

The Karyopherin Kap95 Regulates Nuclear Pore Complex Assembly into Intact Nuclear Envelopes In Vivo[□]

Kathryn J. Ryan,* Yingna Zhou, and Susan R. Wentz

Department of Cell and Developmental Biology, Vanderbilt University Medical Center, Nashville, TN 37232-8240

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Nuclear pore complex (NPC) assembly in interphase cells requires that new NPCs insert into an intact nuclear envelope (NE). Our previous work identified the Ran GTPase as an essential component in this process. We proposed that Ran is required for targeting assembly factors to the cytoplasmic NE face via a novel, vesicular intermediate. Although the molecular target was not identified, Ran is known to function by modulating protein interactions for karyopherin (Kap) β family members. Here we characterize loss-of-function *Saccharomyces cerevisiae* mutants in *KAP95* with blocks in NPC assembly. Similar to defects in Ran cycle mutants, nuclear pore proteins are no longer localized properly to the NE in *kap95* mutants. Also like Ran cycle mutants, the *kap95-E126K* mutant displayed enhanced lethality with *nic96* and *nup170* mutants. Thus, Kap95 and Ran are likely functioning at the same stage in assembly. However, although Ran cycle mutants accumulate small cytoplasmic vesicles, cells depleted of Kap95 accumulated long stretches of cytoplasmic membranes and had highly distorted NEs. We conclude that Kap95 serves as a key regulator of NPC assembly into intact NEs. Furthermore, both Kap95 and Ran may provide spatial cues necessary for targeting of vesicular intermediates in de novo NPC assembly.

INTRODUCTION

Nuclear pore complexes (NPCs) are large, proteinaceous structures that span the pore formed by fusion of the inner and outer nuclear membranes (INM and ONM). They are required for the passive diffusion of small metabolites and proteins and the selective transport of large proteins and RNAs between the nucleus and cytoplasm (Fahrenkrog and Aebi, 2003; Suntharalingam and Wentz, 2003). Structural and proteomic studies of NPCs from several organisms have revealed a highly conserved 40–60-MDa entity with an eightfold axis of rotational symmetry perpendicular to the plane of the nuclear envelope (NE; Yang *et al.*, 1998; Stoffler *et al.*, 2003; Beck *et al.*, 2004). The central core of the complex is formed from a series of spoke and ring-like structures that presumably anchor the NPCs to the NE. Filaments emanate from the cytoplasmic and nuclear NPC faces. Approximately 30 individual proteins, collectively termed nucleoporins or Nups, have been identified (Rout *et al.*, 2000; Cronshaw *et al.*, 2002) and can be isolated in

discrete, evolutionarily conserved subcomplexes (reviewed in Vasu and Forbes, 2001; Suntharalingam and Wentz, 2003). These Nup subcomplexes are believed to be the building blocks for NPC assembly.

In eukaryotic cells that undergo an open mitosis, both the NE and NPCs are disassembled at the onset of mitosis and must be reassembled after chromosome segregation (Burke and Ellenberg, 2002; Hetzer *et al.*, 2005). Others have elegantly demonstrated that this reassembly is a highly ordered process, with temporal recruitment of individual Nups to the reforming NE and nucleus (Buendia and Courvalin, 1997; Bodoor *et al.*, 1999; Haraguchi *et al.*, 2000; Belgareh *et al.*, 2001; Daigle *et al.*, 2001; Loiodice *et al.*, 2004; Rabut *et al.*, 2004). During reassembly, unique membrane vesicles are rapidly recruited to the chromatin. Specific subsets of Nups also become associated with the chromatin at this time, and failure of these Nups to associate with chromatin before NE closure results in an irreversible block in NPC assembly (Harel *et al.*, 2003b; Walther *et al.*, 2003a; Antonin *et al.*, 2005; Franz *et al.*, 2005). The vesicles then fuse to form a closed NE (Franz *et al.*, 2005). Finally, additional Nups are recruited to complete NPC biogenesis and result in a transport competent nucleus (Macaulay and Forbes, 1996; Goldberg *et al.*, 1997).

Ran, a member of the Ras-like family of small GTPases, regulates key events during mitosis and the subsequent nuclear reassembly process (Dasso, 2002; Quimby and Dasso, 2003). The nucleotide-bound state of Ran is controlled by both a specific GTPase-activating protein (RanGAP, Rna1 in yeast; Bischoff *et al.*, 1995), which stimulates GTP hydrolysis to favor the production of RanGDP, and a guanine nucleotide exchange factor (RanGEF, RCC1 in vertebrates and *Schizosaccharomyces pombe*; Prp20 in *Saccharomyces cerevisiae*; Bischoff and Ponstingl, 1991), which promotes nucleotide release to favor RanGTP. During interphase, RanGEF is localized to the nucleus, whereas RanGAP is cytoplasmic. The asymmetric distribution of the RanGEF and RanGAP regulatory fac-

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* Present address: Biology Department, Texas A&M University, 3258 TAMU, College Station, TX 77843-3258.

Address correspondence to: Kathryn J. Ryan (kryan@mail.bio.tamu.edu).

Abbreviations used: GFP, green fluorescent protein; INM, inner nuclear membrane; NE, nuclear envelope; NES, nuclear export sequence; NLS, nuclear localization sequence; NPC, nuclear pore complex; Δ , null; Nup, nucleoporin; ONM, outer nuclear membrane; ts, temperature sensitive.

tors results in RanGTP predominating in the nucleus, whereas RanGDP is favored in the cytoplasm (Hetzer *et al.*, 2002; Kalab *et al.*, 2002). Because RanGEF is tightly associated with chromatin, the concentration of RanGTP remains high near the chromosomes after nuclear envelope breakdown (Li *et al.*, 2003; Li and Zheng, 2004; Kalab *et al.*, 2006).

A cellular role for Ran was first elucidated in nucleocytoplasmic transport wherein RanGTP interactions with the karyopherin β family of transport receptors (also known as importins, exportins, and transportins) control the directionality of transport (Gorlich and Kutay, 1999; Pemberton and Paschal, 2005). During nuclear import, a karyopherin (Kap) recognizes and binds a nuclear localization sequence (NLS) within the imported protein. Once in the nucleus, RanGTP binds to the Kap causing NLS cargo release. In a parallel process, factors to be exported contain nuclear export sequences (NES), which are recognized by different Kap family members. However, for nuclear export, RanGTP is required to stabilize the Kap-NES cargo interaction. The presence of cytoplasmic RanGAP rapidly converts RanGTP to RanGDP resulting in NES cargo release. Thus, RanGTP acts as the molecular switch controlling Kap-cargo interactions with the asymmetric distribution of its regulatory factors providing the critical spatial information.

The mechanisms for RanGTP action in mitotic events also occurs by modulating the interactions of Kaps with key factors (Harel and Forbes, 2004; Mosammamaparast and Pemberton, 2004). For example, importin β blocks spindle assembly by binding and sequestering specific spindle assembly factors (Gruss *et al.*, 2001; Nachury *et al.*, 2001; Wiese *et al.*, 2001; Tsai *et al.*, 2003). However, when these importin β complexes are near chromatin, the locally high concentration of RanGTP promotes RanGTP-importin β interaction and release of these factors. This localized release ensures that spindle assembly occurs only at chromosomes. Likewise, the balance of importin β and RanGTP controls proper NE assembly after mitosis. Addition of excess importin β to an *in vitro* nuclear reassembly assay blocks vesicle fusion and inhibits formation of a closed NE (Harel *et al.*, 2003a; Walther *et al.*, 2003b). In contrast, removal of importin β or addition of RanGTP leads to aberrant membrane fusion and the accumulation of cytoplasmic membranes harboring NPC structures (termed annulate lamellae). The molecular target(s) of importin β during NE assembly and membrane fusion are unknown. Interactions between RanGTP and importin β also appear to regulate the assembly of NPCs after mitosis and during subsequent nuclear growth *in vitro* (Harel *et al.*, 2003a; Walther *et al.*, 2003b; D'Angelo *et al.*, 2006).

Although mitotic NPC reassembly has been extensively studied, there is little known about how NPCs are assembled *de novo* into an intact NE. In cells undergoing a closed mitosis, all NPCs must insert into a closed NE, presumably by coordinated recruitment of Nups during fusion of the INM and ONM to form the pore. For cells with an open mitosis, NPCs also must assemble into an intact NE during interphase when NPC number increases (Maul *et al.*, 1971). Maintenance of NPC number in postmitotic cells also requires NPC biogenesis into a closed NE. To focus on the mechanism of NPC biogenesis in intact NEs, we have used a genetic strategy in the yeast *S. cerevisiae* (Ryan and Wentz, 2002). We identified Ran as a key regulator of NPC assembly (Ryan *et al.*, 2003). Intriguingly, mutants with perturbations at each stage of the Ran GTPase cycle accumulate small, cytoplasmic vesicles. At least one Nup, Nic96, is associated with these vesicles. This led us to propose a model for NPC assembly in which cytoplasmic Ran is necessary for the fusion of a novel, vesicular intermediate to the ONM and nucleation of new NPC formation.

A cytoplasmic function for the Ran GTPase cycle in *de novo* NPC assembly into intact NEs presents a paradox due to the predominant localization of RanGAP to the cytoplasm and RanGEF to the nucleus. However, several studies indicate that microenvironments of Ran and its regulatory factors exist within the cytoplasm and the nucleus. For example, RanGTP has been identified at centrosomes where it is involved in regulating duplication (Keryer *et al.*, 2003; Wang *et al.*, 2005). In addition, despite the high levels of RanGTP that are visualized around mitotic chromatin (Kalab *et al.*, 2002, 2006), RanGAP has been localized to kinetochores and its mislocalization results in defects in kinetochore structure (Joseph *et al.*, 2002; Arnaoutov and Dasso, 2003; Arnaoutov *et al.*, 2005). Strikingly, this requirement for RanGAP activity in kinetochore function is also found during closed mitosis in *S. cerevisiae* (Tanaka *et al.*, 2005), and a temperature-sensitive allele of *S. pombe* RanGAP (*Sprna1^{ts}*) has chromosome segregation and centromeric silencing defects (Kusano *et al.*, 2004).

Here, we characterize a mutant allele of *KAP95*, the yeast orthologue of importin β , that was isolated in our screen for nuclear pore complex assembly (*npa*) mutants. The mutant allele, *kap95-E126K*, results in a protein that is unstable at nonpermissive temperatures. Concatenate with the loss of *Kap95*, cells accumulate extended cytoplasmic membrane sheets and fail to correctly localize Nups to the NE. Taken together, we propose that *Kap95* and Ran function together to spatially restrict fusion of a vesicular intermediate to the ONM for productive *de novo* NPC assembly into an intact interphase NE.

MATERIALS AND METHODS

Yeast Strains and Reagents

Yeast strains used in this study are listed in Table 1. General yeast manipulations were performed according to standard methods. All strains except SWY3189 and SWY3190 (see below) were grown at 23°C in YPD (yeast extract, peptone, 2% glucose) or synthetic complete (SC) media lacking appropriate amino acids and supplemented with 2% glucose unless otherwise indicated. For growth at the nonpermissive temperature, cells were incubated at 34 or 37°C. SWY3189 and SWY3190 (*kap95 Δ* + *pGAL-KAP95*) were grown in YP + 1% galactose + 1% raffinose. To inhibit *KAP95* expression, SWY3189 cells were washed into fresh YPD medium and incubated at 23°C. Cycloheximide (Sigma-Aldrich, St. Louis, MO) was added directly to growing cultures to a final concentration of 10 μ g/ml. To compare strain growth at different temperatures, early log phase cultures were pelleted and resuspended in a minimal volume of 10 mM Tris HCl, 1 mM EDTA, pH 8. The number of cells was counted and then diluted to 2×10^7 cells/ml. A series of fivefold dilutions were made, and 5 μ l of each was spotted to YPD plates and incubated at the appropriate temperature.

Cloning and Plasmid Construction

Cloning of the wild-type allele of *NPA16* was accomplished using a *LEU2/CEN* yeast genomic library as previously described (Ryan and Wentz, 2002). To sequence the *npa16* allele of *kap95*, genomic DNA was isolated from *npa16* and used as a template in PCR to amplify the entire open reading frame. The PCR products from two independent reactions were cloned into pRS315 and sequenced. The results of the sequencing were compared with the yeast genome (www.yeastgenome.org) to identify the mutation.

To place *KAP95* under the *GAL* promoter, oligonucleotides 1084 (5'-ACAA-GATCTACCGCTGAATTGCTC-3') and 1085 (5'-GCGAGATCTTACAG-GATCCCTAAGGATAATTGACGCTTC-3') were used to amplify the *KAP95* open reading frame of pSW503 (Iovine and Wentz, 1997) using PCR. The resulting product was digested with BglII and cloned into the BamHI site of pSW1328 (Verbsky *et al.*, 2002) to make *pGAL-KAP95* (pSW3067). For the plasmid expressing the dominant *kap95 Δ 48* mutant (pSW1037), oligonucleotides 672 (5'-ACCCGGATCCTCGATGAAAATACAAAGCTA-3') and L15-2 (5'-CGCGGATCCATACATTGACTATTAACGCGCAG-3') were used to amplify by PCR a fragment from the *KAP95* open reading frame (in pSW503) for the sequence from the codon for amino acid 49 through the termination codon. The resulting product was digested with BamHI and cloned in frame with the sequence coding for glutathione S-transferase (GST) under the control of the *GAL* promoter cassette in pBj382 (*TRP1*, 2 μ pRS424).

Table 1. Yeast strains used in this study

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)
SWY811	<i>MATα ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100 ade2-1::ADE2:ura3 nup49ΔGLFG::GFP-S65T-TRP1</i>	Bucci and Wentte (1998)
SWY1695	<i>MATa GFP-nic96:HIS3 ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100 ade2-1::ADE2:ura3</i>	Bucci and Wentte (1998)
SWY2089	<i>MATα GFP-nic96:HIS3 nup170-GFP:URA3 lys2 ura3-1 his3-11,15 leu2-3,112 can1-100 ade2-1::ADE2:ura3</i>	Ryan and Wentte (2002)
SWY2090	<i>MATa GFP-nic96:HIS3 nup170-GFP:URA3 trp1-1 ura3-1 his3-11,15 leu2-3,112 can1-100 ade2-1::ADE2:ura3</i>	Ryan and Wentte (2002)
SWY2119	<i>MATα nup170-GFP:URA3 ura3-1 his3-11,15 lys2 leu2-3,112 can1-100 ade2-1::ADE2:ura3</i>	This study
SWY3189	<i>MAT? kap95Δ::HIS3 GFP-nic96:HIS3 nup170-GFP:URA3 trp1-1 ura3-1 his3-11,15 leu2-3,112 can1-100 ade2-1::ADE2:ura3 + pGAL-KAP95-TRP1 (pSW3067)</i>	This study
SWY3190	<i>MATa kap95Δ::HIS3 trp1-1 ura3-1 his3-11,15 leu2-3,112 can1-100 ade2-1::ADE2:ura3 + pGAL-KAP95-TRP1 (pSW3067)</i>	This study
SWY3299	<i>MATα kap95-E126K GFP-nic96:HIS3 trp1-1 ura3-1 his3-11,15 leu2-3,112 can1-100 ade2-1::ADE2:ura3</i>	This study
SWY3300	<i>MATa kap95-E126K nup170-GFP:URA3 lys2 ura3-1 his3-11,15 leu2-3,112 can1-100 ade2-1::ADE2:ura3</i>	This study
SWY3561	<i>MATα kap95-E126K(npa16) nic96-GFP:HIS3 NUP170-GFP:URA3 lys2 ura3-1 his3-11,15 leu2-3,112 can1-100 ade2-1::ADE2:ura3</i>	This study
SWY3562	<i>MATa kap95-E126K trp1-1 ura3-1 his3-11,15 leu2-3,112 can1-100 ade2-1::ADE2:ura3</i>	This study
SWY3609	<i>MATa kap95-E126K nup49ΔGLFG::GFP-S65T-TRP1 ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100 ade2-1::ADE2:ura3</i>	This study

Transport Assays and Microscopy

To assay steady state nuclear import efficiency, strains containing pGAD-GFP (cNLS-GFP; Shulga *et al.*, 1996) or pNS167 (Nab2-NLS-GFP; Shulga *et al.*, 1996) plasmids were grown to early log phase in SC media lacking uracil at 23°C and then shifted to growth at 37°C for 6 h. Fluorescence and differential interference contrast (DIC) microscopy were performed on an Olympus BX50 microscope (Center Valley, PA) using an UPlan 100×/1.3 objective. Images were captured using a Photometrics CoolSnap HQ camera (Tucson, AZ) with MetaVue software. Indirect immunofluorescence was performed as previously described (Wentte *et al.*, 1992) with the mAb118C3 against Pom152 (Strambio-de-Castillia *et al.*, 1995), polyclonal anti-Nup116 C-term antibody (Iovine *et al.*, 1995), or monoclonal anti-Ran antibody (Transduction Laboratories, San Jose, CA) followed by Alexa-594-conjugated anti-mouse or anti-rabbit secondaries (Molecular Probes, Carlsbad, CA). Like-images within a figure were captured for the same exposure time, and all image processing was done in MetaVue. Final figures were assembled in Adobe Photoshop (San Jose, CA). Samples for thin-section electron microscopy were prepared as previously described (Wentte and Blobel, 1993). Sections were viewed on a Philips CM-12 electron microscope (Mahwah, NJ), and images were taken using a CCD digital camera.

Western Analysis of Kap95 Protein Levels

Cultures were grown to early log phase and then shifted to 34 or 37°C for the indicated time or were shifted from growth in galactose to glucose media. Crude cell lysates were prepared by a method adapted from Yaffe and Schatz (1984). Briefly, 30 mg of cells was harvested, washed with water, and resuspended in 160 μl of 1.85 M sodium hydroxide, 7.4% β-mercaptoethanol and incubated on ice for 10 min. An equal volume of 50% trichloroacetic acid was added and incubated on ice for an additional 10 min for protein precipitation. Samples were centrifuged at 15K relative centrifugal force in a microcentrifuge for 2 min. Next, pellets were washed with 500 μl 1 M Tris base and resuspended in SDS sample buffer. The equivalents of 1.5 mg of starting cell pellets were separated by SDS-PAGE and transferred to nitrocellulose. The blots were probed with affinity-purified anti-Kap95 antibody (1:100; Iovine and Wentte, 1997) followed by an alkaline-phosphatase labeled, anti-rabbit secondary antibody.

RESULTS

Identification of a *kap95* Mutant in a Genetic Screen for Defective NPC Assembly

In our previous studies, we isolated a collection of nuclear pore complex assembly (*npa*) mutants in the yeast *S. cerevisiae* (Ryan and Wentte, 2002). The *npa* mutants are temperature sensitive (*ts*) and are characterized by their inability to

correctly localize GFP-Nic96 and Nup170-GFP to the NE/NPCs at the nonpermissive temperature. Nic96 and Nup170 are both associated with NPC subcomplexes that play critical roles in global NPC structure and assembly (Grandi *et al.*, 1993; Aitchison *et al.*, 1995; Zabel *et al.*, 1996; Marelli *et al.*, 1998). Thus, perturbation of GFP-Nic96 and Nup170-GFP localization at NPCs has proven to be an excellent indicator of NPC structural integrity and defects in NPC biogenesis (Zabel *et al.*, 1996; Ryan and Wentte, 2002; Ryan *et al.*, 2003). To further understand the NPC assembly mechanism at the molecular level, we concentrated on the characterization of the *npa16* complementation group, which was represented by a single allele in the mutant collection. The wild-type *NPA16* gene was identified by complementation of the *ts* growth phenotype with a yeast genomic library. By DNA sequence analysis and comparison to the yeast genome database, we found that the library plasmid insert contained *KAP95*. A plasmid harboring only *KAP95* was tested and found to rescue both the growth and GFP-Nup mislocalization phenotypes (data not shown). To delineate the precise mutation in the *npa16/kap95* strain, the mutant allele was cloned and sequenced. A single point mutation that results in an amino acid substitution of Glu to Lys at amino acid 126 was found. This allele was designated *kap95-E126K*.

In the wild-type parental cells expressing both GFP-Nic96 and Nup170-GFP, the GFP-Nup signal forms a punctate pattern uniformly distributed around the NE (Figure 1A). This pattern indicates that these Nups are assembled into NPCs. When the *kap95-E126K* cells were grown at the permissive temperature of 23°C, GFP-Nic96 and Nup170-GFP were also localized to the NE/NPCs. However, after shifting the *kap95-E126K* cells to the nonpermissive growth temperature of 34°C for 5 h, defects in cell growth and perturbations in GFP-Nup localization were detected. At 23°C, the doubling time of the *kap95-E126K* cells was the same as the *KAP95* parental strain. In contrast, shifting to growth at 34°C inhibited *kap95-E126K* cell growth, and cell divisions ceased after ~4 h (data not shown). Coincidentally, the GFP-Nup

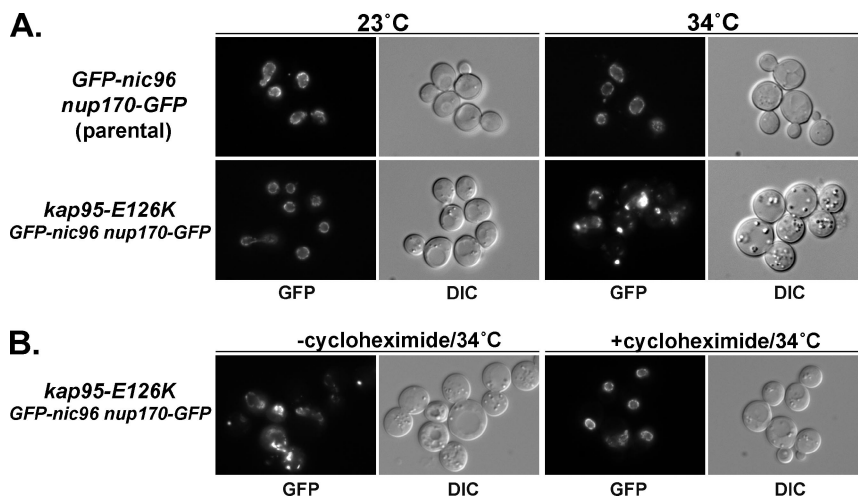


Figure 1. *KAP95* is required for correct assembly of newly synthesized Nups. (A) The parental strain (*GFP-nic96 nup170-GFP*) and the *npa16* mutant (*kap95-E126K GFP-nic96 nup170-GFP*) were grown at 23°C to early log phase and then shifted to 34°C (nonpermissive temperature) for 5 h. GFP-Nic96 and Nup170-GFP were localized by direct fluorescence microscopy of live cells (GFP columns). DIC images show overall cell morphology. (B) GFP-Nup mislocalization requires new protein synthesis in the *npa16-kap95-E126K* cells. Cells were grown as in (A) in the absence (–) or presence (+) of cycloheximide. Parental, SWY2089; *npa16-kap95-E126K*, SWY3561.

signal at the NE rim became markedly diminished in the *kap95-E126K* cells. Most of the GFP-Nup signal was instead mislocalized and often concentrated in bright foci near the nucleus (Figure 1A).

To test if the GFP-Nup mislocalization observed in the *kap95-E126K* cells at 34°C represented a global defect in Nup localization and NPC structure, the integral membrane pore-associated protein Pom152 and the GLFG-repeat containing nucleoporin Nup116 were localized using indirect immunofluorescence microscopy. Similar to GFP-Nic96 and Nup170-GFP, Pom152, and Nup116 were not correctly localized in the *kap95-E126K GFP-nic96 nup170-GFP* cells shifted to growth at 34°C (Figure 2, A and B, top rows). Like the GFP-Nups, these proteins appeared concentrated in cytoplasmic foci and in regions associated with the NE.

The failure of Nups to localize exclusively in NPCs at the nuclear rim could result from either a block in NPC assembly or an increased turnover of pre-existing NPCs. To distinguish between these two possibilities, new protein synthesis was inhibited by addition of cycloheximide during the shift to the nonpermissive growth temperature. Cycloheximide treatment blocked the mislocalization of the GFP-Nups in *kap95-E126K* cells grown at 34°C (Figure 1B). Equally significant is the observation that a weak GFP-Nup signal remained associated with the NE in the absence of cycloheximide. We have previously speculated that the fraction of GFP-Nup signal still associated with the NE and forming the weak nuclear ring in *npa* mutant cells (Figure 1, A and B, –cycloheximide) represents NPCs formed before the temperature shift. Indeed, previous studies of *nic96* mutants have shown that if new assembly is blocked then NPC density in the NE decreases by half with every division (Zabel *et al.*, 1996; Gomez-Ospina *et al.*, 2000). Growth of such *nic96* NPC assembly mutant cells arrests after two or three cell divisions. This correlates directly with the defects in the *kap95-E126K* mutant cells. These results strongly suggested that Nup mislocalization in the *kap95-E126K* cells was the result of a defect in assembling newly synthesized Nups into new NPCs.

The kap95-E126K Mutant Functions as an Effective Null with Complete Loss of Kap95 Protein

Kap95 is an essential member of the karyopherin- β family and required for nuclear import of cargo proteins containing a basic-type NLS (reviewed in Bednenko *et al.*, 2003; Stewart, 2003). The x-ray crystal structure of Kap95 reveals a frame-

work of 19 HEAT repeats (Lee *et al.*, 2005; Liu and Stewart, 2005). These HEAT repeats form a superhelical structure that provides a large surface area for protein–protein interactions. Like other family members, Kap95 is divided into three functional domains. The N-terminal region binds the small GTPase Ran. The central HEAT repeats form a surface that directly interacts with Nups harboring FG, FxFG, or GLFG domains. The C-terminal domain provides a docking site for the adaptor karyopherin- α (Kap60/Srp1/importin α), which directly binds the NLS cargo. E126 is a highly conserved residue, and based on the crystal structure, lies on the outer surface of the protein (Cingolani *et al.*, 1999; Lee *et al.*, 2005; Liu and Stewart, 2005). However, co-crystal structures also indicate that it is not likely to be involved in binding FG-Nups (Bayliss *et al.*, 2000; Bayliss *et al.*, 2002). To examine the molecular defect caused by the *kap95-E126K* mutation, the ability of *kap95-E126K* protein to bind RanGTP, Kap60/Srp1, and GLFG or FxFG nucleoporins was assayed. Using both yeast two-hybrid and in vitro recombinant protein strategies, no qualitative differences in binding were observed between the wild-type Kap95 and *kap95-E126K* proteins and any of these binding partners (data not shown). Interestingly, Western blot analysis showed that the stability of the *kap95-E126K* protein in vivo was dramatically altered when cells were shifted to the nonpermissive temperature. In wild-type cells, Kap95 levels remained constant when cells were grown at 34°C. However, the levels of the *kap95-E126K* protein were dramatically decreased when the *kap95-E126K* cells were cultured at 34°C (Figure 3). This result suggested that E126 residue contributes to the stability of Kap95, especially at higher temperatures.

The decrease in protein levels suggested that the *kap95-E126K* mutant represents a loss of function allele that phenocopies the null allele at the nonpermissive growth temperature. Indeed, the time frame for the protein level decrease was coincident with the time frame for the GFP-Nup mislocalization defect to become apparent. To test if loss of Kap95 was directly responsible for the GFP-Nup mislocalization phenotype, *KAP95* was deleted in *GFP-nic96 nup170-GFP* and wild-type strain backgrounds. A plasmid carrying *KAP95* under an inducible promoter was introduced to allow for *KAP95* expression and cell growth when the *kap95 Δ* strains were grown in galactose. However, addition of glucose to logarithmically growing cells repressed *KAP95* expression and led to the gradual loss of Kap95 protein (Figure 4A). When Kap95 levels decreased in the

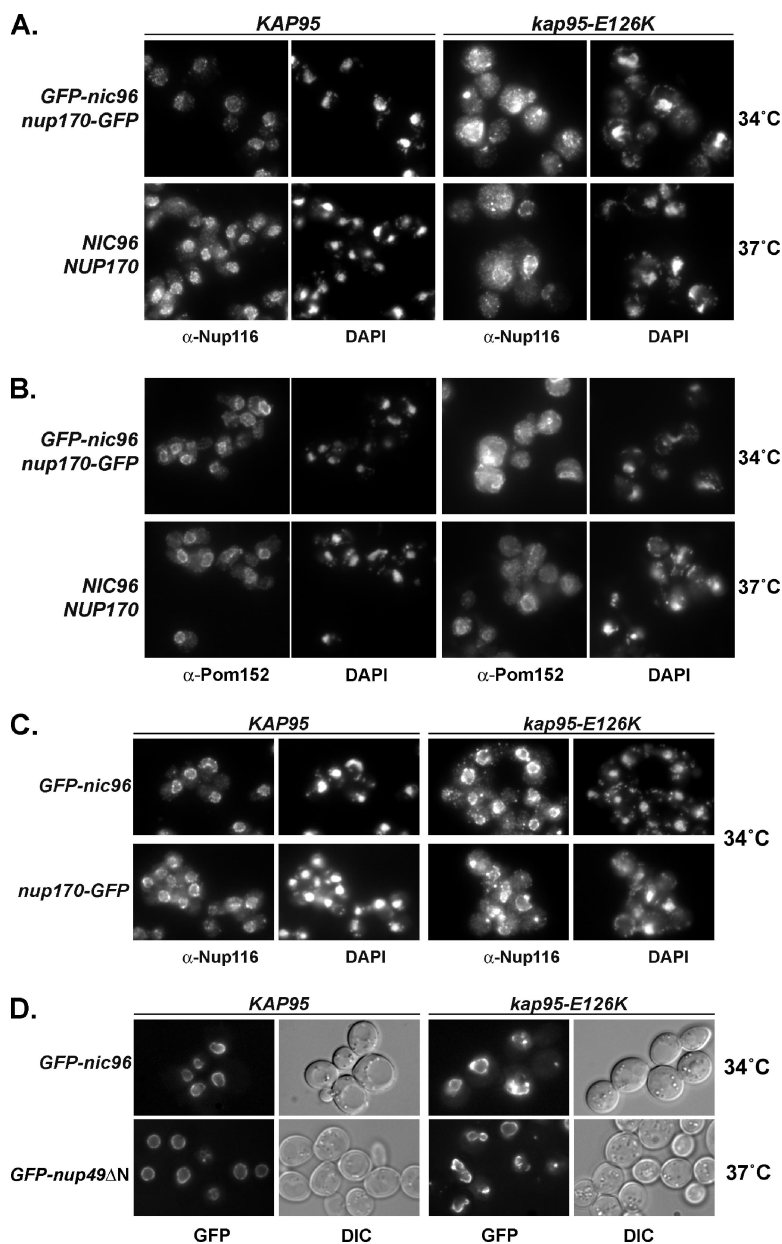


Figure 2. Nup localization defects are observed in *kap95-E126K* cells with multiple combinations of *GFP-nup* alleles. (A and B) Analysis of Nup116 (A) and Pom152 (B) localization in *KAP95* and *kap95-E126K* strains. Early log phase cells with *GFP-nic96* and *nup170-GFP* (SWY2089, SWY3561) or wild-type alleles of both NUPs (SWY518, SWY3562) were shifted to the nonpermissive temperatures, (as determined in Figure 5) for 5 h and processed for indirect immunofluorescence microscopy with an antibody against Nup116 or Pom152. Note DAPI staining detects both nuclear (large spots) and mitochondrial (small spots) DNA. (C) Nup116 localization in wild-type or the *kap95-E126K* mutant cells combined with either *GFP-nic96* (SWY1695, SWY3299) or *nup170-GFP* (SWY2119, SWY3300) was determined after shifting to growth at 34°C. Indirect immunofluorescence with anti-Nup116 antibodies was conducted as in A. (D) Localization of *GFP-nic96* (SWY1695, SWY3299) or *GFP-nup49ΔN* (SWY811, SWY3609) in wild-type and *kap95-E126K* cells was determined after shifting cells at the early log phase of growth to the indicated temperature for 5 and 6 h, respectively. GFP signals were visualized by direct fluorescence microscopy. DIC images show overall cell morphology.

kap95Δ GFP-nic96 nup170-GFP cells, both GFP-Nup and Nup116 mislocalization was observed (Figure 4B). Likewise, in the *kap95Δ* cells lacking GFP-Nups, Pom152, and Nup116 were both mislocalized (Figure 4C). In both the *kap95Δ* and *kap95Δ GFP-nic96 nup170-GFP* cells, the perturbed Nups showed diminished nuclear rim signal and increased cytoplasmic signal with distinct foci. These phenotypes were indistinguishable from the *kap95-E126K* mutant phenotypes. Thus, it is the absence of Kap95 in the *kap95-E126K* mutants that is responsible for the NPC assembly defect.

Functional Connections Revealed by Genetic Interactions between the *kap95-E126K* and *nup* Alleles

For the *npa* screen, the *GFP* sequence was integrated into the genome to generate a parental strain that contained *GFP-nic96* and *nup170-GFP*. The GFP-tagged proteins that result are not fully functional, because the strain expressing both GFP-Nic96 and Nup170-GFP is ts at 37°C but not 34°C

(Figure 5, and Ryan and Wentz, 2002). In our previous analysis of the Ran GTPase cycle in NPC assembly, we found genetic interactions between the Ran GTPase cycle mutants (e.g., *ntf2-H104Y* and *rna1-S116F*) and the *GFP-nic96* and *nup170-GFP* alleles (Ryan *et al.*, 2003). If the Kap95 function in NPC assembly is linked to the role for Ran, we speculated that the *kap95-E126K* mutant might also have genetic interactions with the *nup* alleles. As isolated in the *npa* screen, the *kap95-E126K GFP-nic96 nup170-GFP* strain (*npa16*) failed to grow at 34°C. However, when the *kap95-E126K* mutation was crossed to a strain background containing wild-type alleles of *NIC96* and *NUP170*, the cells were no longer ts at 34°C. The lowest nonpermissive temperature of the *kap95-E126K NIC96 NUP170* mutant strain was instead 37°C (Figure 5). To determine which *GFP-nup* was contributing to the ts phenotype, *kap95-E126K* was combined independently with the *GFP-nic96* and *nup170-GFP* alleles. Neither the *GFP-nic96* nor the *nup170-GFP* strain was ts,

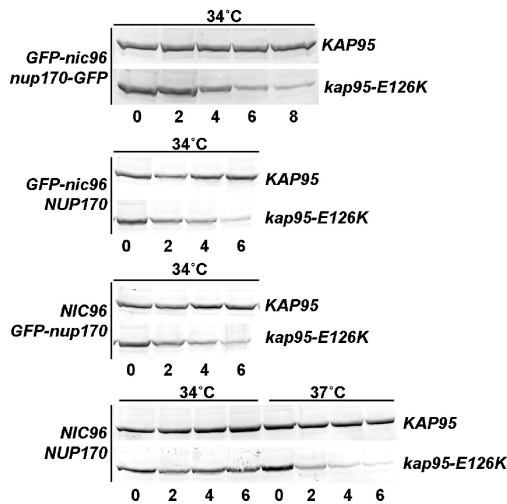


Figure 3. *kap95-E126K* protein is unstable in cells grown at non-permissive temperatures. Strains expressing different combinations of *NIC96* and *NUP170* alleles (left column) with either wild-type *KAP95* or mutant *kap95-E126K* alleles (right column) were grown to early log phase and shifted to the indicated temperatures for various times (in hours). Total cell lysate from each was separated by SDS-PAGE, and immunoblotting with an anti-Kap95 antibody was performed to detect protein levels. Strains, from top to bottom: SWY2089, SWY3561, SWY1695, SWY3299, SWY2119, SWY3300, SWY518, SWY3562.

although growth of the *nup170-GFP* strain was slower at 37°C. However, both the *kap95-E126K GFP-nic96* and the *kap95-E126K nup170-GFP* strains were lethal at 34°C (Figure 5). Thus, the *kap95-E126K* phenotype was exacerbated by either *nup* allele.

Next, we asked whether the genetic interactions between *kap95-E126K* and *GFP-nic96* or *nup170-GFP* were specific or if *kap95-E126K* had enhanced temperature sensitivity with all mutant *nup* alleles. Unlike *GFP-nic96* and *nup170-GFP*, *GFP-nup49ΔN* did not enhance the temperature sensitivity of *kap95-E126K*. The *kap95-E126K GFP-nup49ΔN* double mutant grew in a similar manner as the *kap95-E126K* single mutant (Figure 5).

We also tested for protein stability in the *kap95-E126K GFP-nup* versus *NUP* strains (Figure 3). Wild-type Kap95 protein levels remained constant in the different *GFP-nup* strains and at different growth temperatures. In either the *GFP-nic96* or *nup170-GFP* strains, the levels of the *kap95-E126K* protein decreased over time at 34°C. In contrast, *kap95-E126K* protein was stable in *NIC96 NUP170* cells grown at 34°C. However, when the *kap95-E126K NIC96 NUP170* cells were shifted to growth at 37°C (the nonpermissive growth temperature for this strain) the *kap95-E126K* protein levels decreased. Thus, the temperature sensitivity of the *kap95-E126K GFP-nup* strains was directly correlated with *kap95-E126K* protein levels. Moreover, *Nic96* and *Nup170* both individually contributed to the stabilization of *kap95-E126K* protein at 34°C but were insufficient at 37°C.

To evaluate whether the *GFP-nup* alleles also contributed to the Nup mislocalization defect observed in the *kap95-E126K GFP-nic96 nup170-GFP* mutant, a series of different strains were tested. In the *kap95-E126K GFP-nic96* and *kap95-E126K nup170-GFP* cells, *Nup116* localization was altered after growth at the nonpermissive temperature (Figure 2C). Likewise, in *kap95-E126K GFP-nic96* and *kap95-E126K GFP-nup49ΔN* cells, *GFP-Nic96* and *GFP-nup49ΔN* were each independently perturbed (Figure 2D). It is interesting that the *GFP-nup49ΔN* allele did not show a genetic interaction with the

kap95-E126K mutant, but the *GFP-nup49ΔN* protein was perturbed in the same relative manner as a *GFP-nup* from a gene that was genetically linked (*nic96* and *nup170*). This correlates with the above results in the *kap95Δ* strain where the GFP-tagged Nups were not required for the NPC perturbation (Figure 4C). Thus, the primary defect in NPC assembly in the *kap95-E126K* mutant is independent of but synergistic with the *GFP-nic96 nup170-GFP* mutant phenotype.

Cytoplasmic Membrane Accumulation Is Observed in the *kap95-E126K* Mutant Cells

Thin-section electron microscopy was used to assess the ultrastructural consequences associated with the *kap95-E126K* mutant. At 34°C, cells from the *KAP95 GFP-nic96 nup170-GFP* parental strain had normal morphology with small amounts of cortical ER and a continuous NE with electron-dense NPCs spanning the INM and ONM (Figure 6A). In contrast, nuclear architecture was severely distorted at the nonpermissive temperature in *kap95-E126K GFP-nic96 nup170-GFP* cells. We observed several distinct differences compared with wild-type cells. First, large invaginations of the cytoplasm into the nuclear sphere resulted in the nucleus and NE appearing as layered membrane rings (lines, Figure 6, B–E). Often the NPCs aligned between the different juxtaposed membranes rings (Figure 6, B–E). This alignment likely accounts for the bright foci observed by direct fluorescence microscopy of the GFP-Nups (Figures 1 and 4). Second, interestingly, some of the NPC-like structures failed to span the NE and were associated with just the INM (asterisks, Figure 6, B–E). Third, the accumulation of ~70-nm vesicles was also observed within many of the cytoplasmic invaginations (Figure 6, B, D, and E). Fourth, in addition to nuclear defects, *kap95-E126K GFP-nic96 nup170-GFP* cells also had long stretches of cytoplasmic membranes (arrow, Figure 6, B–D). Unlike annulate lamellae, no electron-dense NPC structures were observed in these cytoplasmic membrane sheets. All of the electron dense NPC-like structures were consistently associated with membranes linked to the NE.

To confirm that the ultrastructural perturbations were directly linked to the Kap95 defect, we also examined the *kap95Δ* mutants by thin-section electron microscopy. After growth of the *kap95Δ GFP-nic96 nup170-GFP* strain under conditions to repress *KAP95*, membrane structures accumulated in the cytoplasm (Supplementary Figure 1). The morphology was indistinguishable from that in the *kap95-E126K GFP-nic96 nup170-GFP* cells. Moreover, electron microscopy analysis of *kap95Δ NIC96 NUP170* cells again showed the same phenotype (Ryan, unpublished data).

Kap95 Role in NPC Assembly Is Linked to Ran Function in Assembly

Given our prior studies showing an *in vivo* role for the RanGTPase cycle in NPC assembly (Ryan *et al.*, 2003), we tested for whether the defects in the *kap95* mutants were directly linked to Ran function. One possibility was that depleting Kap95 may indirectly alter the cellular levels of RanGTP or Ran localization. By indirect immunofluorescence microscopy, we analyzed the localization of Ran in wild-type and *kap95-E126K GFP-nic96 nup170-GFP* cells. No dramatic changes were observed (Supplementary Figure 2). We also noted that Nup localization was not altered when wild-type *KAP95* was overexpressed (Figure 7B).

A further test of global RanGTP function was conducted by monitoring the steady state nuclear import capacity of two independent Kap pathways. If Ran function was indirectly altered, we would expect all Kap-dependent import pathways to be inhibited. Wild-type and *kap95-E126K* cells harboring plasmids expressing GFP reporters for a Kap95

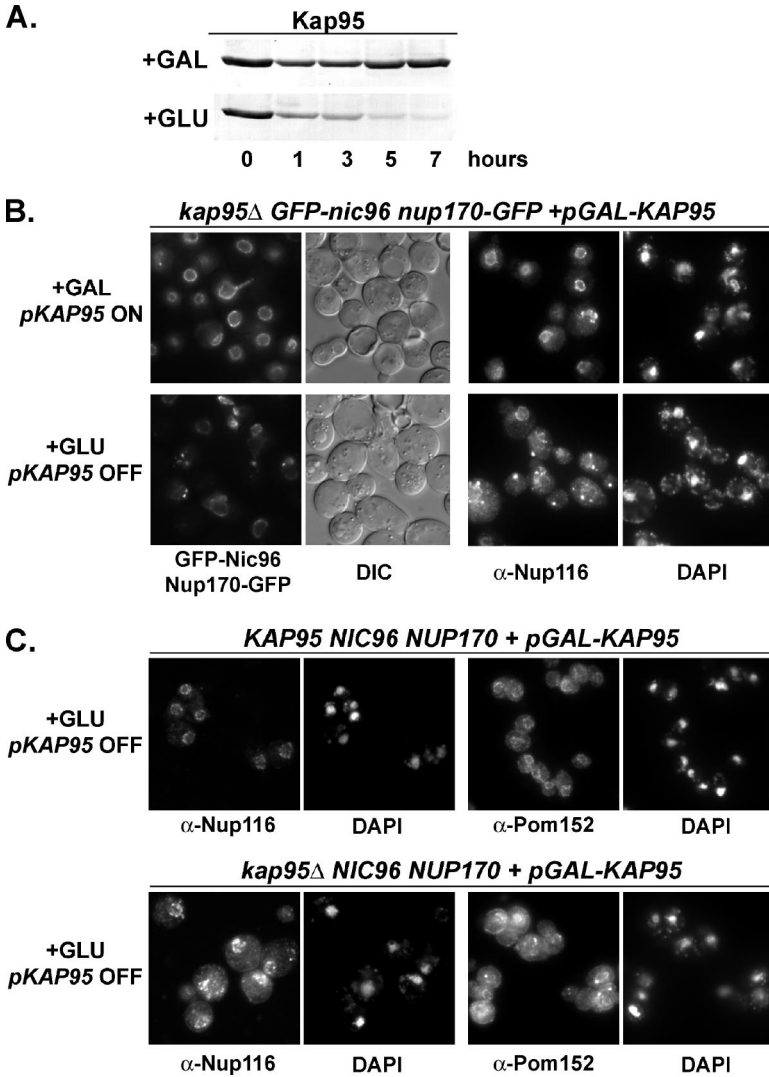


Figure 4. A *kap95* null (Δ) mutant mimics the *kap95-E126K* phenotype. A plasmid containing *KAP95* under control of a *GAL* promoter (pSW3067) allowed for *KAP95* expression in wild-type *KAP95* and *kap95Δ* strains. (A) Immunoblotting with an anti-Kap95 antibody of crude cell lysates harvested from *kap95Δ GFP-nic96 nup170-GFP* (SWY3189) cells grown in 1% galactose + 1% raffinose (+GAL) or after shifting to 2% glucose media (+GLU) for various times was performed to detect loss of Kap95 protein. (B) GFP-Nic96 and Nup170-GFP were visualized by direct fluorescence microscopy in the *kap95Δ GFP-nic96 nup170-GFP* (SWY3189) cells grown in 1% galactose + 1% raffinose (+GAL) or shifted to 2% glucose media (+GLU) for 8 h. In the same cells, indirect immunofluorescence microscopy with an anti-Nup116 antibody was used to detect Nup116 localization. (C) Localization of Pom152 and Nup116 by indirect immunofluorescence microscopy was conducted in the wild-type *KAP95* (SWY518, top row) and the *kap95Δ* strain (SWY3190, bottom row) after shifting the cells from growth in YP 2% galactose to YP 2% glucose.

cargo (cNLS) and a Kap104 cargo (Nab2-NLS) were examined after growth at the nonpermissive temperature. In wild-type cells, the fluorescence signals for both cNLS-GFP and Nab2-NLS-GFP were concentrated in the nucleus. In *kap95-E126K* cells, the nuclear cNLS-GFP signal was markedly diminished reflecting decreased nuclear import (Figure 7A, top rows). This was expected as Kap95 import should be abolished as the *kap95-E126K* protein is degraded. In con-

trast, the Nab2-NLS-GFP remained localized in the nucleus of temperature-arrested *kap95-E126K* cells (Figure 7A, bottom row). This indicated that RanGTP function in the Kap104 import pathway was not perturbed.

To directly test for whether Ran interaction with Kap95 was required for NPC assembly, we made use of a dominant mutant wherein the sequence encoding the N-terminal 48 amino acid residues of Kap95 is deleted (*kap95Δ48*). Others

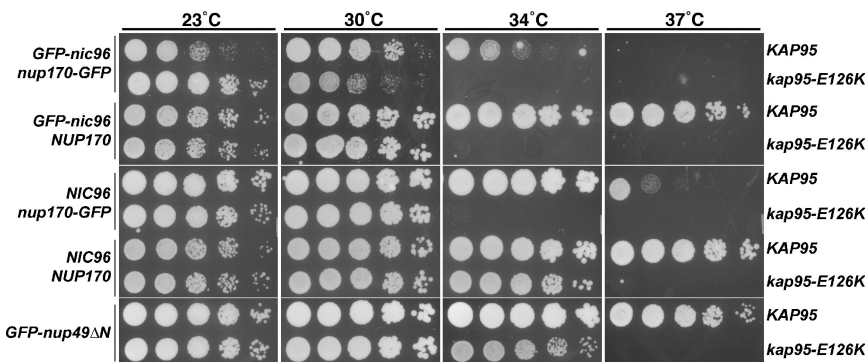


Figure 5. The *kap95-E126K* mutant displays enhanced lethality with *GFP-nic96* and *nup170-GFP* alleles. Serial dilutions of strains with various combinations of *NIC96* and *NUP170* alleles (left column) in either wild-type *KAP95* or *kap95-E126K* mutant backgrounds (right column) were spotted on YPD for growth at the indicated temperature. Strains, from top to bottom: SWY2090, SWY3561, SWY1695, SWY3299, SWY2119, SWY3300, SWY518, SWY3562, SWY811, SWY3609.

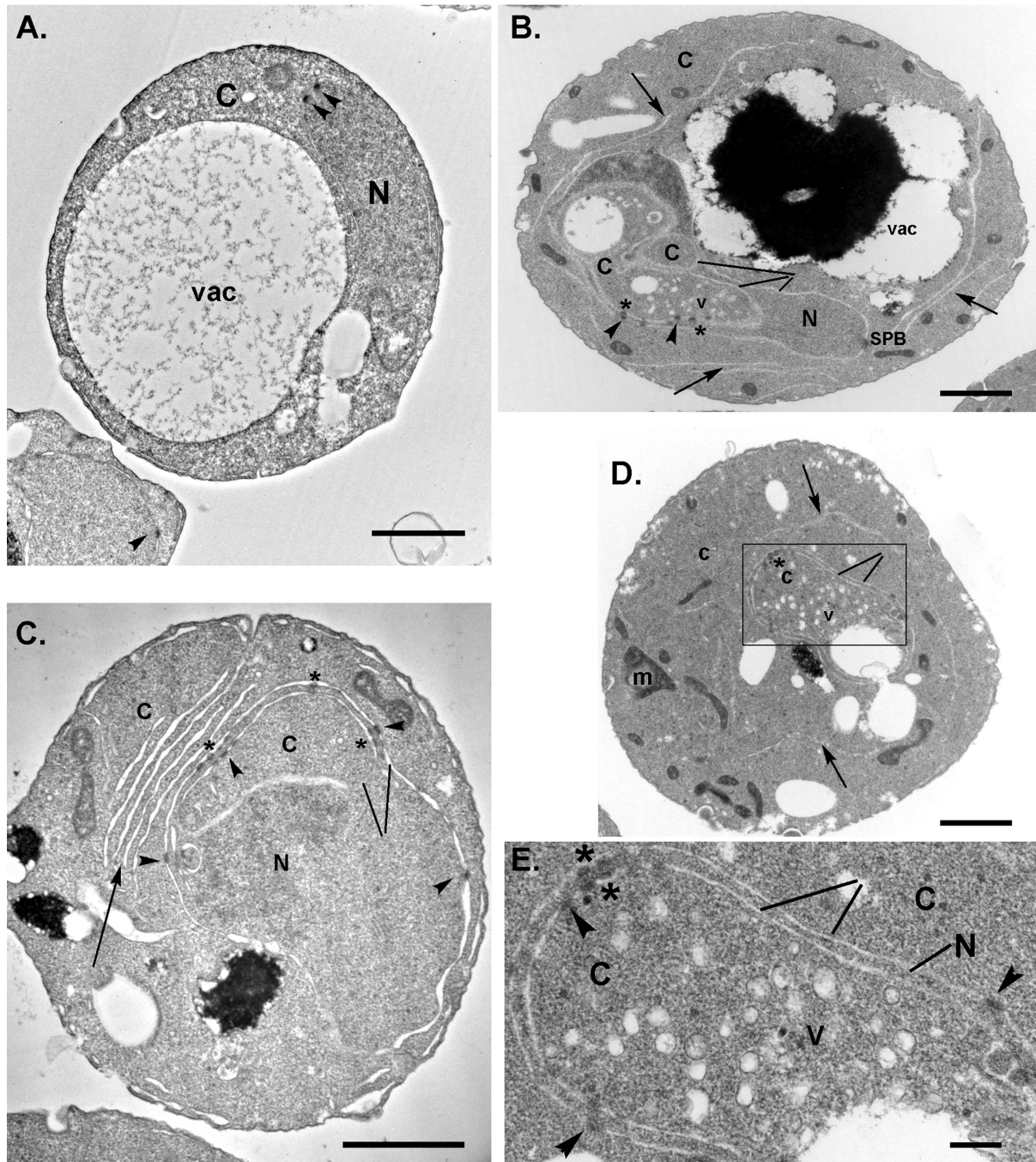


Figure 6. Loss of Kap95 leads to disruption of cellular membrane architecture. Cells in early log phase for (A) the parental strain (SWY2089) and (B–E) the corresponding *kap95-E126K GFP-nic96 nup170-GFP* mutant (SWY3561) were shifted to growth at 34°C for 5 h, then processed for thin-section transmission electron microscopy. Arrowheads denote NPCs that span the NE, asterisks (*) mark areas where NPC-like structures appear restricted to the INM in the *kap95-E126K* mutant. Lines point to NE; arrows point to stretches of cytoplasmic (nonnuclear) membranes. N, nucleus; C, cytoplasm; v, vesicles; m, mitochondria; SPB, spindle pole body; vac, vacuole. Bars, (A–D) 1 μ m; (E) 200 nm.

have shown that the N-terminal 44 amino acids of the vertebrate importin β are necessary for RanGTP binding (Kutay *et al.*, 1997). In *in vitro* import assays, the importin β lacking RanGTP binding acts as a dominant negative, presumably because of the lack of recycling importin α (Kutay *et al.*, 1997). Here, sequence-encoding residues 49–861 of Kap95 was fused in frame with that of GST and placed under the control of the inducible *GAL10* promoter in a multicopy plasmid. The cell growth and GFP-nup localization for *GFP-*

nic96 nup170-GFP cells harboring the *GAL-GST-kap95 Δ 48* plasmid were compared with that for cells harboring a wild-type *GAL-KAP95* plasmid. Induction of *GAL-GST-kap95 Δ 48* expression resulted in a lethal phenotype when cells were grown on media with galactose as the carbon source (data not shown). Moreover, GFP-Nup localization was markedly altered in the galactose-induced *GAL-GST-kap95 Δ 48* cells (Figure 7B). The GFP-Nup signal at the nuclear rim was diminished and cytoplasmic foci were prevalent. Thus, the

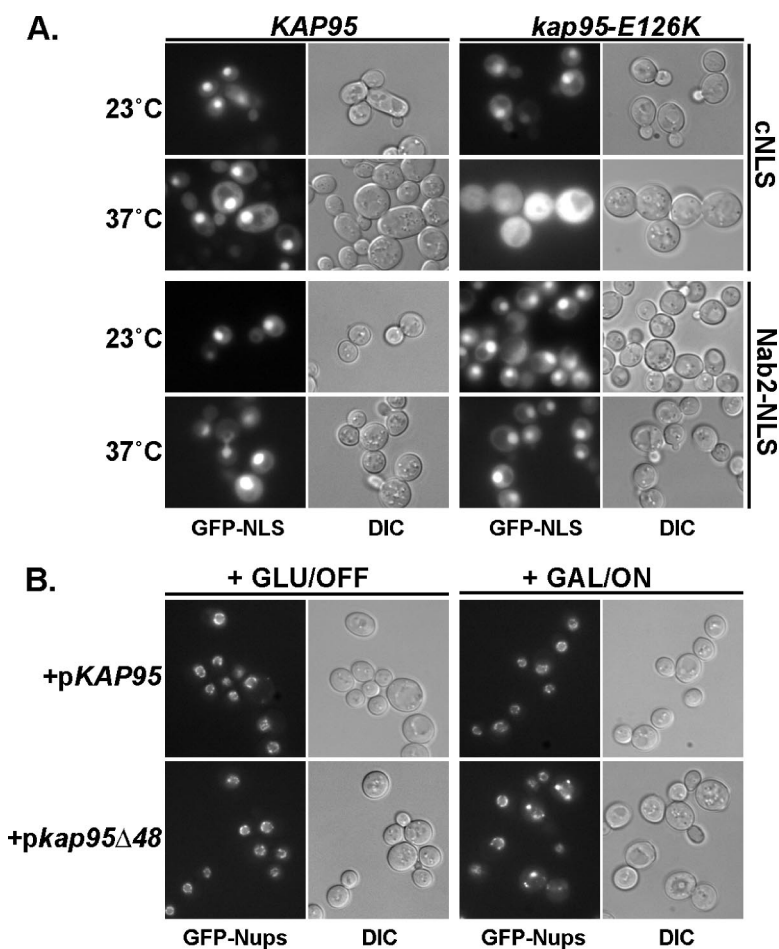


Figure 7. The role for Kap95 in NPC assembly is linked to a Kap95-RanGTP interaction. (A) Nuclear transport is not globally perturbed in the *kap95-E126K* mutant. The effects of the *kap95-E126K* mutant on nuclear import via the Kap95 pathway versus the Kap104 pathway were assayed using GFP reporters in wild-type (SWY518) and *kap95-E126K* (SWY3562) cells. cNLS-GFP (Kap95 pathway) and Nab2-NLS-GFP (Kap104 pathway) fusions were visualized by direct fluorescence microscopy after shifting to growth at 37°C for 6 h. (B) The *kap95Δ48* dominant mutant perturbs GFP-nup localization. Wild-type cells expressing *GFP-nic96* and *nup170-GFP* (SWY2090) transformed with the wild-type p*GAL-KAP95* (pSW3067) or dominant p*GAL-GST-kap95Δ48* (pSW1307) plasmids were grown to early log phase in SC media lacking tryptophan and supplemented with 2% glucose then shifted to 2% galactose media for 20 h. Direct fluorescence microscopy was used to detect the GFP-Nups. DIC shows overall cell morphology.

kap95Δ48 mutant lacking the RanGTP-binding domain results in dominant negative effects on Nup localization. This links the roles for Kap95 and RanGTP in NPC assembly.

DISCUSSION

Here, we have shown that Kap95, the yeast orthologue of importin β , is required for normal nuclear architecture and cellular membrane dynamics. When Kap95 levels decrease, cells have a highly invaginated NE and accumulate extended sheets of cytoplasmic membranes. These membrane perturbations are distinct from the cytoplasmic honeycomb-like appearance of membranes found in the *npa* mutants that block ER-to-Golgi transport and indirectly effect Nup localization and NE morphology (Ryan and Wenthe, 2002). Instead, the membrane defects in *kap95-E126K* cells are more similar to the annulate lamellae and excess NE observed when RanGTP is added to or importin β is depleted from in vitro *Xenopus* nuclear reassembly assays (Harel *et al.*, 2003a; Walther *et al.*, 2003b). Our results suggest that, just as in mitotic nuclear reassembly, Kap95 serves to regulate membrane fusion to shape an intact NE. Because these effects are observed in the absence of NE breakdown in yeast, they raise the interesting possibility that importin β may also have a role in NE dynamics during interphase in metazoan cells.

At the molecular level, Ran functions by modulating interactions between Kaps and other proteins. Previously, we have shown that disrupting the Ran GTPase cycle inhibits

NPC assembly and results in the cytoplasmic accumulation of distinct Nup containing vesicles (Ryan *et al.*, 2003). We proposed that these vesicles target a subset of Nups and NPC assembly factors to the ONM where RanGTP is required for their fusion to the NE. Significantly, the long stretches of nonnuclear membranes that are observed in the *kap95-E126K* loss-of-function mutant directly contrast with the cytoplasmic vesicles that accumulate in the Ran GTPase cycle mutants. We conclude that Kap95 is not simply regulating the cellular levels of RanGTP. Instead, the Kap95-RanGTP function in NPC assembly occurs by an independent mechanism. Overall, our results indicate that Kap95 and Ran have antagonistic effects on membrane dynamics. With these results, we have now refined our model for NPC assembly to include an inhibitory role for Kap95 in the fusion of the novel, Nup-containing vesicles (Figure 8).

We speculate that cytoplasmic Kap95 normally inhibits the fusion of these vesicles until they reach the NE where a highly localized pool of RanGTP binds and sequesters Kap95 (Figure 8A). This would allow specific vesicular fusion to the ONM. In the absence of Kap95, vesicular fusion would not be spatially regulated (Figure 8C). The lack of spatial regulation would result in the indiscriminate fusion of these vesicles into extended sheets of cytoplasmic membranes and the failure to correctly target Nups and other assembly factors to the NE for NPC assembly. Consistent with this hypothesis, both the membrane and Nup mislocalization phenotypes are seen when Kap95 levels decrease in either the *kap95-E126K* or *kap95Δ* mutant backgrounds. In

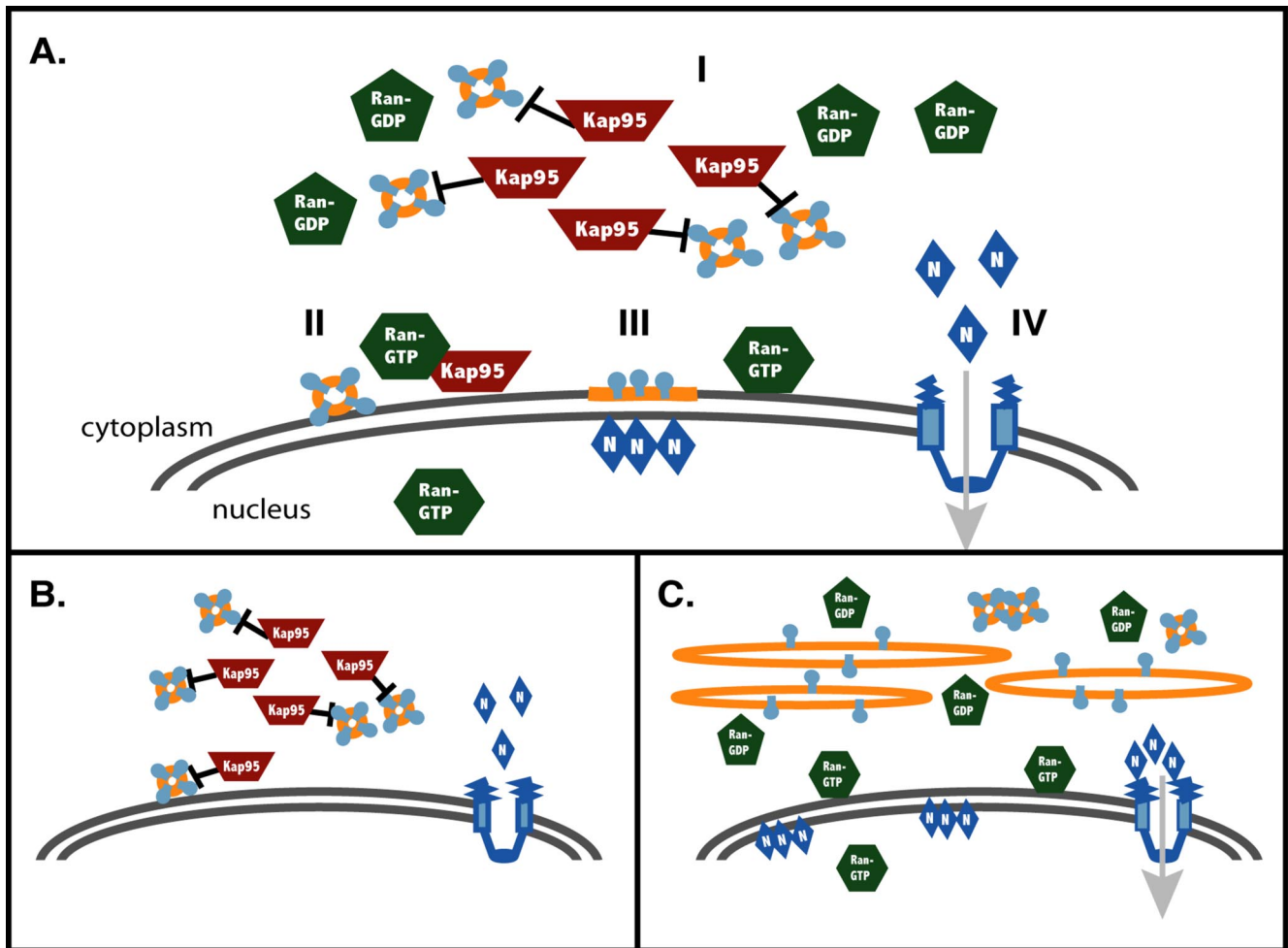


Figure 8. Model for the spatial regulation of NPC assembly. (A) In wild-type cells, Kap95 inhibits vesicle intermediates from fusing in the cytoplasm where RanGDP concentrations are high (I). Near the ONM, binding of RanGTP to Kap95 relieves the inhibitory effect and the assembly vesicle can fuse to the ONM (II). This fusion event provides a concentration of NPC assembly factors at the ONM that is able to recruit additional proteins (designated by N-labeled diamonds) to the INM for NPC assembly from both the nuclear and cytoplasmic faces of the NE (III). Such nuclear assembly factors would be imported through existing NPCs (IV). (B) When the Ran GTPase cycle is disrupted, Kap95 continues to block vesicle fusion even at the NE, and the vesicle intermediates accumulate in the cytoplasm. Factors that would normally be transported into the nucleus also remain in the cytoplasm as nucleocytoplasmic transport becomes disrupted with perturbations of the Ran GTPase cycle. (C) The absence of Kap95 allows for the unregulated cytoplasmic fusion of assembly vesicles to form membrane sheets. This results in a failure to target necessary assembly factors to the ONM. Because nuclear import can still occur, nuclear assembly factors concentrate at the INM and are not available for assembly into the cytoplasmic membranes.

addition, the dominant *kap95 Δ 48* mutant also resulted in GFP-nup perturbations. Although necessary, the loss of Kap95 does not appear to be sufficient for fusion, because vesicles still accumulate in the cytoplasmic invaginations. This suggests that additional fusion machinery, which is not trapped in the invaginations, is required for efficient vesicle fusion. The *kap95 Δ 48* mutant could be acting by sequestering such an assembly factor that needs to be released by RanGTP binding to Kap95. Thus, Kap95 could inhibit fusion through interactions with vesicle proteins or cytoplasmic fusion proteins.

Although a vesicle intermediate during interphase NPC assembly may seem counterintuitive, such an intermediate could be important in specifically targeting NPC assembly to the NE. The annulate lamellae found in oocytes, early embryonic cells, and other rapidly proliferating cells indicate that NPC assembly is not restricted to the NE (Kessel, 1992). However, the rapid proliferation of these cell types means NPCs are continually disassembled during the inherent di-

vision cycles and can be reformed in the NE or new annulate lamellae during postmitotic nuclear reassembly (Stafstrom and Staehelin, 1984; Cordes *et al.*, 1996). Postmitotic cells or cells undergoing a closed mitosis do not have this alternative assembly opportunity and may require that the location of NPC assembly be more tightly regulated. Assembly vesicles would allow essential factors to be concentrated and then removed from membranes to prevent the nucleation of NPCs at nonnuclear membranes. Interactions between Ran and Kap95 at the ONM would provide the critical spatial information for subsequent vesicle fusion and allow NPCs to assemble only in the NE.

Unlike the annulate lamellae formed in *in vitro* *Xenopus* extracts by sequestration of importin β (Harel *et al.*, 2003a; Walther *et al.*, 2003b), the cytoplasmic membranes in *kap95-E126K* or *kap95 Δ* yeast cells do not contain electron dense NPCs structures. A long-standing model for NPC assembly has been that it occurs from both the nuclear and cytoplasmic faces of the NE (Macaulay and Forbes, 1996), and recent

studies have confirmed this model (D'Angelo *et al.*, 2006). During assembly into a closed NE, access of these Nups to the INM could occur by transport through existing NPCs. This has been demonstrated in studies of Nup53 import by Kap121 (Marelli *et al.*, 2001; Lusk *et al.*, 2002). Further, with an intact NE, nuclear import of factors required at INM would make them unavailable for assembly into annulate lamellae in the cytoplasm. This may account for the lack of NPCs structures in the cytoplasmic membranes found in *kap95-E126K* cells. Moreover, the presence of electron dense plaques at the INM when Kap95 is depleted (Figure 6, asterisks) may reflect the accumulation of such INM/NPC assembly components.

Although nuclear import is most likely required for continued NPC assembly during interphase, defects in nuclear import alone cannot fully account for the perturbations in the *kap95-E126K* mutant. Indeed, global nuclear import is not perturbed in the *kap95-E126K* mutant. If defective import were the sole cause, then inhibiting nuclear import by loss of either Ran or Kap95 function should result in the same phenotypes. However, the Ran GTPase cycle mutants and *kap95* loss-of-function mutants have opposite effects on membrane morphology, which are not the consequence of altering overall Ran levels. This is compelling evidence for additional, cytoplasmic roles for Ran and Kap95 during NPC assembly into a closed NE. Furthermore, Ran action in the cytoplasm is consistent with published in vitro nuclear assembly and growth results (Harel *et al.*, 2003a; Walther *et al.*, 2003b; D'Angelo *et al.*, 2006). During the assembly process, two distinct steps are inhibited by GTP γ S (Macaulay and Forbes, 1996). The first is the fusion of chromatin bound vesicles to form a closed NE, which has been shown to be Ran-dependent (Hetzer *et al.*, 2000). In addition, GTP γ S independently blocks the assembly of NPCs into the closed NE (Macaulay and Forbes, 1996), which is a common step in both postmitotic and interphase assembly. This block can be rescued with the addition of fresh extract to the cytoplasmic face of the NE (Macaulay and Forbes, 1996). Addition of importin β also inhibits NPC assembly at this stage (Harel *et al.*, 2003a; Walther *et al.*, 2003b). Continued insertion of NPCs into a growing nucleus was also found to require a balance of importin β and RanGTP at the ONM (D'Angelo *et al.*, 2006). We hypothesize that the *kap95-E126K* mutant identified in the *npa* screen is blocked in the assembly mechanism at the stage before fusion of the inner and outer nuclear membranes is triggered (Figure 8C).

Both Nic96 and Nup170 are highly abundant Nups implicated in NPC assembly and structure (Grandi *et al.*, 1993; Aitchison *et al.*, 1995; Zabel *et al.*, 1996; Marelli *et al.*, 1998); however, neither contains a characteristic FG-repeat domain necessary for karyopherin binding during nuclear import or export through the NPC (Bednenko *et al.*, 2003). Therefore, we were surprised to find that the severity of the ts phenotype of *kap95-E126K* strains was dependent on *NIC96* and *NUP170* alleles. Both *GFP-nic96* and *nup170-GFP* were able to independently exacerbate the temperature sensitivity of *kap95-E126K*. In contrast, *GFP-nup49 Δ N*, a mutant allele of a *FG-NUP*, did not enhance the temperature sensitivity of *kap95-E126K*. Others have demonstrated that efficient targeting of Nup53 to the NPC requires another karyopherin, Kap121. This targeting is dependent on a Kap121-binding domain in a non-FG region of Nup53 (Lusk *et al.*, 2002). It is also interesting to note that a form of importin β with decreased affinity for FG nup binding, importin β I178D, is still able to inhibit NPC assembly in the *Xenopus* system (Walther *et al.*, 2003b). These results indicate that Kap95 may interface with a set of factors during NPC assembly that are

distinct from those that it interacts with for NPC translocation.

Because of the asymmetric distribution of Ran regulatory factors, accepted models have restricted interactions between RanGTP and Kaps to either the nucleus during interphase or near chromatin during an open mitosis. Thus, the cytoplasmic effects of our Ran GTPase cycle mutants were unexpected (Ryan *et al.*, 2003). However, recent in vitro studies using *Xenopus* extracts support our model for a cytoplasmic function of RanGTP in NPC assembly into an intact NE (D'Angelo *et al.*, 2006). Furthermore, Hetzer and coworkers found that RanGTP and importin β had opposite effects on the assembly processes in vitro (D'Angelo *et al.*, 2006). Now, our in vivo characterization of the *kap95-E126K* mutant provides a true parallel to these in vitro results. Exactly how RanGTP is generated at the ONM remains unclear. Because Ran is only 25 kDa in size and within the diffusion limit for the NPC, diffusion of nuclear RanGTP across the NPC may produce a highly localized region of RanGTP at the ONM. Alternatively, localization of Ran regulatory factors to the ONM may establish the correct, local Ran environment. Significantly, these in vitro and in vivo functional requirements for Ran and Kap95 (importin β) during interphase NPC assembly have been discovered in organisms with very distinct nuclear architecture and dynamics (*Xenopus* and budding yeast). We predict that the mechanism for NPC assembly into intact NEs will be conserved in all eukaryotes.

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