The Three Fungal Transmembrane Nuclear Pore Complex Proteins of *Aspergillus nidulans* Are Dispensable in the Presence of an Intact An-Nup84-120 Complex

Hui-Lin Liu, Colin P.C. De Souza, Aysha H. Osmani, and Stephen A. Osmani

Department of Molecular Genetics, The Ohio State University, Columbus, OH 43210

Submitted June 20, 2008; Revised October 20, 2008; Accepted November 10, 2008
Monitoring Editor: Reid Gilmore

In *Aspergillus nidulans* nuclear pore complexes (NPCs) undergo partial mitotic disassembly such that 12 NPC proteins (Nups) form a core structure anchored across the nuclear envelope (NE). To investigate how the NPC core is maintained, we affinity purified the major core An-Nup84-120 complex and identified two new fungal Nups, An-Nup37 and An-ELYS, previously thought to be vertebrate specific. During mitosis the An-Nup84-120 complex locates to the NE and spindle pole bodies but, unlike vertebrate cells, does not concentrate at kinetochores. We find that mutants lacking individual An-Nup84-120 components are sensitive to the membrane destabilizer benzyl alcohol (BA) and high temperature. Although such mutants display no defects in mitotic spindle formation, they undergo mitotic specific disassembly of the NPC core and transient aggregation of the mitotic NE, suggesting the An-Nup84-120 complex might function with membrane. Supporting this, we show cells devoid of all known fungal transmembrane Nups (An-Ndc1, An-Pom152, and An-Pom34) are viable but that An-*ndc1* deletion combined with deletion of individual An-Nup84-120 components is either lethal or causes sensitivity to treatments expected to destabilize membrane. Therefore, the An-Nup84-120 complex performs roles, perhaps at the NPC membrane as proposed previously, that become essential without the An-Ndc1 transmembrane Nup.

INTRODUCTION

Nuclear pore complexes (NPCs) form regulated aqueous channels in the nuclear envelope (NE) allowing the transport of macromolecules, such as RNA and proteins, across the NE. NPCs are massive multiprotein assemblies composed of ~30 nucleoporins (Nups) with an estimated molecular mass of 50–125 MDa (Reichelt *et al.*, 1990; Rout *et al.*, 2000; Alber *et al.*, 2007b). To maintain this structure across the NE, cells need to insert and anchor NPCs into the phospholipid bilayer of the NE as well as stabilize the curved membrane encompassing the overall NPC structure (Devos *et al.*, 2004). Although the fundamental architecture of the NPC is conserved among all eukaryotes (Akey and Radermacher, 1993; Yang *et al.*, 1998; Beck *et al.*, 2007), the composition and dynamics of NPCs varies between lower and higher eukaryotes, particularly during mitosis (Vasu and Forbes, 2001; Tran and Went, 2006). For example, NPCs undergo a cycle of complete disassembly and reassembly during vertebrate open mitosis (Dultz *et al.*, 2008), whereas during the closed mitosis of *Saccharomyces cerevisiae*, NPCs remain intact (Makhnevych *et al.*, 2003). In addition, during the partially open mitosis of *Aspergillus nidulans*, peripheral Nups disassemble from a core NPC structure, which remains in the NE (De Souza *et al.*, 2004; Osmani *et al.*, 2006a; De Souza and Osmani, 2007). Despite these differences, the basic principles involved in maintaining the NPC structure across the NE is expected to be evolutionarily conserved.

The insertion and anchoring of NPCs into the NE membrane is thought to involve transmembrane Nups. Interestingly, transmembrane Nups are not highly conserved and only three transmembrane Nups have been identified in any system so far studied, Pom34, Pom152, and Ndc1 in budding yeast (Wozniak *et al.*, 1994; Chial *et al.*, 1998; Rout *et al.*, 2000; Madrid *et al.*, 2006) and Gp210, Pom121, and Ndc1 in vertebrates and *Caenorhabditis elegans* (Gerace *et al.*, 1982; Hallberg *et al.*, 1993; Mansfeld *et al.*, 2006; Stavru *et al.*, 2006a,b). Of these, Ndc1 is the only transmembrane Nup conserved between yeasts and vertebrates. Ndc1 is essential in yeasts and is required for the duplication and NE insertion of spindle pole bodies (SPBs) (Winey *et al.*, 1993; West *et al.*, 1998). Ndc1 has also been shown to play roles in targeting nonmembrane Nups to NPCs (Lau *et al.*, 2004) in a manner that is partially redundant with Pom152 (Madrid *et al.*, 2006). However, Ndc1 is not essential in either *C. elegans* (Stavru *et al.*, 2006a) or *A. nidulans* (Osmani *et al.*, 2006a). It is thought that functional redundancies between transmembrane Nups allow organisms to survive when individual transmembrane Nups are deleted (Madrid *et al.*, 2006; Stavru *et al.*, 2006a,b).

In addition to transmembrane Nups, the highly conserved major core subcomplex of the NPC, referred to as yNup84 in *S. cerevisiae* and vNup107-160 in vertebrates, has been proposed to play roles in the establishment and maintenance of nuclear pore membrane curvature (Devos *et al.*, 2004). Components of the yNup84 complex and components of vesicle coat complexes share a unique combination of domain structures, suggesting that yNup84 components functionally interact with the NPC membrane (Devos *et al.*, 2004). Notably, Drin *et al.*, 2007 have shown that the N-terminal domain of Nup133, a component of the vNup107-160 complex, can bind to in vitro-synthesized liposomes in a size-dependent manner. This suggests that the yNup84/vNup107-160 com-

This article was published online ahead of print in *MBC in Press* (<http://www.molbiolcell.org/cgi/doi/10.1091/mbc.E08-06-0628>) on November 19, 2008.

Address correspondence to: Stephen A. Osmani (osmani.2@osu.edu).

plex might be able to directly interact with the nuclear membrane. In support for this concept, mutations in yNup84 components cause clustering of NPCs within the plane of the NE (Siniossoglou *et al.*, 1996; Bai *et al.*, 2004). Additionally, genetic analysis in budding yeast has identified synthetic lethal interactions between null alleles of the yNup84 components (*nup120* or *nup133*) and null alleles of *pom152* or *pom34* (Loeillet *et al.*, 2005; Miao *et al.*, 2006). Such genetic interactions suggest that the yNup84 complex might be functionally linked to the transmembrane Nups in a manner critical for NPC structure.

During vertebrate open mitosis the vNup107-160 complex disassembles from NPCs but partially locates to mitotic kinetochores (Belgareh *et al.*, 2001; Loiodice *et al.*, 2004) and spindle poles (Orjalo *et al.*, 2006). As cells exit mitosis, the vNup107-160 complex is involved in the early stages of NPC and nuclear envelope reassembly and is thought to form a prepore structure to which other Nups are then recruited (Belgareh *et al.*, 2001; Harel *et al.*, 2003; Walther *et al.*, 2003; Gillespie *et al.*, 2007; Dultz *et al.*, 2008; Rasala *et al.*, 2008). We have demonstrated previously that during the partially open mitosis of *A. nidulans*, a similar prepore structure containing An-Nup84-120 complex (equivalent to the *S. cerevisiae* yNup84 and vertebrate vNup107-160 complex) components exists throughout mitosis even though 14 peripheral Nups mitotically disassemble from NPCs (De Souza *et al.*, 2004; Osmani *et al.*, 2006a). In addition to the An-Nup84-120 complex, two transmembrane Nups, An-Ndc1 and An-Pom152, together with An-Nup170 and An-Gle1, remain at the NE throughout *A. nidulans* mitosis (De Souza *et al.*, 2004; Osmani *et al.*, 2006a). However, as in other organisms (Antonin and Mattaj, 2005; Antonin *et al.*, 2008), the relationship between the An-Nup84-120 complex and transmembrane Nups is not well understood.

Although the Nup84-120 complex is conserved among eukaryotes, its composition varies between yeasts and vertebrates (Vasu and Forbes, 2001). The vertebrate Nup107-160 complex contains three additional Nups, Nup37, Nup43, and ELYS, which are not present in yeasts that undergo closed mitosis (Loiodice *et al.*, 2004; Rasala *et al.*, 2006). Although we previously identified most components of the An-Nup84-120 complex based on sequence similarities, because some nucleoporins have very low primary sequence conservation, the NPC component inventory of *A. nidulans* was not complete. Here, using affinity purification of the *A. nidulans* Nup84-120 complex, we identify two new fungal Nups encoding the *A. nidulans* orthologues of Nup37 and ELYS, which were thought previously to be vertebrate specific. We show that these proteins, as well as others in the An-Nup84-120 complex, affect the mitotic NPC behavior and interact functionally with transmembrane Nups, particularly An-Ndc1. We have also identified the third *A. nidulans* transmembrane Nup, An-Pom34. Most significantly we show that all three fungal transmembrane Nups are dispensable as long as the An-Nup84-120 complex is complete.

MATERIALS AND METHODS

General Techniques, Strains, and Plasmids

Media, general techniques for culture of *A. nidulans*, Western analysis, transformation, immunofluorescence, 4,6-diamidino-2-phenylindole (DAPI) staining, and confocal microscopy imaging were as described previously (Osmani *et al.*, 2006a). Gene deletions, endogenous green fluorescent protein (GFP) tagging and mCherry tagging (chRFP) were completed as described using fusion polymerase chain reaction (PCR) and Ku70-deleted strains (Yang *et al.*, 2004; Szewczyk *et al.*, 2006) except that newly generated central cassette plasmids were used. Plasmid pHL81 contains the GA5-S-Tag::pyrG^{AF} targeting cassette. Plasmid pHL82 contains GA5-GFP::riboB^{AF}. pHL83 contains

GA5-GFP::loxp-pyrG^{AF}-loxp. pHL84 contains GA5-GFP::pyroA^{AF}. pHL85 contains GA5-chRFP::pyroA^{AF}. All constructs are designed such that one set of fusion PCR primers can be used to generate any of the replacement constructs and using the shared general primers previously as described for pFNO3 (Yang *et al.*, 2004). All constructs will be made available to the community at the Fungal Genetics Stock Center (<http://www.fgsc.net/>). The GFP-tagged versions of An-Nup37 and An-ELYS are functional as Δ An-nup37+An-ELYS-GFP and Δ An-elys+An-Nup37-GFP strains (Δ indicates null alleles) are not temperature sensitive, whereas Δ An-nup37 Δ An-elys strains are temperature sensitive (Supplemental Figure S2). Strains used in this study are listed in Supplemental Table S1.

Protein Extraction, S-Tag Affinity Purification, and Mass Spectrometry

The S-Tag has been demonstrated to be an excellent fusion peptide for affinity purification of proteins (Kim and Raines, 1993). We have developed an efficient S-Tag affinity purification protocol in *A. nidulans* to purify endogenously S-Tagged proteins in this study. Briefly, cells (2×10^6 spores/ml) were grown in 1 l of YG medium (0.5% yeast extract, 1% glucose, and 10 mM MgSO₄) at 28°C overnight until the packed cell volume reached 0.3–0.4 ml per 10 ml culture in 15-ml conical tubes. Cells were harvested by filtering through miracloth (catalog no. 475855; Calbiochem, San Diego, CA) in a Buchner Flask and washed with 100 ml of ice-cold stop buffer (0.9% NaCl, 1 mM NaN₃, 10 mM EDTA, and 50 mM NaF). The cell mat was immediately frozen in liquid nitrogen and lyophilized overnight. One liter of culture generates 0.7–1 g of dried cell mass. Protein extracts were prepared from lyophilized cells by adding ice-cold HK buffer (Osmani *et al.*, 1991) (all HK buffer contained 300 mM NaCl) to dry cell material that had been ground to a fine powder in a mortar and pestle at room temperature (1.3 ml of HK buffer/0.1g dry cell mass). After brief mixing, cell debris was removed by centrifugation at 21,000 rpm, 4°C, for 30 min in a Beckman Avanti-Je centrifuge and 300 μ l S-Protein agarose slurry (150 μ l of packed bead volume; cat. no. 69704-4, Novagen, Madison, WI) was added to 100 mg of total protein and incubated in an ice package on a rocker for 2 h. The beads were washed with 10 ml of HK buffer six times. Samples were run on 10% SDS-polyacrylamide gel electrophoresis (PAGE). After electrophoresis, gels were fixed with 50% ethanol/10% acetic acid overnight. Proteins were visualized with Bio-Safe Coomassie (Bio-Rad, Hercules, CA). Mass spectrometry analysis was done at The Ohio State University Campus Chemical Instrument Center. Protein bands within gels were trimmed and digested with trypsin. Eluted peptides were analyzed using capillary-liquid chromatography-nanospray tandem mass spectrometry.

Benzyl Alcohol (BA) Sensitivity

Spores were spread evenly on MAG (rich media) and 0.15% BA/MAG plates (20–40 spores per plate). Cells on MAG were incubated at 36 and 42°C for 48 h, whereas cells on 0.15% BA/MAG plates were incubated at 36°C for 70 h to allow the wild-type control to reach a comparable size. Diameters of well separated colonies were measured and normalized to wild type (100%) to quantitate tolerance to BA.

Image Acquisition and Analysis

All confocal images are maximal intensity projections unless indicated. Representative images of at least 13 nuclei followed by live cell imaging are shown. Mitotic stages of cells are aligned according to the onset of anaphase. For Figure 4A, relative grayscale levels were measured along the indicated cross section using ImageQuant software (PerkinElmer Life and Analytical Sciences, Boston, MA). For Figures 4C and 6G and Supplemental Figure S3, net fluorescence intensities of interphase and mitotic nuclei ($n = 25$ each) defined by DAPI staining were quantified from cells treated with 2.4 μ g/ml benomyl. The degree of mitotic dispersal of GFP-tagged Nups was normalized using the net fluorescence of interphase nuclei as 100%. All error bars in the figures represent standard deviations.

Southern Blot Analysis of the Triple Transmembrane Nup-deleted Cells

Genomic DNA of cells with the indicated genetic background (Figure 7D) were digested with EcoRI and probed with the coding regions of An-pom34, An-pom152, An-ndc1, and An-histone H1. Histone H1 probe was used as a control. All four probes were used in the first panel of Figure 7D, whereas two probes were used in the second and the third panels to distinguish An-ndc1 and An-pom34 deletions.

RESULTS

Identification of Two New Nups via Affinity Purification of *A. nidulans* SonBc^{Nup96}

Using affinity purification and mass spectrometric analysis of endogenously S-Tagged SonBc^{Nup96}, we consistently purified SonBc^{Nup96}, An-Nup85, An-Nup120, An-Sec13, and

Figure 1. Identification of two novel proteins of the An-Nup84-120 complex. (A) Endogenously S-Tagged SonBc^{Nup96} was affinity purified and resolved using SDS-PAGE. Proteins within the major bands visualized by Coomassie Blue staining were identified using mass spectrometric analysis. Protein identifications are as indicated. d, degradation products. (B) Size and domains of An-Nup37 and An-ELYS are indicated. A WD40-like domain was found in An-Nup37 (see text). (C) Homologues of An-Nup37 and An-ELYS are conserved in filamentous fungi but are absent in *S. cerevisiae*. n.d., not detected. *, Mans *et al.* (2004) detected similarity between vertebrate Nup37 and *S. pombe* SPAC4F10.18. ^s, predicted gene structure of Afu2g02160 is incorrect. Our predicted length of the An-ELYS homologue in *A. fumigatus* is indicated. (D) PSI-BLAST analysis indicates that full-length An-ELYS is similar to part of mammalian ELYS (5E-94, ENSP 0000354780, amino acids 681–988), but not to *C. elegans* MEL-28. The figure is modeled after Galy *et al.* (2006).

two new Nups we termed An-Nup37 (AN2773.3) and An-ELYS (AN4596.3) (Figures 1 and 2). From cumulative experiments, we obtained 39–79% mass spectroscopy sequence coverage for each of these proteins (Table 1). Neither An-Nup37 nor An-ELYS orthologues exist in *Saccharomyces cerevisiae*, but they are conserved in filamentous fungi, and, more weakly, in *Schizosaccharomyces pombe* (Figure 1C). Weak homology (Zdobnov and Apweiler, 2001) to the WD40 motif was identified in An-Nup37, but no domains were found in An-ELYS (Figure 1B). Mans *et al.* (2004) previously detected weak similarity between vertebrate Nup37 and An-Nup37.

The protein in vertebrates with highest similarity to An-ELYS is vELYS (Mans, personal communication). Surpris-

ingly, similarity between An-ELYS and vELYS (Rasala *et al.*, 2006) is restricted to a region that is not present in MEL-28 the *C. elegans* orthologue of ELYS (Fernandez and Piano, 2006; Galy *et al.*, 2006) (Figure 1D). Furthermore, no MEL-28 homologues can be detected in *A. nidulans*. In addition, no proteins similar to An-ELYS, or the region of vELYS with similarity to An-ELYS, could be detected in *C. elegans*.

An-Nup37 and An-ELYS Are Components of the An-Nup84-120 Complex

Both An-Nup37 and An-ELYS were endogenously S-Tagged and affinity purified. The same set of proteins were purified as those in association with SonBc^{Nup96} (Figure 2A and Supplemental Figure S1). We find the An-Nup84-120 com-

Figure 2. An-Nup37 and An-ELYS copurify core An-Nup84-120 complex components. (A) Endogenously S-Tagged nucleoporins, as indicated, were affinity purified. Protein extracts of wild-type strain R153, which does not express an S-Tagged protein, were similarly treated to demonstrate specificity of the purifications. Purified proteins were resolved by SDS-PAGE and the gels stained with Coomassie Blue. Bands 1–11 were identified using mass spectroscopy. Band 1, an-Nup120; band 2, an-Nup85; band 3, SonBc^{Nup96}; band 4, degradation product of An-Nup120; band 5, an-Nup37; band 6, an-Sec13; band 7, an-ELYS; band 8, an-Nup133; band 9, an-Nup84; and bands 10 and 11, degradation product of An-Nup84. Percent mass spectrometric sequence coverage is detailed in Table 1. S-Tagged proteins are labeled with asterisk (*). (B) An-Nup84-120 components are present in An-Nup37-S-Tag pull downs from an *An-nup133*-deleted strain but not an *An-nup120*-deleted strain. An-Nup84-120 components are not present in an An-ELYS-S-Tag pull down from an *An-nup120*-deleted strain.

plex is composed of two very stable subcomplexes, An-Nup84-133 and An-Nup37-85-96-120-ELYS. Moreover, deletion of *An-nup120*, but not *An-nup133*, disrupts the integrity of the complex containing An-Nup120 (Figure 2B). This result is similar to *S. pombe* in which orthologues of Nup84 and Nup133 are purified in a separate complex from the

Nup85-120 complex (Bai *et al.*, 2004). However, by affinity purification of An-Nup37-S-Tag by using highly concentrated (45 mg/ml) protein extracts (normal is ~20 mg/ml) the An-Nup84-120 complex as a whole was purified (Table 1). Unexpectedly, we were unable to detect An-Seh1 in any affinity purifications of An-Nup84-120 components even

Table 1. Summary of proteins identified in the An-Nup84-120 complex affinity purification experiments by LC MS/MS

	Unique peptides identified in the mass spectrometric analysis of S-Tag affinity purified Nups						Total unique peptides (% mass spec. coverage)
	sonBc ^{Nup96a}	ELYS ^a	Sec13 ^a	Nup84 ^a	Nup133 ^a	Nup37 ^{a,b}	
An-Nup84	0	0	0	34	28	25	38 (39)
An-Nup133	0	0	0	25	47	2	50 (50)
An-Nup37	8	37	14	0	0	13	37 (65)
An-ELYS	7	44	6	0	0	5	44 (79)
An-Nup85	12	44	28	0	0	5	44 (55)
An-Nup120	18	71	28	0	0	25	71 (70)
SonBc ^{Nup96}	17	33	18	0	0	14	33 (51)
An-Sec13	0	4	15	0	0	2	15 (55)
An-Sec31	0	0	75	0	0	0	75 (68)
An-Sec16	0	0	29	0	0	0	29 (20)

^a Endogenously S-Tagged Nups.

^b This affinity purification was done using highly concentrated protein extracts (45 mg/ml).

Figure 3. An-Nup37 and An-ELYS are stable NPC core components and are also present on SPBs during mitosis. (A) Endogenously GFP-Tagged An-Nup37 and An-ELYS cells were fixed and stained with DAPI to visualize DNA. A single nonconfocal image is shown. (B–F) The location of endogenously GFP-Tagged Nups as well as SPB and kinetochore markers were followed using live cell spinning disk confocal microscopy. (B) An-ELYS and An-Nup120 colocalize at the NPCs during mitosis. See Supplemental Video 1. (C) An-ELYS remains at NPCs during mitosis when peripheral Nups such as An-Nup49 are dispersed (Supplemental Video 2). (D and E) During mitosis, An-ELYS does not locate to the kinetochores (An-Ndc80) but accumulates at the SPBs (An-Gcp3), which are indicated by arrowheads (Supplemental Video 3). (F) SonBc^{Nup96}, An-Nup37, and An-Ndc1 (Supplemental Videos 5–7) remain at the NPCs throughout the cell cycle and also transiently locate to the SPBs during mitosis (indicated by arrowheads). Bar, 5 μ m.

though Seh1 orthologues are components of the comparable yNup84 and vNup107-160 complexes. An endogenously C-terminally tagged GFP version of An-Seh1 did not locate to NPCs and an S-Tagged version did not copurify with NPC components nor did native An-Seh1 copurify with other Nups.

An-Nup37 and An-ELYS Are Stable Components of the NPC Core Structure and Concentrate at SPBs during Mitosis

To determine whether An-Nup37 and An-ELYS are stable core components of the NPCs, we followed them throughout the cell cycle using functional endogenously GFP-tagged alleles (Supplemental Figure S2). Both An-Nup37-GFP and

An-ELYS-GFP localized around the nuclear periphery in a manner typical of NPC proteins (Figure 3A) and remained at the nuclear periphery throughout mitosis. This is clearly revealed when An-ELYS is compared with the core An-Nup120 (Figure 3B and Supplemental Video 1) or the peripheral An-Nup49 NPC proteins during mitosis (Figure 3C and Supplemental Video 2). We conclude An-Nup37 and An-ELYS are structural components of the NPC and remain as part of the core NPC structure during mitosis. In higher eukaryotes all components of the vNup107-160 NPC sub-complex relocate in part to kinetochores (Belgareh *et al.*, 2001; Loiodice *et al.*, 2004) as well as to the spindle poles (Orjalo *et al.*, 2006) during mitosis. To determine whether a pool of the related Nups locate similarly during *A. nidulans*

mitosis, we followed the localizations of these Nups in relationship to the kinetochore marker An-Ndc80 (Yang *et al.*, 2004). As in *S. pombe* and *S. cerevisiae*, the kinetochores of *A. nidulans* cluster near the spindle pole body (SPB) during interphase (Wigge and Kilmartin, 2001; Yang *et al.*, 2004) (Figure 3D, G2 and G1). During mitotic progression kinetochores no longer associate at the SPBs but become distributed between the separating SPBs on the mitotic spindle (Figure 3D, M and Supplemental Video 3). However, An-ELYS does not concentrate on kinetochores but instead becomes concentrated at the mitotic SPBs, a location confirmed using the SPB marker An-Gcp3 (Figure 3E and Supplemental Video 4). These studies suggest that like vertebrate Nup107-160 components (Orjalo *et al.*, 2006), An-ELYS concentrates at the spindle poles during mitosis (Figure 3E). However, it does not noticeably locate at kinetochores or on the mitotic spindle. This is not specific to An-ELYS but is true for all components of the An-Nup84-120 complex as well as An-Ndc1-GFP (Figures 3, B, E, and F and 4B. Example videos are shown for SonBc^{Nup96} (Supplemental Video 5), An-Nup37 (Supplemental Video 6) and An-Ndc1 (Supplemental Video 7).

An-Nup37 and An-ELYS Are Required to Maintain the Mitotic Location of An-Nup84-120 Components to the NE

Δ An-nup37 and Δ An-elys strains are viable and grow like wild-type strains from 20 to 42°C (Supplemental Figure S2; data not shown). Because An-Nup37 and An-ELYS are non-essential core NPC components we investigated whether either of their deletions had effects on NPCs during the cell cycle. When either An-nup37 or An-elys are deleted, NPC core components were observed to locate around the nuclear periphery in interphase but surprisingly partially dispersed from NPCs during mitosis (Supplemental Figure S3A, compare wild type [Supplemental Video 9] to Δ An-nup37 [Supplemental Video 11] and Δ An-elys [Supplemental Video 12]). This suggests that An-Nup37 and An-ELYS are not required for NPC formation but may be required to maintain the NPC core structure during mitosis when peripheral Nups are dispersed. We investigated the effects of lack of both An-Nup37 and An-ELYS on NPC structure expecting that the double deletion might cause a more dramatic effect as the double mutant displays temperature sensitivity (Figure 6C and Supplemental S2). Importantly, however, these experiments were carried out at 25°C at which temperature the double mutant grew normally. During interphase SonBc^{Nup96}-GFP locates around the nuclear periphery of the double mutant in a manner identical to wild-type strains. However, as cells enter mitosis SonBc^{Nup96}-GFP dispersed throughout the cell (Figure 4A). We have never observed such dispersal in wild-type strains. As nuclei complete mitosis the dispersed SonBc^{Nup96}-GFP returns back to the NPC. This dispersal and return is reflected in the grayscale profile of such dividing nuclei (Figure 4A and Supplemental Video 8). Thus, quite remarkably, when both An-Nup37 and An-ELYS are missing, SonBc^{Nup96}-GFP behaves like a peripheral Nup. Not only is SonBc^{Nup96}-GFP dispersed from the NPC during mitosis in the absence of An-Nup37 and An-ELYS but the other components of the An-Nup84-120 complex also disperse (Figure 4B, compare wild-type [Supplemental Video 9] with Δ An-nup37 Δ An-elys [Supplemental Video 10]). To quantitate the degree of dispersal at a specific mitotic stage, cells were arrested in a pseudometaphase using benomyl to activate the spindle assembly checkpoint. Cells were stained with DAPI to locate nuclei and allow measurement of the amount of GFP-Nups around the nuclear periphery in interphase and mitotically arrested cells. More than 80% of the

An-Nup84-120 complex remains around the nuclear periphery in wild-type mitotic cells, whereas only 10% of the complex remains in Δ An-nup37 Δ An-elys cells (Figure 4C). This remaining 10% is probably an overestimation because nuclei exclude vesicles and mitochondria, leading to an overestimation of the relative concentration of freely dispersed protein within nuclei, as discussed previously (Ovechkina *et al.*, 2003). Mitotic dispersal of the core NPC components is not specific to the absence of An-Nup37 and An-ELYS as deletions of An-Nup133 or An-Nup85 also caused varying degrees of mitotic dispersal of the An-Nup84-120 complex (Supplemental Figure S3B, compare wild type [Supplemental Video 13] with Δ An-nup133 [Supplemental Video 14] and Δ An-nup85 [Supplemental Video 15]).

Spindle Formation Is Not Affected by the Absence of the An-Nup84-120 Complex at the SPB but Mitosis Is Slightly Extended

In Δ An-nup37 Δ An-elys cells the An-Nup84-120 complex fails to locate to the mitotic SPBs (Figure 4B), and it is known that the orthologous complex of vertebrates is required for normal spindle formation (Orjalo *et al.*, 2006). To characterize potential mitotic defects caused by lack of the An-Nup84-120 complex at the SPB, we measured the duration of mitosis by following the mitotic spindle using GFP-tagged tubulin (Ovechkina *et al.*, 2003) and nuclear transport by following NLS-DsRed in Δ An-nup37 Δ An-elys cells (Figure 4, D and E, compare wild-type GFP-tubulin in Supplemental Video 16 with Δ An-nup37 Δ An-elys in Supplemental Video 17 as well as NLS-DsRed in wild type [Supplemental Video 18] to Δ An-nup37 Δ An-elys [Supplemental Video 19]). No major mitotic defects were detected either in the formation or structure of the mitotic spindle, or in the changes in nuclear transport characteristic of *A. nidulans* mitosis. However, the duration of mitosis in the double Δ An-nup37 Δ An-elys mutant was reproducibly slightly longer than the comparable wild-type strain (Figure 4E).

Other Mitotic Defects Caused by the Absence of An-Nup37 and An-ELYS

Because of the dramatic effects caused by the double Δ An-nup37 Δ An-elys mutant on the mitotic behavior of the core subcomplex of the NPC, we wanted to define what other mitotic changes occur in this mutant background. An-Nup2 displays a unique behavior during mitosis moving from the NPC to chromatin and back to the NPC (Figure 5A and Supplemental Video 20). This distribution is not affected by lack of An-Nup37 and An-ELYS (Figure 5A and Supplemental Video 21). Similarly the mitotic behavior of two transmembrane Nups, An-Pom152 (Figure 5B, compare wild type [Supplemental Video 22] to Δ An-nup37 Δ An-elys [Supplemental Video 23]) and An-Ndc1 (Figure 5C, compare wild type [Supplemental Video 7] to Δ An-nup37 Δ An-elys [Supplemental Video 24]), was not markedly perturbed by lack of An-Nup37 and An-ELYS.

In contrast to An-Nup2, An-Pom152, and An-Ndc1, the mitotic behavior of An-Nup170-GFP is modified in Δ An-nup37 Δ An-elys cells. Typically, An-Nup170-GFP locates in a relatively smooth pattern around the nuclear periphery throughout interphase and mitosis. However, in the absence of An-Nup37 and An-ELYS, An-Nup170 becomes more punctate during mitosis (Figure 5F). This difference is particularly clear when comparing the live cell imaging for wild-type Nup170 in Videos 29 to the mutant in Supplemental Video 30.

An-Gle1 shows a smooth pattern around the NE during normal mitotic progression similar to An-Erg24, which

Figure 4.

locates to the NE but also to the ER (Figure 5, D and E). Using An-Gle1-GFP and An-Erg24-GFP, we have demonstrated that during *A. nidulans* mitosis, two nuclei are generated from one by a double restriction of the NE (Ukil, De Souza, Liu, and Osmani, unpublished data). This phenomenon can be seen in Figure 5, D and E, in which the points of restriction and subsequent NE fission are marked by arrowheads. However, in the absence of An-Nup37 and An-ELYS, the mitotic behavior of both An-Gle1-GFP and An-Erg24-GFP are modified and the double NE restriction does not occur normally. Instead a transient aggregation of both An-Gle1-GFP (62% of the nuclei imaged, $n = 13$, and Supplemental Video 26) and An-Erg24-GFP (82% of the nuclei imaged, $n = 61$, and Supplemental Video 28) occurs during mitotic progression in $\Delta\text{An-nup37}\Delta\text{An-elys}$ cells (Figure 5, D and E, arrows), which is not observed in wild-type cells (An-Gle1-GFP, 0%, $n = 13$, Supplemental Video 25; and An-Erg24-GFP, 0% $n = 42$, and Supplemental Video 27).

Together, these data suggest that interaction of the An-Nup84-120 complex with other core *A. nidulans* Nups and the NE is compromised specifically during mitosis if individual An-Nup84-120 complex components are missing.

An-Nup84, An-Nup120 or An-Nup133 Nulls Require the Transmembrane Nup An-Ndc1 for Viability

Because the Nup84-120 complex is predicted to form a membrane coat within the NPC structure (Devos *et al.*, 2004; Hsia *et al.*, 2007), we looked for potential genetic interactions between the transmembrane Nups An-Ndc1 and An-Pom152 and components of the An-Nup84-120 complex. Neither An-Ndc1 nor An-Pom152 is essential (Osmani *et al.*, 2006a). We therefore generated a double $\Delta\text{An-ndc1}\Delta\text{An-pom152}$ strain, which was surprisingly found to be viable and without overt defects (Figure 6A). In contrast to this, individual transmembrane *nup* deletions displayed marked genetic interactions with *An-nup84-120* mutants. First, deletion of *An-pom152* enhanced the temperature sensitivity of the *An-nup84* deletion (Osmani *et al.*, 2006a) to the point where the double mutant was barely viable at 32°C (Figure 6A). In addition, lack of *An-pom152* also increases the tem-

perature sensitivity of the $\Delta\text{An-nup120}$ and $\Delta\text{An-nup133}$ Nup deletions (Figure 6A).

Even more pronounced genetic interactions were uncovered with the *An-ndc1* null allele. Most impressively, $\Delta\text{An-ndc1}$ causes synthetic lethality when combined with either $\Delta\text{An-nup84}$, $\Delta\text{An-nup120}$, or $\Delta\text{An-nup133}$ (Figure 6B). We initially could not generate such double mutants by genetic crosses (crosses were sterile) and therefore individually deleted *An-nup84*, *An-nup120*, or *An-nup133* in a $\Delta\text{An-ndc1}$ background. In each case, we could not recover haploid double mutants but could recover the $\Delta\text{An-nup84}$, $\Delta\text{An-nup120}$, and $\Delta\text{An-nup133}$ alleles through heterokaryon rescue (Figure 6B) (Osmani *et al.*, 2006b), indicating the double mutants cause lethality. When conidia (asexual uninucleated spores) from the heterokaryons were propagated, those with the deleted alleles could not grow (Figure 6B, UU) confirming that deletion of *An-nup84*, *An-nup120*, or *An-nup133* in a $\Delta\text{An-ndc1}$ background causes lethality. Additionally, combining $\Delta\text{An-ndc1}$ with deletions of other components of the An-Nup84-120 complex ($\Delta\text{An-nup85}$, $\Delta\text{An-nup37}$, and $\Delta\text{An-elys}$) resulted in varying degrees of enhanced temperature sensitivity at 42°C (Figure 6C).

Deletion of All An-Nup84-120 Components That Cause Temperature Sensitivity Cause Sensitivity to the Membrane Fluidizer Benzyl Alcohol

To test whether defects caused by deletions of An-Nup84-120 complex components might involve membrane stability, cells were treated with the membrane fluidizer BA. BA has been shown to affect membrane dynamics in vitro and in vivo (Colley and Metcalfe, 1972; Gordon *et al.*, 1980) and has been used previously to investigate membrane fluidity and NPC formation (Scarcelli *et al.*, 2007). We found concentrations of BA above 0.35% are lethal to *A. nidulans*, whereas 0.15% BA is tolerated by wild-type cells at temperatures tested from 25 to 42°C. We then compared the tolerance of wild type and various deletion strains to 0.15% BA at 36°C. The transmembrane *nup* deletions $\Delta\text{An-pom152}$ and $\Delta\text{An-ndc1}$ were not BA sensitive. Interestingly however, the An-Nup84-120 complex mutants which caused temperature sensitivity ($\Delta\text{An-nup84}$, $\Delta\text{An-nup120}$, and $\Delta\text{An-nup133}$) also displayed marked sensitivity to BA at 36°C (Figure 6, C and D). This is not a general feature of temperature sensitive mutations as the *nimA1* and *nimT23* temperature sensitive alleles are not sensitive to BA at 36°C (Figure 6, C and D).

Our data indicate that if deletion of An-Nup84-120 complex components (either singly or in combination) causes temperature sensitivity, it also causes BA sensitivity and results in mitotic-specific dispersal of the remaining core An-Nup84-120 components from the NE. This suggests that these three phenotypes might be linked through a common mechanism perhaps involving membrane stability. Enhanced temperature and BA sensitivity are also phenotypes of the genetic interactions between An-Nup84-120 complex and An-Ndc1. For example $\Delta\text{An-elys}\Delta\text{An-ndc1}$ double mutants display greatly enhanced temperature sensitivity and BA sensitivity (Figure 6C). We therefore wished to determine whether mitotic disassembly of core Nups was also more extensive in such double mutants. We thus followed the distribution of the An-Nup84-120 complex (using An-Nup37-GFP) in various deletion backgrounds. Deletion of *An-ndc1* on its own did not affect An-Nup37 mitotic NPC location in comparison to wild-type cells (Figure 6E and Supplemental Video 31). Noticeably, however, *An-elys* deletion enhanced the mitotic dispersal of An-Nup37 (Figure 6, E and F, and

Figure 4 (cont). The An-Nup84-120 complex disassembles from NPCs during mitosis in $\Delta\text{An-nup37}\Delta\text{An-elys}$ cells at permissive temperature. (A) Endogenously GFP-Tagged SonBc^{Nup96} remains at the NE during wild-type mitosis (Figure 3F) but is reversibly dispersed from the NE during mitosis in a $\Delta\text{An-nup37}\Delta\text{An-elys}$ background (Supplemental Video 8) at 25°C. The dispersal of SonBc^{Nup96} can be seen in the micrographs and is reflected in the pixel profiles through the G2, M, and G1 nucleus. (B) Similarly, as shown, endogenously GFP-tagged An-Nup120, An-Nup85, An-Nup133 (see wild type [Supplemental Video 9] and $\Delta\text{An-nup37}\Delta\text{An-elys}$ [Supplemental Video 10] for comparison), and An-Nup84 remain at the NE during mitosis in wild-type cells but mitotically disperse in a $\Delta\text{An-nup37}\Delta\text{An-elys}$ double-mutant background. (C) Graphs showing nuclear levels of the Nups from the strains indicated in B during interphase (I) or mitosis (M). The remaining GFP-tagged Nup (green) at the NE was quantified by measuring the net fluorescence intensity of interphase and mitotic nuclei defined by DAPI staining (red). For mitotic samples, cells were treated with 2.4 $\mu\text{g}/\text{ml}$ benomyl to arrest cells in a pseudometaphase state. (D and E) GFP-tubA and NLS-DsRed were followed to compare spindle morphology and nuclear transport in wild-type and $\Delta\text{An-nup37}\Delta\text{An-elys}$ cells. The duration of mitosis was measured and plotted in the bar graph shown (E). The time difference between wild-type and $\Delta\text{An-nup37}\Delta\text{An-elys}$ cells is significant ($p < 0.05$ for tubA-GFP and $p < 0.01$ for NLS-DsRed). Data are represented as mean $\pm$ SD. Bar, 5 μm .

Figure 5. Locations of core NPC components are modified in $\Delta An-nup37\Delta An-elys$ cells. (A–F) Endogenously GFP-tagged An-Nup2, An-Pom152, An-Ndc1, An-Gle1, An-Erg24, and An-Nup170 were analyzed in both wild-type and $\Delta An-nup37\Delta An-elys$ cells at 25°C. (A–C) The distribution of An-Nup2 (Supplemental Videos 20 and 21), An-Pom152 (Supplemental Videos 22 and 23), and An-Ndc1 (Supplemental Videos 7 and 24) are similar in wild-type and $\Delta An-nup37\Delta An-elys$ cells. (D) An-Gle1 reveals two restrictions of the NE, which generates two

Supplemental Video 33), whereas cells lacking both An-Ndc1 and An-ELYS, almost completely disassembled An-Nup37 from mitotic NPCs (Figure 6, E and F, and Supplemental Video 34). This phenotype is more severe than the deletion of the two transmembrane Nups An-Ndc1 and An-Pom152, which does not cause temperature sensitivity or BA sensitivity, and caused An-Nup37 to be more punctate but did not greatly affect its location around the nuclear periphery during mitosis (Figure 6E and Supplemental Video 32). Thus, the temperature sensitivity, BA sensitivity and mitotic disassembly phenotypes of An-Nup84-120 complex mutants are shared with mutants having the double $\Delta\text{An-ndc1}\Delta\text{An-elys}$ deletions.

In addition, as An-Nup37 still localized to SPBs in the absence of An-Ndc1, these experiments also demonstrate that the SPB localization of An-Nup84-120 complex components is not dependent on An-Ndc1 which is known to be a component of both NPCs and SPBs (Winey *et al.*, 1993).

Triple Transmembrane Nup-deleted Cells Are Viable

Collectively, the data presented above indicate the An-Pom152 and An-Ndc1 transmembrane Nups are dispensable in the presence of all An-Nup84-120 complex components. However, in all organisms so far studied three transmembrane Nups are present, suggesting the possible existence of a third transmembrane Nup in *A. nidulans*, which might provide the essential transmembrane functions in the double $\Delta\text{An-ndc1}\Delta\text{An-pom152}$ strain. In the Ascomycete fungi, the third known transmembrane Nup is Pom34 (Miao *et al.*, 2006). Mans *et al.* (2004) identified a sequence within chromosome III ANcontig1.161 of the genome sequence of *A. nidulans* encoding a protein related to Pom34. This sequence has subsequently been reannotated as AN11123.3 (http://www.broad.mit.edu/annotation/genome/aspergillus_group/MultiHome.html). This predicted protein contains the Pfam domain 08058 (Finn *et al.*, 2008) characteristic of Pom34 proteins and, like Pom34, has two predicted transmembrane domains (Figure 7A). Endogenously tagged AN11123 protein locates to the nuclear periphery colocalizing with An-Nup49 in interphase (Figure 7B). During a mitotic arrest imposed by spindle assembly checkpoint activation, the peripheral An-Nup49 protein dispersed from NPCs, whereas AN11123 remained at the nuclear periphery indicating it to be a core NPC component. Given the sequence similarities to Pom34, and its localization at the NPC at mitosis, we have named AN11123 An-Pom34.

We deleted An-pom34 from a $\Delta\text{An-ndc1}\Delta\text{An-pom152}$ strain and most surprisingly obtained viable haploid triple mutants. The mutations in this strain were confirmed both by diagnostic PCR (Figure 7C) and by Southern blot analysis by using the coding region of each transmembrane Nup as probe (Figure 7D). Colony growth tests of single, double, and the triple transmembrane Nup mutants at various tem-

peratures (Figure 7E) revealed the $\Delta\text{An-ndc1}\Delta\text{An-pom34}$ double mutant to cause relatively mild cold and heat sensitivity. These defects were more pronounced in the triple mutant. The rate of colony formation was measured over a 5-d period at 32°C and the triple mutant displayed a slightly decreased growth rate (Figure 7F). Analysis of mitosis, following spindle morphology and nuclear transport, failed to reveal any marked defects caused by the triple transmembrane Nup deletions (Figure 7G). We conclude the three fungal transmembrane Nups are not essential in *A. nidulans* when the An-Nup84-120 complex is intact.

DISCUSSION

Nup37 and ELYS Are Not Vertebrate-specific Nups

We have identified two new fungal Nups previously thought to be vertebrate specific. Surprisingly, although both An-ELYS and vELYS were identified as components of an orthologous NPC subcomplex, An-ELYS is much smaller than vELYS and lacks the AT hook motif characteristic of vELYS proteins that play critical roles in initiating NPC assembly on AT-rich chromatin by recruiting Pom121- and Ndc1-containing vesicles (Rasala *et al.*, 2008). Even more surprising is the fact that MEL-28, the *C. elegans* orthologue of vELYS, has no sequence similarity to An-ELYS. This is because the domain within vELYS to which An-ELYS has homology is lacking from MEL-28. This indicates that the domain shared between vELYS and An-ELYS might be sufficient to target these proteins to the vNup107-160/An-Nup84-120 complex and suggests that MEL-28 may not be a component of a similar NPC subcomplex. vELYS has been shown to play a critical role in NPC reassembly (Rasala *et al.*, 2006; Franz *et al.*, 2007; Gillespie *et al.*, 2007; Rasala *et al.*, 2008), whereas our data indicate An-ELYS, although involved, is not absolutely required for NPC reassembly in *A. nidulans*. It is therefore likely, given its increased size and domain complexity, that vELYS has important functions that are not shared with its smaller fungal orthologue.

Peripheral Nups May Help Stabilize the Mitotic NPC Architecture

Both An-Nup37 and An-ELYS are nonessential, similar to numerous other NPC genes in *A. nidulans* and other species. However, deletion of both together causes temperature sensitivity and dramatic changes in the mitotic behavior of the core NPC at permissive temperature (Figure 8). During mitosis, peripheral Nups disperse, whereas the An-Nup84-120 complex remains associated with the NE and is thought to act as a scaffold to which the dispersed Nups return during mitotic exit (Osmani *et al.* 2006a). However, in $\Delta\text{An-nup37}\Delta\text{An-elys}$ cells, the remaining An-Nup84-120 components disperse, along with the peripheral Nups, specifically during mitosis. In essence, the double deletion causes An-Nup84-120 components to act like peripheral Nups (Figure 8). This effect also occurs in other An-Nup84-120 complex mutants, which, similar to $\Delta\text{An-nup37}\Delta\text{An-elys}$ cells, are sensitive to membrane destabilization as well as elevated temperature. These data might be explained if NPC stability is compromised when key An-Nup84-120 complex components are missing, but peripheral Nups stabilize the whole structure during interphase. However, during mitosis, when the peripheral Nups disperse from NPCs, their supporting role in NPC stability is removed causing the An-Nup84-120 complex to also disperse.

Figure 5 (cont). nuclei during wild-type (Supplemental Video 25) mitosis (Ukil, De Souza, Liu, and Osmani, unpublished data) indicated by two arrowheads. In $\Delta\text{An-nup37}\Delta\text{An-elys}$ cells (Supplemental Video 26), the double restrictions were not detected but instead a transient bright focus (indicated by an arrow) was observed. (E) An-Erg24-GFP also reveals the double NE restriction in wild-type mitosis (indicated by two arrowheads, Supplemental Video 27), but in $\Delta\text{An-nup37}\Delta\text{An-elys}$ mitotic cells a bright focus accumulates (indicated by an arrow, Supplemental Video 28). (F) An-Nup170 distribution becomes more punctate during mitosis in $\Delta\text{An-nup37}\Delta\text{An-elys}$ cells (Supplemental Video 30) compared with the wild-type (Video 29) mitotic distribution. Bar, 5 μm .

Figure 6. Interactions between An-Nup84-120 complex components and transmembrane Nups. (A) Cells with the indicated genetic backgrounds were spot inoculated on MAG plates and incubated at 32, 36, and 38 °C for 40 h. The white, green, or yellow color of the colonies is caused by different spore (conidia) colors of the strains, which does not affect growth. Bar, 1 cm. The double $\Delta An-nup84 \Delta An-pom152$ mutations cause synthetic growth defects at all temperatures but can be propagated on 1 M sucrose plates (data not shown). (B) Heterokaryon rescue analysis. After deletion of either *An-nup84*, *An-nup120*, or *An-nup133* in the $\Delta An-ndc1$ background allowed recovery of the double mutants only through heterokaryon rescue (Osmani *et al.*, 2006b). Conidia (asexual uninucleated spores) from the heterokaryons were streaked on selective (–UU) and nonselective (+UU) plates and incubated at 32 °C for 24 h before photography. Results of diagnostic PCR of wild-type cells, heterokaryotic cells grown on selective media, and spores grown from the heterokaryon on nonselective media are shown, as indicated in the cartoon. The combination of the growth tests and diagnostic PCR results proves (Osmani *et al.*, 2006b) that deletion of *An-ndc1* in combination with either $\Delta An-nup84$, $\Delta An-nup120$, or $\Delta An-nup133$ alleles causes lethality. (C) Spores from strains with null alleles of the indicated nonessential NPC core components were spread on MAG or 0.15% BA/MAG plates to test their sensitivity to BA and temperature. Cells on MAG plates were incubated at 36 and 42 °C for 48 h. Cells on 0.15% BA/MAG plates were incubated at 36 °C for 70 h. Representative colonies are shown for each strain. Bar, 1 cm. (D) Quantification of the tolerance to 0.15% BA at 36 °C. Well-separated colonies were chosen for measurement. Colony diameters were measured and plotted in the bar graph. Data are represented as mean $\pm$ SD. (E) Representative location of endogenously GFP-tagged An-Nup37 in various genetic backgrounds of cells progressing through mitosis (Supplemental Videos 31–34). Bar, 5 μ m. (F) The net intensity of An-Nup37-GFP (green) around interphase and mitotic nuclei defined by DAPI staining (red) in the strains indicated in E, quantified as in Figure 4C. The difference between $\Delta An-elys$ and $\Delta An-ndc1 \Delta An-elys$ is significant ($p < 0.0001$). Data are represented as mean $\pm$ SD.

We find it quite remarkable that the dispersed An-Nup84-120 complex is reassembled efficiently into NPCs during mitotic exit. It normally does not disperse, even in pseudometaphase-arrested cells, and we had thought it provided a resident prepore structure important for NPC

reassembly. Clearly, however, *A. nidulans* has a mechanism to reassemble the An-Nup84-120 complex into NPCs when it becomes mitotically dispersed. The transmembrane Nups An-Ndc1 and An-Pom152, along with An-Nup170 and An-Gle1, are likely important for this as they

Figure 7. Triple transmembrane Nup-deleted cells are viable. (A) Size and domains of An-Pom34 and Sc-Pom34 are indicated. Two transmembrane domains predicted using the TMPRED software locate at comparable positions (Miao *et al.*, 2006). (B) Endogenously GFP-tagged An-Pom34 localizes around the nuclear periphery similar to An-Nup49 in interphase. An-Pom34 is part of the mitotic NPC core as, unlike the peripheral Nup An-Nup49, it does not disperse during mitotic arrest imposed by SAC activation. (C and D) Both diagnostic PCR and Southern blot analysis were used to confirm deletion of all three transmembrane Nup genes in the same strain. External primers were used to confirm the deletion of *An-pom34*, *An-pom152*, and *An-ndc1* loci. Southern blot analysis using the coding regions of *An-pom34*, *An-pom152*, *An-histone H1* and *An-ndc1* as probes was performed to confirm that none of the known fungal transmembrane Nups are present in triple deleted cells. All four probes as indicated were used (top), whereas the indicated two probes were used (middle and bottom) to distinguish *An-ndc1* and *An-pom34* deletions whose bands run at near identical size. (E) Cells with the indicated genetic backgrounds were spot inoculated on MAGUU plates and incubated at 20°C for 5 d and at 32, 36, and 42°C for 39 h. Bar, 1 cm. (F) Wild-type and transmembrane Nup-deleted cells were spot inoculated on MAGUU plates at 36°C to follow their growth curve (1, wild type; 2, Δ*An-pom34*; 3, Δ*An-ndc1*; 4, Δ*An-pom152*; 5, Δ*An-ndc1*Δ*An-pom152*; 6, Δ*An-pom34*Δ*An-pom152*; 7, Δ*An-ndc1*Δ*An-pom34*; and 8, Δ*An-ndc1*Δ*An-pom34*Δ*An-pom152*). The diameter of cells was measured every 24 h for 5 d. (G) Spindle morphology (GFP-tubA) and the nuclear transport (NLS-DsRed) of wild type (Supplemental Video 35), and triple transmembrane Nup-deleted (Supplemental Video 36) cells were followed using live cell spinning disk confocal microscopy at 25°C.

do not disperse under these conditions, suggesting that they may have an early role in NPC reassembly (Figure 8).

The An-Nup84-120 Complex and Membrane Stability

Because transmembrane Nups have been shown to play early roles in NPC assembly in other systems (Madrid *et al.*,

2006; Mansfeld *et al.*, 2006; Stavru *et al.*, 2006a; Rasala *et al.*, 2008), and because they are not dispersed in An-Nup84-120 mutants, we had expected that deletion of the three *A. nidulans* transmembrane Nups would cause lethality. As we demonstrate here, this is not the case. This suggests other Nups function redundantly with the transmembrane Nups.

Figure 8. Extreme mitotic changes in the composition of the *A. nidulans* NPC core structure in Δ An-nup37 Δ An-elys cells. Two new nucleoporins, An-Nup37 and An-ELYS, have been identified as components of the An-Nup84-Nup120 complex of *A. nidulans*. These nucleoporins are lettered in yellow within the overall proposed structure of the NPC based upon our data in *A. nidulans* and the extensive studies of others. Both An-Nup37 and An-ELYS are core NPC components, along with An-Nup85, An-Nup120, An-Nup84, An-Nup133, SonBc^{Nup96}, An-Pom152, An-Ndc1, An-Pom34, An-Gle1, and An-Nup170 (red), which all remain at NPCs during interphase and mitosis. The peripheral Nups, including SonA^{Gle2}, SonBn^{Nup98}, An-Nup42, An-Nup49, An-Nup57, An-Nup82, An-Nup159, An-Nup188, An-Nup192, An-Nic96, An-Nsp1, An-Mlp1, An-Sac3, and An-Nup2 (blue), disassemble from NPCs during mitosis. In the absence of An-Nup37 and An-ELYS, the basic composition of the NPC is not changed during interphase. However, during mitosis the entire An-Nup84-120 complex is dispersed from the NPC, demonstrating normal composition of the An-Nup84-120 complex is required to maintain the core NPC structure intact during mitosis. Surprisingly, NPCs can be reassembled normally without the prepore structure when the An-Nup84-120 complex is mitotically dispersed. Although the transmembrane Nups An-Pom152, An-Pom34, and An-Ndc1 as well as An-Nup170 and An-Gle1 are not dispersed in this condition, they are unlikely to form a prepore structure in the NE throughout mitosis and their interactions with each other remains to be determined. However, that An-Pom152, An-Pom34, An-Ndc1, An-Nup170, and An-Gle1 do not disperse during mitosis indicates that they are positioned to play an early role in the NPC reassembly in *A. nidulans*.

Because the An-Nup84-120 complex has been proposed to coat, and perhaps bend, the NE membrane within the NPC structure, we looked for genetic interactions between An-Nup84-120 components and An-Ndc1 and An-Pom152 transmembrane Nups. This revealed that deletions of An-Nup84-120 components are either synthetically sick or synthetically lethal in combination with the An-*ndc1* deletion, suggesting the An-Nup84-120 complex and An-Ndc1 play essential overlapping roles. To test whether the synthetic interactions between An-Ndc1 and the An-Nup84-120 complex might involve interactions with membrane, we tested the effects of the membrane fluidizer BA on single and double mutants. This revealed that mutations that are temperature sensitive are also sensitive to BA. Such mutations also cause mitotic specific dissociation of the An-Nup84-120 complex from the NE. Interestingly, in *S. cerevisiae*, *nup84Δ* has been shown to be synthetically lethal with *spo7Δ* and *nem1Δ*, two genes involved in phospholipid biosynthesis and nuclear membrane growth (Siniossoglou *et al.*, 1998; Santos-Rosa *et al.*, 2005), implicating the yNup84 complex in nuclear membrane stability. In addition, although physical interactions between membrane and the whole An-Nup84-120/vNup107-160 complex has not been demonstrated, Drin *et al.* (2007) have shown that in vitro the N-terminal domain of Nup133 can bind to liposomes in a size-dependent manner. We therefore suggest that An-Ndc1, a transmembrane

Nup, and the An-Nup84-120 complex might play overlapping roles at the membrane of the NE. Interestingly, in the absence of An-Nup37 and An-ELYS, the NE forms transient aggregates during mitosis suggesting that an intact An-Nup84-120 complex is also important for normal mitotic NE morphology in *A. nidulans*.

One function ascribed to the transmembrane Nups is anchoring of NPCs within the NE. In vertebrates NE anchoring of NPCs involves Ndc1 interactions with the soluble Nup93, Nup53 and Nup205 NPC proteins (Mansfeld *et al.*, 2006). Our findings suggest that An-Ndc1 function involves the An-Nup84-120 complex. This is in keeping with biochemical and bioinformatic analyses predicting the Nup84-120 complex will bind and perhaps bend the NE at NPCs and the concept, derived from recent structural analysis of NPC architecture, that this complex forms a NPC membrane coat (Alber *et al.*, 2007a; Alber *et al.*, 2007b; Drin *et al.*, 2007; Hsia *et al.*, 2007).

Our finding that cells are viable in the absence of all three known fungal transmembrane Nups indicates that *A. nidulans* can assemble NPCs normally, and anchor them in the NE, without transmembrane Nups. Despite extensive studies, to date only three transmembrane Nups have been identified in any one system, but there remains the possibility

that *A. nidulans* contains additional transmembrane Nups. However, even if additional transmembrane Nups exist, these, in combination with An-Pom152 and An-Pom34, are unable to complement An-Ndc1 functions when components of the An-Nup84-120 complex are missing.

CONCLUSIONS

It has recently been suggested that several Nups, with varying capacities to interact with the NE membrane, will likely act in a synergistic manner to deform the NPC membrane, as occurs in other cellular processes involving membrane curvature (Antonin *et al.*, 2008). Transmembrane Nups intuitively would be expected to help anchor NPCs within the NE and the An-Nup84-120 complex has been suggested to play a role in membrane curvature. Our studies provide additional experimental evidence for interactions between transmembrane and nontransmembrane Nups to enable NPCs to interact functionally with the NPC membrane. In fact, our analysis further suggests that transmembrane Nup function might be redundant with Nups traditionally thought of as being soluble. One pressing challenge is to define what the respective roles of the transmembrane and soluble Nups are in anchoring the NPC within the NE membranes.

ACKNOWLEDGMENTS

We thank all members of the Osmani laboratory for help and input to this work, especially Leena Ukil. We thank Berl Oakley and Yi Xiong for sharing reagents and unpublished data. We also thank Ben Mans for insights to the sequence similarities between An-ELYS and vELYS and Anita Hopper for insight and critique of the manuscript. We thank Liwen Zhang and Kari Green-church at The Ohio State University-CCIC for help and mass spectrometry sample analysis. This work was supported by National Institutes of Health grant SAO-GM-042564.

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