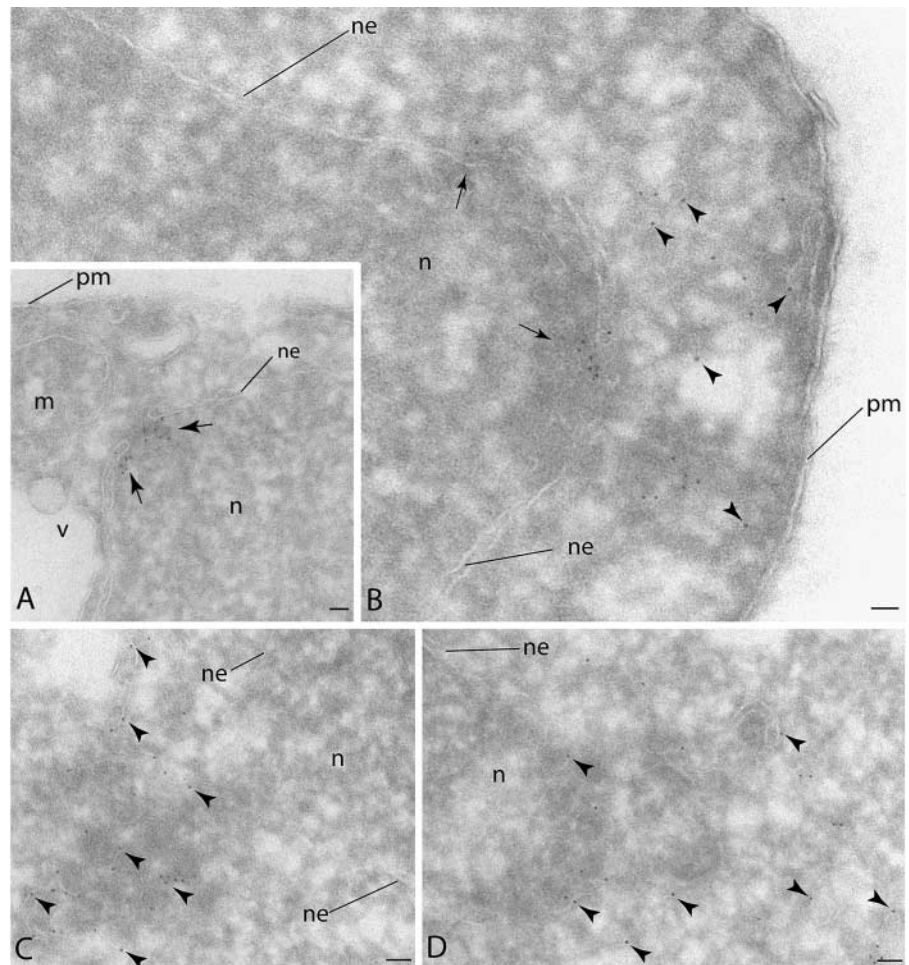
activity in the six *sec13* mutants previously isolated in this screen ranged from 14–35% of wild type (Ryan and Wentz, 2002). Thus, the Ran GTPase cycle mutants did not have significant defects in secretion.

We speculated that the vesicles reflected the accumulation of an NPC assembly intermediate. To test this hypothesis, we biochemically analyzed whether nups were associated with a vesicular fraction. Using RanGEF (*prp20-G282S*) mutant cells, lysates were separated into a nuclear/large or-

ganelle fraction, a cytoplasmic fraction, and a high speed membrane fraction by differential centrifugation. The fractions were analyzed by immunoblotting for GFP-Nic96 (Fig. 7). In the wild-type parental cells shifted to 34°C, GFP-Nic96 fractionated with nuclei, indicating that the majority of the protein was assembled into NPCs in the NE (Fig. 7, top row). In contrast, in lysate from *prp20-G282S* cells shifted to 34°C, a significant portion of GFP-Nic96 was isolated in the high speed membrane fraction (Fig. 7, middle row). The presence of GFP-Nic96 in the high-speed membrane fraction is consistent with vesicle association. The nucleolar protein Nop1 remained in the nuclear fraction, indicating that the nuclei remained intact in mutant cells and during the fractionation (Fig. 7, bottom row).

To confirm that nups were associated with vesicles *in vivo*, GFP-Nic96 and Nup170-GFP were localized after growth at 34°C using immunogold labeling of ultrathin cryosections with anti-GFP primary and gold-conjugated secondary antibodies. In the parental strain, GFP-Nup labeling was localized almost exclusively to NPCs in the NE (Fig. 8 A). Moreover, gold particles were absent from the nucleoplasm and cytoplasm even when additional membrane structures were present. In contrast, in each of the Ran GTPase cycle mutants grown at 34°C, a significant fraction of the GFP-Nup labeling was found in the cytoplasm associated with vesicles/membranes (Fig. 8, B–D; unpublished data). The

Figure 8. Nups associate with cytoplasmic membranes and vesicles *in vivo*. (A) Parental (SWY2090) or (B–D) RanGEF (*prp20-G282S*, SWY2515) strains were grown for 5 h at 34°C and processed for immunoelectron microscopy with antibodies against GFP followed by a gold-conjugated secondary. m, mitochondria; pm, plasma membrane; ne, nuclear envelope; n, nucleus. Arrows indicate NPC labeling; arrowheads indicate label on cytoplasmic membranes/vesicles. Bars, 0.1 μ m.



vesicles were similar to those observed by conventional EM. This strongly supports the conclusion that nups associate with vesicles in vivo. Such nup-containing vesicles have not been previously reported in *S. cerevisiae*. They may result from the defect in NPC assembly and reflect accumulation of a novel vesicular NPC assembly intermediate.

Discussion

Using a genetic approach, we have identified a role for the Ran GTPase cycle in yeast NPC assembly. Individual mutations in genes encoding *gyp1* (Ran) and each of Ran's regulatory factors (*ntf2*, *prp20*, and *rna1*) result in cells that do not properly localize newly synthesized nups to the NE. Moreover, the mutant cells coincidentally accumulate small nup-containing vesicles. Together, our results suggest that the Ran GTPase cycle is required for a vesicular-mediated step in new NPC assembly into the intact NE.

Macaulay and Forbes (1996) originally documented two distinct GTP γ S-sensitive steps during in vitro pore formation. The first entails the fusion of nuclear vesicles to form the NE. Extensive studies have recently documented that this step is mediated by Ran (Hetzer et al., 2000; Zhang and Clarke, 2000, 2001). The second involves the transition from an intact double membrane to the formation of a nuclear pore by intermembrane fusion of the inner and outer nuclear membranes. We speculate that Ran is the GTPase inhibited by GTP γ S in the second nuclear pore/NPC assembly step. Based on the ordered assembly pathway, the Ran GTPase step required for NPC assembly should be distinct and before the NPC assembly step inhibited by the Ca²⁺ chelator, BAPTA. These conclusions impact the models for NPC assembly into intact nuclear membranes in all organisms.

In the proposed NE/NPC biogenesis pathway, organisms with a closed mitosis would not require the Ran GTPase-dependent steps for vesicle fusion during NE formation. However, the NE membrane perturbations observed in the Ran GTPase cycle mutants suggest that Ran may also function in *S. cerevisiae* NE growth and maintenance. A potential requirement for Ran in NE maintenance during closed mitosis was first suggested by results in the fission yeast *S. pombe*. Ran GTPase cycle mutants in *S. pombe* show nup mislocalization and NE fragmentation in either RanGEF (*pim1*) mutants (Demeter et al., 1995) or cells overexpressing RanGAP (*rna1*⁺; Matynia et al., 1996); however, these effects are secondary to a block in mitotic exit and failure to reestablish an interphase nucleus (Demeter et al., 1995). Unlike the *S. pombe* mutants, the particular alleles we have characterized from *S. cerevisiae* have a primary defect in NPC assembly and do not appear to be blocked in mitosis. This conclusion is based on the following observations: first, the *S. cerevisiae* mutant cells undergo multiple rounds of cell division at the nonpermissive temperature, whereas the *S. pombe* mutant strains arrest immediately after the first mitosis (Demeter et al., 1995). Second, NPCs already at the NE are stable for at least 6 h at the nonpermissive temperature in the *S. cerevisiae* mutants (Fig. 3; unpublished data). Thus, the mislocalized nups are not derived from the breakdown of existing NPCs and result from new protein synthesis. Finally, the GFP-Nup mislocalization defects in *S. cerevisiae*

require at least two doublings before detection by fluorescence. This time course is similar to that observed for the NPC assembly phenotype observed in a *nic96* mutant strain (Zabel et al., 1996). Overall, the results presented here provide the first evidence that the Ran GTPase cycle is required specifically for NPC assembly.

As with NE formation, the exact role of the Ran GTPase in NPC assembly remains to be elucidated. We speculate that during the entire life cycle of budding yeast and during interphase in vertebrate cells, Ran is required for at least two independent NPC assembly steps: (1) a nuclear event based on Ran-dependent protein import; and (2) a cytoplasmic event involving vesicular fusion (see next paragraph). In regard to a role for transport in assembly, early association of some nups with chromatin after mitosis suggests that some components of the NPC are assembled from the nuclear side (Chaudhary and Courvalin, 1993; Bodoor et al., 1999; Haraguchi et al., 2000; Belgareh et al., 2001). In organisms with a closed mitosis and during interphase in higher eukaryotes, this would require active transport across the NE for NPC assembly. Such transport reactions may require RanGTP to dissociate karyopherin–nup complexes once they reach the nucleus. In fact, recent studies in yeast have shown that Nup53 is imported by the transport factor Kap121 (Marelli et al., 2001; Lusk et al., 2002). Interestingly, our EM studies of the *S. cerevisiae* Ran GTPase cycle mutants show a striking accumulation of nup-associated membranes and vesicles in the cytoplasm. This cytoplasmic phenotype is distinct from the transport-dependent phenotype reported in Nup53-overexpressing cells wherein stacks of double membranes, which contain empty pores and potentially partially assembled NPCs, accumulate in the nucleus (Marelli et al., 2001). Thus, although part of the function of Ran in NPC assembly may be related to its role in transport, the results presented here indicate that there is most likely an additional or different requirement for Ran in NPC biogenesis.

A role for the Ran GTPase cycle in a cytoplasmic vesicular-mediated NPC assembly step is based on the defects in the Ran GTPase cycle mutants. The accumulation of cytoplasmic nup-associated vesicles was coincident with a defect in new NPC assembly. This suggests that nups, or a defined subset, may be trafficked to the NE by vesicles. Only three integral membrane proteins biochemically fractionate with yeast NPCs (Rout et al., 2000). Pom152 and Pom34 are unique to the NPC, whereas Ndc1 is also a component of the spindle pole body (Chial et al., 1998). As the outer nuclear membrane is continuous with and forms part of the ER, these three membrane proteins should be able to diffuse through the ER network to the NE. However, the nup-containing vesicles in the Ran GTPase cycle mutants may reflect an alternative mechanism by which certain nups are effectively concentrated. When these vesicles fuse with the outer nuclear membrane, a critical, localized nup concentration would trigger pore formation and nucleate new NPC assembly. This model is also supported by vertebrate cell experiments showing that the second GTP γ S-sensitive step results in intact double nuclear membranes that lack pores or NPC structures (Macaulay and Forbes, 1996). This intermediate can be converted to a NE with NPCs on the addition of

fresh cytosol (Macaulay and Forbes, 1996), suggesting a critical event at the outer nuclear membrane was inhibited. Moreover, the absence of preexisting NPCs indicates that assembly did not require ongoing protein import. NPC assembly at the end of mitosis in vertebrate cells may only require the Ran-mediated vesicular step because certain nups can associate with chromatin before the NE is closed.

It remains unclear how RanGTP could be generated at the cytoplasmic face of the NE for NPC assembly and how the Ran GTPase could mediate vesicular fusion/trafficking. In all organisms, the RanGAP (Rna1 in yeast) is localized in the cytoplasm, whereas the RanGEF (Prp20 in yeast; RCC1 in other organisms) is nuclear (Görlich and Kutay, 1999). This asymmetric distribution of regulatory factors is an inherent tenet of models concerning Ran function. Recently, *BUD5*, a cytoplasmically localized GEF, was identified as a multicopy suppressor of *prp20* (RanGEF), and its ability to multicopy suppress *prp20* mutations is dependent on *GSP1* (Ran) overexpression (Clément et al., 2001). Further work showed that Bud5 and Ran interact in vivo and in vitro (Clément et al., 2001). As *BUD5* is not essential, it cannot be performing the essential function required in NPC assembly, but this does raise the possibility of additional, cytoplasmic RanGEFs. Alternatively, Prp20 may have an as yet unreported cytoplasmic pool.

Recent studies of Ran-dependent processes have led to models where the general function of the Ran GTPase is to provide a signal of chromosome position (Dasso, 2001; Hetzer et al., 2002). The association of RanGEF with chromatin results in high RanGTP concentrations near chromosomes (Kalab et al., 2002). This high concentration of RanGTP allows dissociation of import complexes and assembly of export complexes in the nucleus, spatial regulation for the release of inhibitory karyopherins from key compo-

nents in spindle assembly, and positioning information for post-mitotic NE reassembly (Hetzer et al., 2002). If the Ran GTPase is responsible for a NPC assembly step triggered at the outer nuclear membrane, this would effectively uncouple RanGTP function from the chromosomes in both vertebrates and yeast.

The isolation and characterization of these *ntf2*, *prp20*, *rna1*, and *gsp1* mutants provides genetic and in vivo proof of Ran function in yeast NPC assembly. Furthermore, the finding of multiple, conserved nuclear processes controlled by RanGTP in organisms with such diverse nuclear structures suggests that there is a common, early nuclear assembly pathway from yeasts to vertebrates. Our future efforts will be focused on identifying the mediators of NPC assembly that are regulated by Ran. The gene(s) encoding the factor(s) may correspond to one of the uncharacterized *npa* mutants (Ryan and Wentte, 2002). In addition, the nup-associated vesicles in the Ran GTPase cycle mutants may be similar to the mitotic nup-associated vesicles in vertebrate cells. Thus, based on the elegant vertebrate in vitro studies, these results also set the stage for the development of a yeast-in vitro NPC assembly assay. In combination with the wealth of biochemical knowledge about the yeast NPC and the extensive collections of nup mutants, this future direction should provide greater insight into the NPC assembly mechanism in both yeast and higher eukaryotes.

Materials and methods

Yeast strains, reagents, and assays

All yeast strains used in this work are listed in Table I. General yeast manipulations were conducted by standard methods with transformations by the lithium acetate method (Ito et al., 1983). All strains were grown at 23°C in YPD (yeast extract, peptone, and 2% glucose) unless otherwise noted.

Table I. Yeast strains

Strain	Genotype	Source
SWY518	<i>MATa ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100 ade2-1::ADE2:ura3</i>	(Bucci and Wentte, 1997)
SWY519	<i>MATα ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100 ade2-1::ADE2:ura3</i>	(Bucci and Wentte, 1997)
SWY2089	<i>MATα lys2 ura3-1 his3-11,15 leu2-3,112 can1-100 ade2-1::ADE2:ura3 GFP-nic96:HIS3 nup170-GFP:URA3</i>	(Ryan and Wentte, 2002)
SWY2090	<i>MATa trp1-1 ura3-1 his3-11,15 leu2-3,112 can1-100 ade2-1::ADE2:ura3 GFP-nic96:HIS3 nup170-GFP:URA3</i>	(Ryan and Wentte, 2002)
SWY2514	<i>MATα ntf2-H104Y(npa11) lys2 ura3-1 his3-11,15 leu2-3,112 can1-100 ade2-1::ADE2:ura3 nic96-GFP:HIS3 nup170-GFP:URA3</i>	backcross of <i>npa11</i> xSWY2089
SWY2515	<i>MATa prp20-G282S(npa14) trp1-1 ura3-1 his3-11,15 leu2-3,112 can1-100 ade2-1::ADE2:ura3 nic96-GFP:HIS3 nup170-GFP:URA3</i>	backcross of <i>npa14</i> xSWY2089
SWY2516	<i>MATa rna1-S116F(npa13) trp1-1 ura3-1 his3-11,15 leu2-3,112 can1-100 ade2-1::ADE2:ura3 nic96-GFP:HIS3 nup170-GFP:URA3</i>	backcross of <i>npa13</i> xSWY2089
SWY2517	<i>MATa ntf2-H104Y(npa11) lys2 ura3-1 his3-11,15 leu2-3,112 can1-100 ade2-1::ADE2:ura3</i>	SWY2514 xSWY518 haploid
SWY2518	<i>MATa prp20-G282S(npa14) trp1-1 ura3-1 his3-11,15 leu2-3,112 can1-100 ade2-1::ADE2:ura3</i>	SWY2515 xSWY519 haploid
SWY2519	<i>MATa rna1-S116F(npa13) trp1-1 ura3-1 his3-11,15 leu2-3,112 can1-100 ade2-1::ADE2:ura3</i>	SWY2516 xSWY519 haploid
SWY2520	<i>MATα gsp1-P162L(npa15) lys2 ura3-1 his3-11,15 leu2-3,112 can1-100 ade2-1::ADE2:ura3 GFP-nic96:HIS3 nup170-GFP:URA3</i>	<i>npa15</i> ; original screen isolate
SWY2541	<i>MATa prp20-1 leu2 his3 ura3 trp1-1 GFP-nic96:HIS3 nup170-GFP:URA3</i>	SWY2090 x <i>prp20-1</i> haploid
SWY2542	<i>MATa rna1-1 ura3 lys2 his3? his4? his7? tyr? GFP-nic96:HIS3 nup170-GFP:URA3</i>	SWY2089 x <i>rna1-1</i> haploid
N43-6c-gsp1-1757	<i>MATa Δgsp1::HIS3::gsp1-1757::LEU2 ade2 leu2 trp1 ura3</i>	(Oki and Nishimoto, 1998)
<i>rna1-1</i>	<i>MATa rna1-1 ura3-52 tyr1 his4 his7</i>	(Traglia et al., 1989)
<i>prp20-1</i>	<i>MATa prp20-1 leu2 his3-200 ura3-52</i>	(Amberg et al., 1993)

For growth at the nonpermissive temperature, cells were incubated at 34°C. For cycloheximide treatment of cells, 10 mg/ml cycloheximide (Sigma-Aldrich) was added to a final concentration of 10 µg/ml. Invertase assays were conducted as described previously (Ryan and Wentte, 2002).

Plasmid construction and cloning

Plasmids of wild-type *NTF2*, *PRP20*, and *RNA1* were generated by cloning PCR products into the *Xba*I/*Bam*HI site of pRS315 (Sikorski and Hieter, 1989). The oligo pairs are as follows: *NTF2*, 5'-GCCTCTAGAGCGTAGC-CTTGTACC-3' (*NTF2/Xba*), and 5'-ATTGGATCCCTATCGCCTTATACAT-3' (*NTF2/Bam*); *PRP20*, 5'-TAATCTAGACCTATAGCACTGGGGC-3' (*PRP20/Xba*), and 5'-AGGAGATCTCATGAGAATTTGTCGGT-3' (*PRP20/Bgl*II); *RNA1*, 5'-TTATAGATCTCCGGAAGAAGCTTG-3' (*RNA1-5*), and 5'-AGCATCTAGACGATAATGCT-3' (*RNA1-3*); *GSP1*, 5'-TAGATCTA-GAGCCTTTAAAGGCTTACACC-3' (*GSP1-5*), and 5'-ATAAGGATCCG-TATACAGTTTAGGGT-3' (*GSP1-3*). Appropriate library-isolated plasmids or genomic DNA (Promega) were used as templates. The *NTF2* and *GSP1* products were digested with *Xba*I/*Bam*HI. The *PRP20* and *RNA1* products were digested with *Xba*I/*Bgl*III.

Selected mutants were backcrossed until the *ts* phenotype segregated 2:2 and was linked to the GFP-Nup mislocalization phenotype. A *LEU2/CEN* yeast genomic library (American Type Culture Collection) was used for complementation of the *ts* phenotypes. Inserts capable of rescuing the *ts* growth defect were isolated, and sequence comparison to the *S. cerevisiae* Genome Database identified candidate genes.

Identification of *npa15* as a *gsp1* allele was accomplished by mating all *MATa npa* strains to the *ts gsp1* strain N43-6c-gsp1-1757 (Oki and Nishimoto, 1998). Diploids were selected and tested for growth at 34°C. Previous complementation grouping of mutants showed that there were no alleles of *npa15* in the *MATa* collection (Ryan and Wentte, 2002).

To obtain the sequence of mutant alleles, genomic DNA was prepared from each strain and used as templates in PCR with the above oligo pairs. Two independent PCR trials were completed for each mutant allele, cloned, and independent isolates were sequenced.

Microscopy

Fluorescence and differential interference contrast microscopy was performed on a microscope (model BX50; Olympus) using an UPlan 100×/1.3 objective. Images were captured using a camera (Photometrics Cool-Snap HQ™; Roper Scientific) with MetaVue™ software (Universal Imaging Corp.). Indirect immunofluorescence was performed as described previously (Wentte et al., 1992) with the antibody mAb118C3 against Pom152 (Strambio-de-Castillia et al., 1995) followed by a Cy3-conjugated anti-mouse secondary. All images within a figure were captured for the same length of time. Thin-section electron microscopy was conducted as described previously (Wentte and Blobel, 1993).

Immunoelectron microscopy was performed essentially as described previously (Rieder et al., 1996). Cells were fixed in 1× PBS containing 4% PFA overnight at 4°C, washed, and resuspended in 2% low temperature agarose. Trimmed mm³ blocks, cryo-protected in 2.3 M sucrose containing 20% polyvinyl pyrrolidone, were mounted on aluminum cryo-pins and frozen in liquid N₂. Ultrathin cryosections were cut on an ultramicrotome (UCT; Reichert) with an FCS cryo-stage, and were collected onto 300 mesh, formvar/carbon-coated nickel grids. Grids were washed, blocked in 10% FCS, and incubated overnight with primary rabbit anti-GFP (~10 µg/ml; Molecular Probes, Inc.). After washing, grids were incubated with 5 nm gold-conjugated secondary donkey anti-rabbit antibody (Jackson ImmunoResearch Laboratories) for 2 h, washed, and embedded in 3.2% polyvinyl alcohol (10 K molecular weight), 0.2% methyl cellulose (400 centipoises), and 0.2% uranyl acetate. Sections were viewed on a transmission electron microscope (model EM 410; Philips); and images were collected with a digital camera (MegaView III; Soft Imaging System).

Biochemical fractionation

Parental (SWY2090) and *prp20-G282S* (SWY2515) strains were grown in 1-l cultures to OD₆₀₀ 0.05–0.10 at 23°C. The cells were shifted to 34°C for 5 h, and were then collected by centrifugation. Cell pellets were washed once with water followed by pretreatment in 100 mM Tris, pH 9.4, 50 mM β-mercaptoethanol, and 10 mM sodium azide for 10 min at RT. Cells were washed in spheroplast wash buffer (SWB; 1.4 M sorbitol, 50 mM Kpi, pH 7.5, and 10 mM sodium azide) and spheroplasted in 40 ml SWB with 225 µg/ml zymolyase 20T for 1.5 h at 30°C. After spheroplasting, cells were washed twice with SWB, resuspended in 6 ml S-lysis buffer (250 mM sucrose, 50 mM KCl, 2.5 mM MgCl₂, and 1 mM DTT; Newmeyer and Wilson, 1991), and incubated on ice for 20–30 min. Cells were lysed using a Dounce homogenizer with B-type pestle. Total lysate was centrifuged at

1,000 g for 10 min to obtain a pellet (P1) and supernatant (S1). The P1 fraction was washed with 5 ml lysis buffer and recentrifuged to yield the nuclear and large organelle fraction. The S1 fraction was further separated by centrifugation at 135,000 g for 1.5 h in a TLS-55 rotor into supernatant (S2) and membrane pellet (P2; Newmeyer and Wilson, 1991). The S2 fraction was recentrifuged at 200,000 g to remove residual membranes, and the resulting supernatant (S3) was the cytoplasmic fraction. P2 was washed in membrane wash buffer (S-lysis buffer plus 50 mM Hepes, pH 7.0) and centrifuged at 200,000 g to obtain the high speed membrane fraction. Equal numbers of cell equivalents from each fraction, as determined by OD₆₀₀ after the temperature shift, were separated by SDS-PAGE and transferred to nitrocellulose for Western analysis with anti-GFP or anti-Nop1 D77 antibodies (Henriquez et al., 1990) and alkaline phosphatase-conjugated secondary antibodies.

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