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Systematic Deletion and Mitotic Localization of the Nuclear Pore Complex Proteins of *Aspergillus nidulans* [D] [V]

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To define the extent of the modification of the nuclear pore complex (NPC) during *Aspergillus nidulans* closed mitosis, a systematic analysis of nuclear transport genes has been completed. Thirty genes have been deleted defining 12 nonessential and 18 essential genes. Several of the nonessential deletions caused conditional phenotypes and self-sterility, whereas deletion of some essential genes caused defects in nuclear structure. Live cell imaging of endogenously tagged NPC proteins (Nups) revealed that during mitosis 14 predicted peripheral Nups, including all FG repeat Nups, disperse throughout the cell. A core mitotic NPC structure consisting of membrane Nups, all components of the An-Nup84 subcomplex, An-Nup170, and surprisingly, An-Gle1 remained throughout mitosis. We propose this minimal mitotic NPC core provides a conduit across the nuclear envelope and acts as a scaffold to which dispersed Nups return during mitotic exit. Further, unlike other dispersed Nups, An-Nup2 locates exclusively to mitotic chromatin, suggesting it may have a novel mitotic role in addition to its nuclear transport functions. Importantly, its deletion causes lethality and defects in DNA segregation. This work defines the dramatic changes in NPC composition during *A. nidulans* mitosis and provides insight into how NPC disassembly may be integrated with mitosis.

INTRODUCTION

The defining feature of eukaryotic cells is the nucleus, which is a subcellular compartment containing the genome separated from the rest of the cell by the nuclear envelope (NE). This compartmentalization requires that a mechanism exist to transport RNA and protein between the nucleoplasm and cytoplasm (Gorlich and Mattaj, 1996). This transport is mediated by the nuclear pore complex (NPC), which provides the sole conduit across the NE and acts to limit and regulate transport in and out of nuclei (see Tran and Wente, 2006 for recent review).

From yeasts to higher eukaryotes, the NPC is composed of multiple copies of about 30 different proteins, some of which have significant sequence conservation, whereas others with little sequence conservation are thought to carry out similar functions (Weis, 2003; Mans *et al.*, 2004; Devos *et al.*, 2006). Many Nups have been identified in *Saccharomyces cerevisiae* using genetic approaches (Ryan and Wente, 2000). In addition, large-scale purifications and protein identification using mass spectroscopy have been used to identify all the Nups of this species (Rout *et al.*, 2000) and their physical interactions (Allen *et al.*, 2002; Lutzmann *et al.*, 2005). Similar proteomic approaches have been used to identify the Nup complement of mammalian cells (Cronshaw *et al.*, 2002). Subsequent to these analysis, the Nup genes of several species, including *Caenorhabditis elegans* (Galy *et al.*, 2003) and *Schizosaccharomyces pombe* (Chen *et al.*, 2004) have been identified based on sequence conservation.

During mitosis the NPCs of higher and lower eukaryotes behave very differently (Rabut *et al.*, 2004). In higher eukaryotes, the most dramatic change to occur in the mitotic cell is the complete dissolution of the nucleus with a concomitant stepwise disassembly of the NPC (Beaudouin *et al.*, 2002; Salina *et al.*, 2002; Lenart *et al.*, 2003). During exit from mitosis, nuclei are rebuilt around segregated DNA, and NPCs are reassembled to reestablish regulated nuclear transport (Margalit *et al.*, 2005).

In cells that undergo mitosis in the absence of a nucleus, mitosis is said to be "open" (Weis, 2003). However, in lower eukaryotic cell types the NE does not breakdown, and DNA segregation occurs within intact nuclei. In these so called "closed" mitoses, the NPC remains intact and continues to mediate regulated nuclear transport during mitosis (Makhnevych *et al.*, 2003). Because proteins, such as tubulin, need to enter nuclei only during mitosis, it has long been assumed that at least some aspects of nuclear transport are under mitotic control. Indeed, there is evidence that the interactions between specific Nups of *S. cerevisiae* are modified during mitosis to change certain transport pathways (Makhnevych *et al.*, 2003). However recent work, described below, in *Aspergillus nidulans* suggests that the NPCs of some fungi are modified in a more dramatic manner during mitosis and that these modifications are regulated by the Cdk1 and NimA kinases. Similarly, during mitosis of the corn smut fungus *Ustilago maydis,* major restructuring of the mitotic NPC and nucleus also occurs (Straube *et al.*, 2005).

Mitosis in *A. nidulans* requires the activation of the highly conserved Cdk1/cyclinB protein kinase complex (Osmani *et al.*, 1991; O'Connell *et al.*, 1992; Ye *et al.*, 1995), a polo-like

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Abbreviations used: NPC, nuclear pore complex; NE, nuclear envelope.

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kinase (Bachewich *et al.*, 2005) and the NimA mitotic kinase (Ye *et al.*, 1995; see O'Connell *et al.*, 2003 for review). Mitotic exit requires the inactivation of these mitotic kinases (Pu and Osmani, 1995; Ye *et al.*, 1998; Bachewich *et al.*, 2005). A link between mitotic regulators and the NPC of *A. nidulans* came from studies aimed at understanding how the NimA kinase promotes initiation and completion of mitosis. Several approaches have been used in this endeavor including two-hybrid (Osmani *et al.*, 2003; Davies *et al.*, 2004) and extragenic suppressor screens (Wu *et al.*, 1998; De Souza *et al.*, 2003). In the extragenic suppressor screen two genes were identified and both encode proteins ($SonA^{Gle2/Rae1}$ and $SonBn^{Nup98}$). These two proteins physically interact with each other in *A. nidulans* (De Souza *et al.*, 2003) as found in yeast and higher eukaryotic cells (Bailer *et al.*, 1998; Blevins *et al.*, 2003).

The fact that mutations in two interacting Nup genes suppress a loss-of-function *nimA* G2 arrest mutation suggested that NimA may influence mitotic regulation by modifying the transport properties of the NPC (Wu *et al.*, 1998; De Souza *et al.*, 2003). In further support of this concept, it has been shown that without NimA function cyclin B and Cdk1 are unable to enter nuclei but Cdk1 is tyrosine dephosphorylated and fully activated as an H1 kinase (Osmani *et al.*, 1991). On activation of NimA, Cdk1/cyclinB can enter nuclei, indicating that NimA function is required in G2 to allow this mitotic kinase entry into nuclei through the NPC (Wu *et al.*, 1998).

Several other experimental observations suggest that the transport properties of the NPC are changed during *A. nidulans* mitosis. For instance, if microtubules are depolymerized by drug treatment, tubulin dimers are excluded from nuclei until cells enter mitosis, at which point tubulin equilibrates between the nucleoplasm and the cytoplasm (Ovechkina *et al.*, 2003). DsRed tagged with a nuclear localization sequence (NLS-DsRed) is efficiently transported into nuclei in interphase, leaving no observable protein in the cytoplasm. During mitosis, NLS-DsRed disperses from nuclei and equilibrates throughout the cell. As daughter nuclei are formed NLS-DsRed is transported back exclusively within nuclei (Suelmann *et al.*, 1997). Collectively these data suggest that during mitosis the transport properties of the *A. nidulans* NPC undergo significant modifications, presumably as part of mitotic regulation of nuclear function.

Major insight into how the *A. nidulans* NPC is regulated during mitosis came from studies of the mitotic behavior of $SonA^{Gle2/Rae1}$ and $SonBn^{Nup98}$ endogenously tagged with GFP (De Souza *et al.*, 2004). Both proteins locate to the NPC during interphase but are dramatically dispersed from the NPC during mitosis. An additional three Nups (An-Nsp1-GFP, An-Nup159-GFP, and An-Nup42-GFP) were also found to disperse from the NPC during mitosis, whereas three core Nup proteins (SonBc-mRFP, An-Pom152-GFP, and An-Nup133-GFP) remained at the NPC (De Souza *et al.*, 2004). In addition, the RanGTP gradient across the NE, which is essential for regulated nuclear transport (Richards *et al.*, 1997; Izaurralde *et al.*, 1997), is compromised during *A. nidulans* mitosis (De Souza *et al.*, 2004).

Activation of both Cdk1 and NimA is required for mitotic dispersal of Nups from the NPC. Further, ectopic induction of NimA was found to release $SonBn^{Nup98}$ from the NPC, cause dispersal of NLS-DsRed from within nuclei, and allow cytoplasmic tubulin access to nuclei out of cell cycle phase during DNA replication arrest (De Souza *et al.*, 2004). The NimA kinase is therefore necessary and, when over expressed out of cell cycle phase, sufficient to modify both the composition of the NPC and its transport properties. $SonBn^{Nup98}$ is extensively phosphorylated during mitosis after activation of NimA, indicating this could perhaps act as a trigger for the mitotic-specific modification of the NPC (De Souza *et al.*, 2004). Higher eukaryotic Nup98 is also phosphorylated specifically during mitosis (Macaulay *et al.*, 1995; Miller *et al.*, 1999), and NimA expressed in higher eukaryotic cells also modifies the NPC (Lu and Hunter, 1995a, 1995b). Together, these data suggest that the mitotic modification of NPC transport involves partial NPC disassembly during *A. nidulans* closed mitosis and provide a new paradigm for how closed mitosis can be regulated (De Souza *et al.*, 2004).

There is thus good evidence that the NPC of *A. nidulans* is subject to partial disassembly during mitosis such that NPCs are "opened" each time nuclei progress through mitosis. In contrast, the mitotic modifications of the NPC of *S. cerevisiae* mentioned above are more subtle and do not affect global regulation of nuclear transport (Makhnevych *et al.*, 2003). This situation indicates *A. nidulans* is an evolutionary intermediate between the closed mitosis of yeasts and the open mitosis of higher eukaryotes.

With the recently completed sequence analysis of *A. nidulans* (Galagan *et al.*, 2005) and two related species, *A. oryzae* (Machida *et al.*, 2005) and *A. fumigatus* (Nierman *et al.*, 2005), it has become feasible to identify the Nups of the aspergilli and to study at a global level the mitotic regulation of the NPC. We have, as reported here, identified the majority of the Nup genes of *A. nidulans*, deleted, and endogenously GFP-tagged them. These studies have allowed us to define the extreme mitotic changes that occur in the composition of the NPC in a species undergoing an evolutionary intermediary between open and closed mitosis.

MATERIALS AND METHODS

Gene deletions and GFP-tagging was completed as described (Yang *et al.*, 2004). Tagging with the monomer cherry red variant (mCherry) of mRFP (Campbell *et al.*, 2002; Shaner *et al.*, 2004) was completed using identical methods. Deleted genes that were essential were recovered as heterokaryons and identified by replica streaking conidia (uninucleate asexual spores) from putative heterokaryotic colonies onto selective (−UU) and nonselective (+UU) plates for the $pyrG^+$ marker of the deletion cassette (Osmani *et al.*, 1988). If transformants produced spores able to grow on nonselective media, but not selective media, this indicated the deleted allele can only propagate in a heterokaryon and suggests that the deleted gene is essential (Osmani *et al.*, 1988). To confirm such heterokaryons harbored both wild-type and deleted alleles, diagnostic PCR reactions were completed using primers positioned outside the deletion cassette (Yang *et al.*, 2004). Heterokaryons were propagated by transfer of mycelia pads from the leading edge of colonies growing on selective agar plates. To obtain heterokaryotic material for DNA isolation, a section (~2 cm long) of the growing edge of heterokaryotic colonies was removed, being careful to take only the top growing surface 5 mm from the leading edge of colony growth. Using tweezers, the strip of the leading edge of the colony was broken into many pieces and used to inoculate 30 ml of liquid selective media in a Petri dish. This material was grown for 24 h at 32°C, and the resulting cells harvested by filtering through Miracloth and immediately frozen in liquid nitrogen before being lyophilized. For strains with nonessential gene deletions and strains expressing fluorescent protein chimeras, the growth procedure was identical but Petri dishes were inoculated with a loop of conidia.

For preparation of DNA, 20–30 mg of lyophilized material was removed to a fresh Eppendorf tube, pulverized briefly with a sterile toothpick, and resuspended in 200 μl of Cell Lysis Solution from a Promega Mini Prep kit (A7510; Madison, WI). An equal volume of Neutralizing Solution from the kit was added and this material then put through the DNA purification protocol described in the kit. The final volume of DNA was 50 μl in H_2O. Five microliters of this DNA was subjected to PCR amplification using the Expand Long Template PCR System (Roche Applied Science, Indianapolis, IN) in 25-μl reactions.

In addition to diagnostic PCR, strains tagged with fluorescent proteins were confirmed by Western blot analysis using anti-fluorescent protein antibodies. Lyophilized mycelia prepared as above were resuspended in 40 μl urea protein sample buffer/mg dry weight, boiled, and spun in an Eppendorf for 5 min. Twenty microliters of the protein supernatant sample was resolved by SDS PAGE, transferred to nitrocellulose, and probed with appropriate commercial specific antibodies.

To screen for conditional phenotypes, deleted strains were tested on agar growth plate tests in response to a range of NaCl (0.25–2 M), sucrose (0.5–1.5 M), nocodazole (0.05–0.2 μg/ml), MMS (0.005–0.02%), DEO (0.005–0.01%), HU (2–10 mg/ml) and camptothecin (2–5 μg/ml). In addition, growth at 20, 30, 32, 37 and 42°C was tested.

All standard classical genetic methodologies for *A. nidulans* were in essence as described previously (Pontecorvo, 1953). A table of the strains utilized in these studies is included in the supplementary materials (Supplementary Table S1), and a list of primers is available from the authors. Standard methodologies, as described previously, were used for live cell imaging using a spinning disk confocal system (De Souza *et al.,* 2004).

RESULTS

Identification of the Nup Genes of A. nidulans

Using Nup protein sequences from *S. cerevisiae* (Rout *et al.,* 2000), *S. pombe* (Chen *et al.,* 2004), *C. elegans* (Galy *et al.,* 2003) and mammals (Cronshaw *et al.,* 2002) the genome of *A. nidulans* (Galagan *et al.,* 2005) was queried using blastp (Altschul *et al.,* 1997) to identify putative Nup genes in addition to those we had previously identified (De Souza *et al.,* 2004). Numerous putative Nups were identified (Table 1), some of which were highly conserved and others less so. One Nup, Nup188, was not identified using blastp because the automated gene calling process did not predict this protein (Galagan *et al.,* 2005). However, using tblastn along with the gene calling of *A. fumigatus* Nup188 (Nierman *et al.,* 2005), the coding region of An-Nup188 was predicted which was highly similar to Nup188p (e-value of 2e-47).

We also considered the protein domains present in different classes of Nups in our searches and for our gene designations. For instance, *S. cerevisiae* Nup2p is a multidomain protein (Loeb *et al.,* 1993; Booth *et al.,* 1999; Solsbacher *et al.,* 2000; Hood *et al.,* 2000; Denning *et al.,* 2001, 2002; Dilworth *et al.,* 2001, 2005; Galy *et al.,* 2003; Matsuura *et al.,* 2003) containing an N-terminal Kap60p (importin α) binding domain followed by a NPC-targeting domain, an FG repeat domain, and a C-terminal Ran binding domain. All of these structural features were identified within the An-Nup2 predicted sequence, which has a similar overall domain structure even though An-Nup2 is predicted to be almost twice as large as Nup2p. Nup2 orthologues identified in other filamentous fungi were similarly large proteins.

In a subsequent bioinformatics study Mans *et al.* (2004) predicted the Nup orthologues of *A. nidulans* and defined the same proteins as our independent predictions. All of the predicted Nup proteins of *A. nidulans* were found to locate to the nuclear periphery (see below) and affinity purifications of several of the Nups indicate the conserved core Nup84/Nup107 complex (Siniossoglou *et al.,* 2000; Loiodice *et al.,* 2004) is also conserved in *A. nidulans* (Liu and Osmani, unpublished results). From the current work, along with our previous studies of *A. nidulans* Nups (Wu *et al.,* 1998; De Souza *et al.,* 2003, 2004) we have identified 26 Nups (Table 1), which constitute the majority of the NPC proteins of *A. nidulans*. We also identified an orthologue of Cdc31p, which has been found associated with the NE and to play a role in mRNA export in *S. cerevisiae* (Fischer *et al.,* 2004) and importinβ (Harel and Forbes, 2004), as well as orthologues of Ran and Rcc1 (Fahrenkrog *et al.,* 2004; Table 1). We were unable to identify orthologues of budding yeast Nup1p, Nup53p, Nup59p, Nup60p, or Pom34p or vertebrate Nup358, Nup43, or Aladin.

Deletion of A. nidulans Nuclear Transport Genes and Identification of Conditional Phenotypes

To begin to define the function of *A. nidulans* transport genes, a systematic gene knockout program was completed. In addition, these null alleles helped confirm the functionality of tagged versions of these genes. For each gene a null allele was generated using homologous integration (Table 1) to replace its coding region with the *A. fumigatus pyrG* nutritional marker (the *sonB* gene was previously deleted and shown to be essential; De Souza *et al.,* 2003). This process was facilitated using fusion PCR to rapidly generate deletion constructs (Yang *et al.,* 2004) and an Δ*nkuA* (ΔAn-*ku70*) recipient strain, which has reduced rates of heterologous integrations (Nayak *et al.,* 2005).

Strains carrying confirmed deletions of the 12 nonessential genes (Table 1) were crossed away from the ΔAn-*ku70* mutation. Resulting progeny were further out-crossed to generate strains with no nutritional markers. During this analysis the ΔAn-*mlp1* mutant was found to be sterile in the outcross and was subsequently found to be also self-sterile. No viable ascospores were recovered although rudimentary cleistothecia (enclosed round structures in which ascospores are made) were formed. Thus An-Mlp1 plays an essential role during the sexual life cycle, perhaps during karyogamy or meiosis. Because we could not cross the ΔAn-*Mlp1* allele away from the ΔAn-*Ku70* allele, it is possible that sterility is a synthetic phenotype cause by the two deletions.

Mlp1p and Mlp2p are large coiled-coil repeat filamentous proteins that stretch from the NPC into the nucleoplasm (Strambio-de-Castillia *et al.,* 1999) and are considered orthologues of the TPR protein of vertebrates (Bangs *et al.,* 1998). Both Mlp1p and Mlp2 have similar localization and overall structure. However, An-Mlp1 is more similar to Mlp1p (e value 5e-110) than Mlp2p (e value 1e-52). Mlp proteins play a role in telomere maintenance (Hediger *et al.,* 2002a, 2002b), and Mlp1p is required for RNA biogenesis and nuclear retention of unspliced mRNAs (Green *et al.,* 2003; Galy *et al.,* 2004; Casolari and Silver, 2004; Vinciguerra *et al.,* 2005), whereas Mlp2p physically interacts with components of the spindle pole body (SPB) and is required for SPB assembly and function (Niepel *et al.,* 2005). Therefore, deletion of An-Mlp1 could potentially cause nonessential defects in telomere maintenance, mRNA biogenesis, or SPB function to cause sterility or it might play a more specific role in the *A. nidulans* sexual cycle.

The original, and out-crossed deletion strains, along with endogenously GFP-tagged strains (see below) were tested under an array of growth conditions (see material and methods) to uncover conditional phenotypes caused by deletion or GFP tagging. Deletion of five nonessential genes caused temperature sensitivity in the original ΔAn-*ku70* background and in out-crossed strains (Figure 1) but grew normally at 32°C (unpublished data). Deletion of An-*nup120*, An-*nup84,* or An-*trm1* caused tight temperature sensitivity at 42°C, whereas deletion of An-*nup133* or An-*nup42* caused restricted growth at 42°C (Figure 1). Deletion of An-*nup49* or An-*nup57* caused measurable cold sensitivity (Figure 1) but allowed normal growth at 32°C (unpublished data). No difference in these conditional phenotypes were observed in the ΔAn-*ku70* strain background compared with out-crossed strains (Figure 1).

In addition to the growth tests, we also set self-crosses to determine if any of the nonessential deletions caused self-sterility. This experiment is possible because *A. nidulans* is homothallic and encodes the two *Aspergillus* mating type genes (Galagan *et al.,* 2005). The self-crosses were set at permissive growth temperatures for the cold- and heat-sensitive mutations. Of the 11 strains tested (note ΔAn-*mlp1* causes both self and outcross sterility) four were self sterile, including ΔAn-*trm1*, ΔAn-*nup84*, ΔAn-*nup120,* and ΔAn-*nup133* (Table 1). During each self-sterile mutant cross, very

Table 1. Nuclear transport genes of *Aspergillus nidulans*

A. nidulans							
Protein name	Predicted gene	*S. cerevisiae*-human	e-value[a]	Mitotic location	Deletion	Deletion: Sc-Sp-Ce[b]	Domains
An-Nup2	AN5485[c]	Nup2p-Nup50	4e-06	Chromatin	Lethal	Viable-Viable-Viable	21 FG + Ran BD
An-Nup42	AN4595	Nup42p-Nlp1	5e-13	Dispersed[d]	Viable[ts]	Viable-Viable-?	19 FG + C3H1 zinc finger
An-Nup49	AN2431	Nup49p-Nup58	1e-0.05	Dispersed	Viable[cs]	Lethal-Lethal-Lethal	8 FG N-term
An-Nup57	AN1064	Nup57p-Nup54	9e-36	Dispersed	Viable[cs]	Lethal-Lethal-Lethal	9 FG N-term
An-Nup82	AN6143	Nup82p-Nup88	Mans *et al.*[e]	Dispersed	Lethal	Lethal-?-?	C-Term coiled coils
An-Nup84	AN1190[f]	Nup84p-Nup107	5e-16 (Nup107)	NPC	Viable[ts] + ss[g]	Viable-Lethal-Viable	pfam04121
An-Nup85	AN9109[h]	Nup85p-Nup75/85	Mans *et al.*[e]	NPC	Viable	Viable-Lethal-37% Lethal	
An-Nup120	AN1238	Nup120p-Nup160	7e-04	NPC	Viable[ts] + ss	Viable-Viable-Lethal	
An-Nup133	AN4293	Nup133p-Nup133	1e-17	NPC[d]	Viable[ts] + ss	Viable-Viable-Viable	pfam04044
An-Nup159	AN2086	Nup159p-Nup214	1e-24	Dispersed[d]	Lethal	Lethal-Lethal-Viable	25 FG + C-term coiled coils
An-Nup170	AN6738	Nup170p-Nup155	1e-130	NPC	Lethal	Viable-?-Lethal	pfam03177
An-Nup188	tBLASTn[i]	Nup188p-Nup188	2e-47	Dispersed	Lethal	Viable-?-?	
An-Nup192	AN0037[j]	Nup192p-Nup205	2e-74	Dispersed	Lethal	Lethal-Lethal-Lethal	
An-Brr6	AN7182	Brr6p-ND	9e-13	Not defined	Lethal	Lethal-?-?	COG5085 Membrane protein
An-Cdc31	AN5618	Cdc31p-Centrin	6e-34	Not tagged	Lethal	Lethal-Lethal-Viable	EF-hand calcium-binding motif
An-Gle1	AN1157	Gle1p-Gle1	2e-19	NPC	Lethal	Lethal-?-?	N-term Coiled coils
KapB[ImportinB1]	AN0906	Kap95p-Importin B1	3e-164	ND	Lethal	Lethal-Lethal-Lethal	COG5215 KAP95
An-Mlp1	AN5499	Mlp1p-TPR	5e-110	Dispersed	Viable + Sterile	Viable-Lethal-?	Coiled coils
An-Ndc1	AN4417	Ndc1p-Ndc1	0.00031[k]	NPC + SPB	Viable	Lethal-Lethal-Viable	5 Transmembrane
An-Nic96	AN6980	Nic96p-Nup93	3e-99	Dispersed	Lethal	Lethal-Viable-Lethal	pfam04097
An-Nsp1	AN4499	Nsp1p-Nup62	6e-47	Dispersed[d]	Lethal	Lethal-Lethal-Lethal	14 FG + C-Term Coiled coils
An-Pom152	AN3454	Pom152p-ND	4e-145	NPC[d]	Viable	Viable-?-?	Transmembrane
An-Ran	AN5482	Gsp1p-Ran	6e-106	Not tagged	Lethal	Lethal-Lethal-Lethal	Small GTPases
An-RanGAP	AN4862	Rna1p-RanGAP1	1e-68	Dispersed[d,l]	Lethal	Lethal-Lethal-Lethal	COG5238 RNA1
An-Rcc1	AN6978	Srm1p-Rcc1	1e-85	Chromatin	Lethal	Lethal-Lethal-Lethal	COG5184 RCC1
An-Sac3	AN7726	Sac3p-Shd1	3e-37	Dispersed	Lethal	Viable-?-?	pfam03399
An-Sec13	AN4317	Sec13p-Sec13	1e-102	NPC and vesicles	Lethal	Lethal-Lethal-Lethal	WD40
An-Seh1	AN5889[m]	Seh1p-Seh1	2e-40	Not tagged	Viable	Viable-Viable-Vaible	WD40
SonA[Gle2]	AN1379	Gle2p-Rae1	4e-99	Dispersed[d]	Lethal	Viable-Lethal-Viable	WD40
SonBc[Nup96]	AN5627	c-Nup145p-Nup96	6e-10	NPC[d]	Lethal	Viable-Lethal-?	
SonBn[Nup98]	AN5627	Nup116p-Nup98	2e-09	Dispersed[d]	Lethal	Lethal-Lethal-?	53 FG + GLEBS domain
An-Trm1	AN9406[n]	Trm1p-Trm1	2e-96	Dispered[o]	Viable[ts] + ss	Viable-Viable-?	pfam02005
Not present	—	Nup1p-Not present					
Not present	—	Nup53p-Nup35					
Not present	—	Nup59p-Nup35					
Not present	—	Nup60p-Not present					
Not present	—	Pom34p-Not present					

[a] Values are for comparison to the *S. cerevisiae* protein at NCBI unless otherwise indicated.

[b] Sc, *S. cerevisiae*; Sp, *S. pombe*; Ce, *C. elegans*.

[c] 5′ RACE indicates the protein has an additional 21 aa.

[d] Location determined previously in De Souza *et al.* (2004).

[e] Very low similarity but published as orthologues in Mans *et al.* (2004).

[f] 3′ RACE indicates nonexistent intron called 2 bp before the stop codon.

[g] ss, self sterile.

[h] 3′ RACE indicates the real stop is within an incorrectly called intron.

[i] A gene not called by automated annotation. The gene structure was predicted using *A. oryzae* and *A. fumigatus* gene structures. The 3′ end was confirmed by RACE.

[j] 3′ RACE indicates that two genes merged. Only the correct coding region was used for BLAST analysis.

[k] Blast at SGD WU-BLAST2.

[l] An-Ran-GAP-GFP is excluded from nuclei during interphase and disperses throughout the cell at mitosis.

[m] 3′ RACE indicates an intron two bases before the predicted stop codon, generating an extra exon encoding 38 aa.

[n] 3′ RACE shows an incorrect intron called removing the correct coding for the C terminus.

[o] An-Trm1-GFP locates within nuclei during interphase and disperses throughout the cell during mitosis.

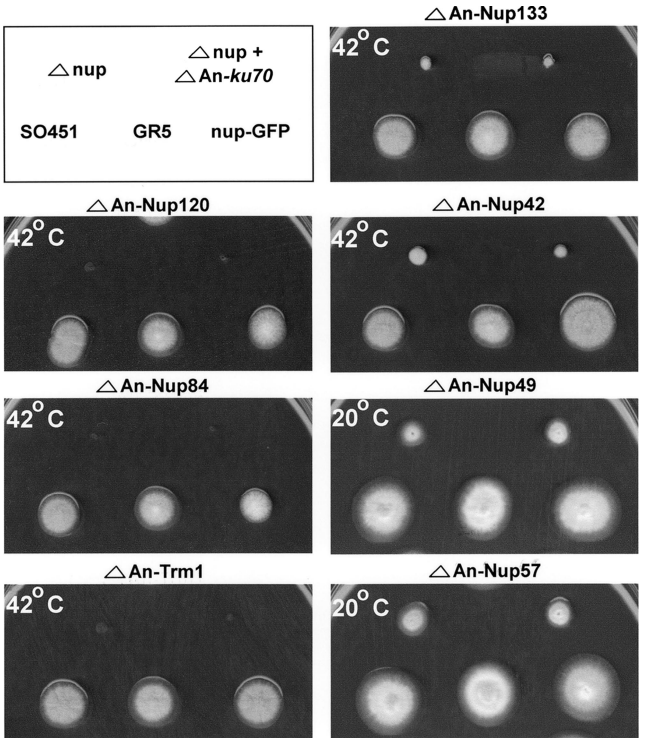

Figure 1. Conditional phenotypes associated with Nup deletions. Strains were spot-inoculated and incubated as indicated. The temperature sensitivity of strains at 42°C (ΔAn-*nup120*, ΔAn-*nup84*, ΔAn-*trm1*, ΔAn-*nup133*, and ΔAn-*nup42*) and the cold sensitivity of strains at 20°C (ΔAn-*nup49* and ΔAn-*nup57*) are shown after 2 d (42°C) or 5 d of growth (20°C). As indicated by the key, in each plate the original deletion in the ΔAn-*ku70* strain and out-crossed strains have been inoculated. As controls, ΔAn-*ku70* and wild-type strains, along with strains carrying the GFP-tagged version of the deleted gene, are also inoculated. All strains grew similarly to wild type at 32°C.

small rudimentary cleistothecia were formed that contained no viable ascospores. This suggests these four genes could play direct roles in the sexual cycle. However, these affects could be indirect because it is known that some autotrophic markers in *A. nidulans* cause recessive self-sterility even on supplemented media. This phenomenon has been interpreted to suggest that a selection process occurs during the sexual cycle so that dikaryons carrying partially deleterious mutations are not able to proliferate and produce sexual spores. This ensures that reproductive energy is invested in dikaryons, and thus sexual spores, of good genetic quality (Bruggeman *et al.*, 2004). Regardless of whether these genes play a direct role in the sexual life cycle or trigger a system that prevents generation of ascospores, it is interesting that the only Nup deletions found to cause self-sterility were components of the *A. nidulans* Nup84 complex. The other deletion causing self-sterility, ΔAn-*trm1*, encodes a 2,N2-dimethylguanosine tRNA methyltransferase (Murthi and Hopper, 2005).

Endogenously tagged versions of the Nup deletions that caused conditional temperature sensitivity or lethality grew at a rate comparable to control strains under normal growth conditions or at the restrictive temperatures (Figure 1 and unpublished data). This provides direct evidence that the majority of the tagged proteins in this study are functional fusions.

Effects of Essential Gene Deletions on Germination and Growth

After transformation of targeted deletion cassettes (Δ*nup*::*pyrG*), essential genes were identified using the heterokaryon rescue technique (Osmani *et al.*, 1988) as described in *Materials and Methods.* Heterokaryons can form in filamentous fungi when two genetically distinct nuclei occur in the same cytoplasm and selection is imposed for each type of nucleus. Importantly, because asexual spores of *A. nidulans* (conidia) are uninucleate, the heterokaryotic state cannot propagate through conidia. A representative example of the heterokaryon growth test is shown in Figure 2A for deletion of the essential An-*nup2* gene. Diagnostic PCR reactions (Yang *et al.*, 2004) confirmed the presence of both wild-type and ΔAn-*nup2* alleles in the heterokaryons formed after deletion of An-*nup2* (Figure 2B). The data demonstrate the deleted allele can only survive in heterokaryons in which the deleted nuclei provide the nutritional marker gene and the nondeleted nuclei the essential gene function. Conidia from the ΔAn-*nup2*/An-*nup2* heterokaryons were streaked three times on nonselective media. Diagnostic PCR on the resulting colonies revealed only the parental allele (Figure 2B, Het. +UU) survived streaking, indicating the deleted allele was lost when selection for the deletion marker was removed. These data demonstrate An-*nup2* is an essential gene.

Using heterokaryon rescue, we identified 18 essential *A. nidulans* genes (Table 1) including An-*nup2*, An-*nup82*, An-*nup159*, An-*nup170*, An-*nup188*, An-*nup192*, An-*brr6*, An-*cdc31*, An-*Gle1*, An-*nic96*, An-*nsp1*, *kapB*$^{\text{importin}\beta}$, An-*ran*, An-*ranGAP*, An-*rcc1*, An-*sac3*, An-*sec13,* and *sonA*$^{\text{gle2/rae1}}$. To characterize the terminal growth phenotype, we determined the amount of growth that could occur in the absence of these essential genes. For *pyrG89* cells grown on nonselective +UU media for 1 d large germlings can form, and after 3 d confluent growth occurs (Figure 3A, Parent +UU). However, these parental *pyrG89* conidia are unable to elaborate a germtube on selective −UU plates even after 3 d growth (Figure 3A, Parent −UU). Therefore, by germinating the mixed conidia of the heterokaryons on selective media we were able to identify the parent *pyrG89* spores because they are unable to send out germtubes (Figure 3A, Parent −UU), whereas the Δ*nup*::*pyrG* conidia could germinate but arrested, dependent on their terminal phenotype.

There was considerable variability in the growth phenotypes of the null conidia (Figure 3B). At one extreme, ΔAn-*sac3* and ΔAn-*nup188* cells grew very slowly but eventually formed microcolonies (unpublished data). This indicates that An-*sac3* and An-*nup188* are not absolutely essential for growth. However, both are clearly required for normal growth and development, and their deletions provide enough selective pressure to form and maintain heterokaryons in which the deletions are covered by the wild-type allele. At the other extreme, exemplified by ΔAn-*sec13* and Δ*kapB*$^{\text{importin}\beta}$ cells, virtually no growth occurred. This phenotype suggests these genes may have an essential role in polarized growth. Between these two extremes, deletion of most of the remaining genes still allowed successful polarized germination and some degree of short-term growth. This is true for ΔAn-*nup192*, ΔAn-*rcc1*, ΔAn-*nup2*, ΔAn-*gle1*, ΔAn-*cdc31*, ΔAn-*nic96*, ΔAn-*nsp1*, ΔAn-*nup182*, Δ*sonA*, ΔAn-*ranGAP*, ΔAn-*nup159*, ΔAn-*brr6*, ΔAn-*nup170,* and ΔAn-*ran* cells. However, deletion of any of these genes does not allow significant growth beyond 1 d. This can be seen by the amount of growth after 1 d compared with 3 d (Figure 3B). Because ΔAn-*nup192,* ΔAn-*rcc1*, ΔAn-*nup2*, ΔAn-*gle1*, ΔAn-*cdc31*, ΔAn-*nic96*, ΔAn-*nsp1*, ΔAn-*nup182*, Δ*sonA*, ΔAn-*ran*-

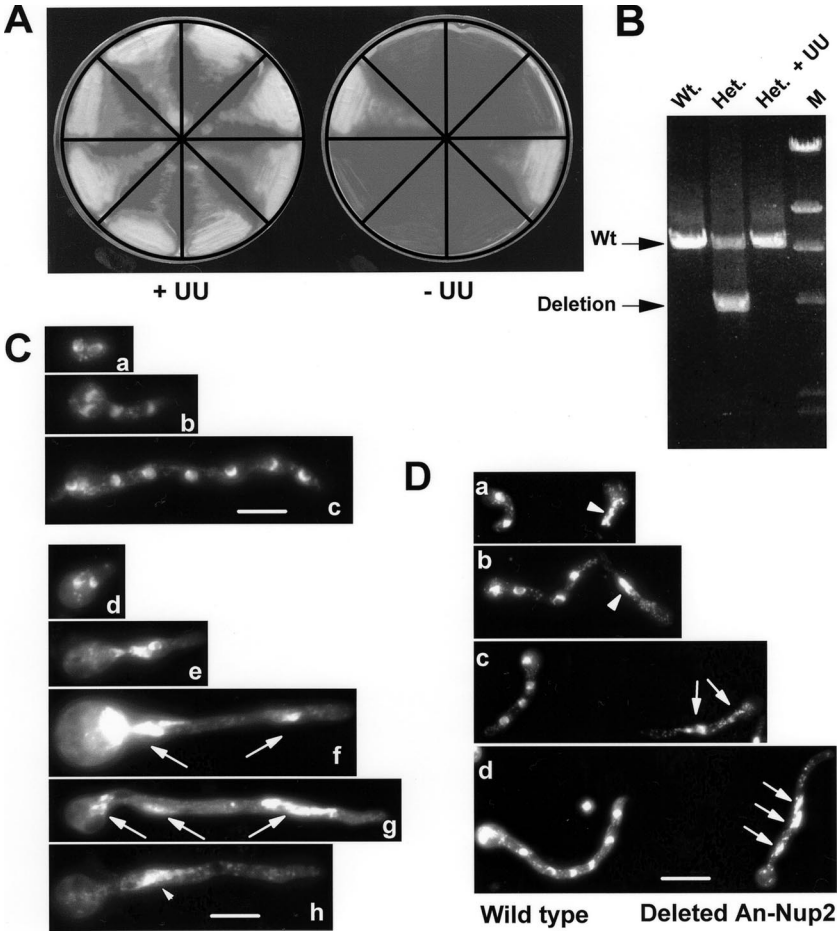

Figure 2. Gene deletion of An-*nup2*. (A) Conidia from *pyrG*$^+$ control or Δ*nup2*::*pyrG* deletion transformants were replica streaked onto nonselective (+UU) and selective plates (−UU) and grown for 2 d before photography. Conidia that generate colonies on +UU and −UU media are from *pyrG*$^+$ nonheterokaryon colonies, whereas conidia that form colonies on +UU but not −UU media are from ΔAn-*nup2*::*pyrG*/An-*nup2 pyrG89* heterokaryons. (B) Diagnostic PCR reactions using DNA isolated from a wild-type strain (Wt.), a putative Δ*nup2*/*nup2*$^+$ heterokaryon (Het.), and from a colony after streaking conidia from the heterokaryon on nonselective media (Het. +UU). The marker (M) is λ*HindIII*. (C) Wild-type germlings with 2, 4, or 8 nuclei stained with DAPI are shown (a–c). Also shown are Δ*nup2* germlings (d–h) stained with DAPI indicating miss-segregated DNA (arrows) or a single large nucleus (arrow head). (D) Germlings grown from conidia of the Δ*nup2*/*nup2*$^+$ heterokaryon on nonselective media for the *pyrG89* marker after DAPI staining are shown. In each case to the left are *pyrG89* cells able to grow normally on the +UU supplemented media. Cells to the right are Δ*nup2* cells and show the same phenotypes as seen on selective media (C, e–h) with miss-segregated DNA (arrows) or single large nuclei (arrow head). Scale bars, ~5 μm.

GAP, ΔAn-*nup159*, ΔAn-*brr6*, ΔAn-*nup170*, and ΔAn-*ran* cells cannot grow beyond the germling stage, we conclude that they are also essential genes.

Nuclear Defects Caused by Deletion of Essential Genes

To characterize the types of nuclear defects caused by lack of the 18 essential genes (Table 1), spores from heterokaryon rescues were grown on selective media, fixed, and stained by DAPI. This allowed visualization of general nuclear structure and also the status of mitotic progression. Typically DAPI-stained *A. nidulans* nuclei are round to ovoid in shape with a less bright hemispherical region that represents the poorly DAPI stained nucleolus (well represented in apical nuclei of germling in Figure 2Cc). During germination, spores of *A. nidulans* first undergo limited isotropic growth and mitosis to generate near-round binucleate cells (Figure 2Ca). The initial isotropic growth is followed by highly polarized growth, typically initiated from a single point, and mitosis occurs, generating polarized germlings with four nuclei (Figure 2Cb). Further polarized growth ensues, linked to rounds of synchronous mitoses, generating increasingly longer cells with 8 (Figure 2Cc) and then 16 nuclei (unpublished data) in the next two cell cycles.

To help explain the analysis of the nuclear defects caused by lack of the 18 essential genes, the nuclear phenotype of ΔAn-*nup2* cells is explained in detail. On germination on selective media, ΔAn-*nup2* spores were able to break dormancy and undergo germtube emergence similar to wild-type spores. However, mitotic progression and nuclear structure were dramatically compromised in the absence of An-*nup2* (Figure 2C, d–h). In some cells, completion of mitosis was defective, which generated cells with single large DNA masses (Figure 2C, e and h). Alternatively, mitotic segregation of DNA was defective resulting in the generation of uneven DNA masses (Figure 1C, f and g). Identical mitotic defects were observed when spores were germinated on nonselective media. In this case those spores derived from the heterokaryon rescue that do not have An-*nup2* deleted were able to germinate and grow as expected of *pyrG89* mutant cells complemented with uridine and uracil (Figure 2D, a–d, cells to the left of each panel). In contrast, spores derived from nuclei carrying the ΔAn-*nup2* allele displayed mis-segregation of DNA or failed to complete mitosis (Figure 2D, a–d, cells to right of each panel). Because the cells in each panel of Figure 2D, a–d, have grown next to each other, it is clear that the rate of germination and short-term growth is similar with or without An-*nup2*. In conclusion, although lack of An-*nup2* does not arrest cell cycle progression or affect short-term growth, lack of this essential Nup does appear to cause mitotic defects.

A similar analysis was performed on the other essential genes with representative cells shown in Figure 4. Although single cells are shown in Figure 4, analysis of the various defects seen for any given deletion was variable with the exception of ΔAn-*cdc31*, which caused a uniform cell cycle arrest. An-*cdc31* is expected to play a role in SPB duplication (Spang *et al.*, 1993) and accordingly, the majority (85%) of ΔAn-*cdc31* cells displayed a classical cell cycle arrest (Morris,

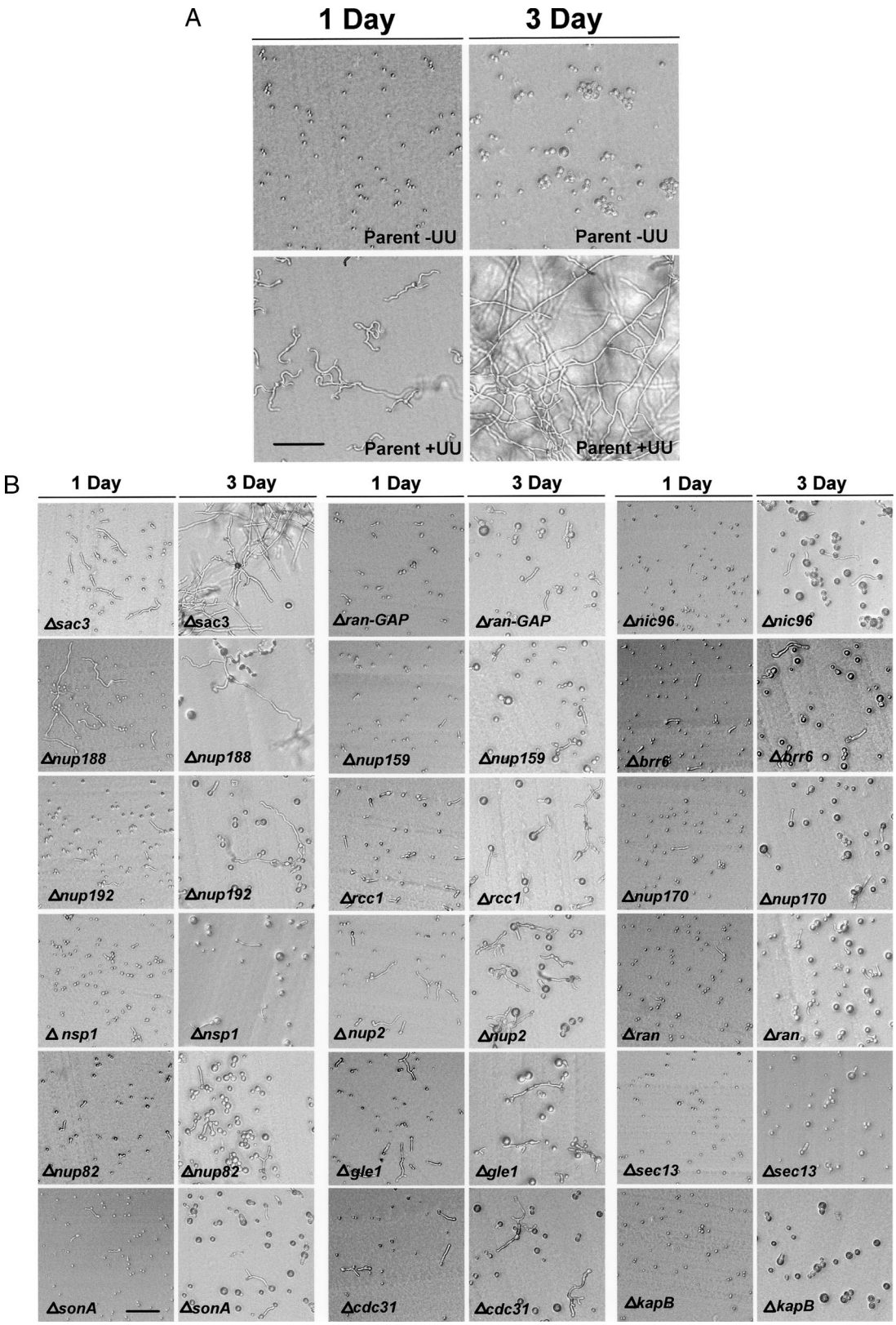

Figure 3. Growth characteristics after essential gene deletions. (A) Depicted is a bright field image of conidia from a *pyrG89* strain (GR5) grown on selective (−UU) or nonselective media for *pyrG89* (+UU) for 1 or 3 d at 22°C. Note the germinating *pyrG89* conidia are unable to form a germtube even after 3 d growth on selective media, although they swell considerably. (B) Growth characteristics of conidia from 18 different essential gene deletions derived from heterokaryons containing nuclei with the deleted allele (Δ*gene X*::*pyrG*$^+$) and *pyrG89* nuclei with the wild-type allele (*Gene X*$^+$; *pyrG*$^-$). Growth was for 1 or 3 d at 22°C on media selective for *pyrG*$^+$. In each panel the *pyrG89* cells are the unpolarized cells, and the null cells are the germlings with varying growth capabilities. Scale bar, ~20 μm.

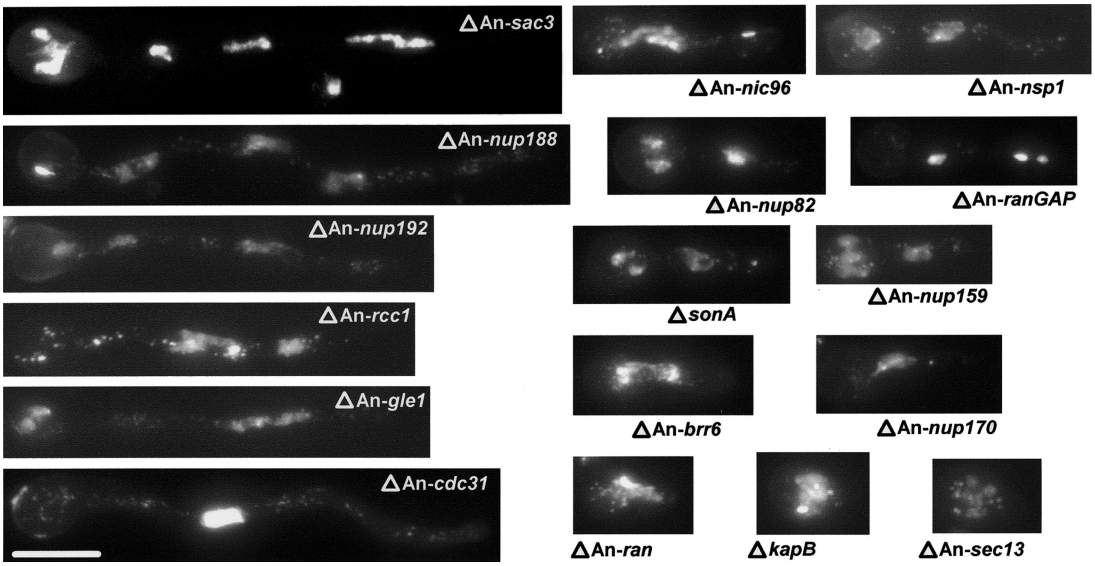

Figure 4. Nuclear defects after nuclear transport gene deletions. Shown are micrographs of representative germlings with the indicated gene deletions stained with DAPI to reveal nuclear number and morphology after 24 h growth at 22°C. Note varying amounts of growth and nuclear morphologies compared with wild-type cells (Figure 2C, a–c). Scale bar, ~5 μm.

1976), with a single discrete, if somewhat large, nucleus present in each germling (Figure 4, ΔAn-*cdc31*). This demonstrates the fact that *A. nidulans* can undergo germination and significant growth without completing mitosis. For the remaining deletions, four main classes of nuclear defects were observed: 1) Cells with odd numbers of nuclei (Figure 4 ΔAn-*nup82* cell for example). This defect was often observed in ΔAn-*sac3*, ΔAn-*nup192*, ΔAn-*nup188*, ΔAn-*rcc1*, ΔAn-*gle1*, ΔAn-*nup82*, ΔAn-*ranGAP*, and ΔAn-*nup159* cells. 2) Cells with nuclei that were difficult to distinguish because of faint DAPI staining (Figure 4, ΔAn-*Sec13,* for example). This defect was observed in ΔAn-*nup192*, ΔAn-*rcc1*, ΔAn-*nic96*, ΔAn-*nup82*, ΔAn-*nsp1*, ΔAn-*ranGAP*, ΔAn-*nup159*, ΔAn-*ran*, ΔAn-*sec13*, and Δ*kapB*$^{\text{importin}\beta}$ cells. 3) Cells with nuclei connected side-to-side or by DNA bridges (Figure 4, ΔAn-*gle1*, for example). This defect was observed in (ΔAn-*nup170*, Δ*sonA*, ΔAn-*nup188*, ΔAn-*rcc1*, ΔAn-*gle1*, ΔAn-*nic96*, ΔAn-*nsp1*, ΔAn-*nup159*, ΔAn-*brr6*, ΔAn-*sec13*, Δ*kapB*$^{\text{importin}\beta}$). 4) Finally, cells containing small DAPI bright structures (Figure 4, ΔAn-*nic96,* for example). This defect was observed in ΔAn-*nup192*, ΔAn-*nup188*, ΔAn-*rcc1*, ΔAn-*gle1*, ΔAn-*nup82*, ΔAn-*ranGAP*, ΔAn-*nup159*, ΔAn-*sec13*, and Δ*kapB*$^{\text{importin}\beta}$. We suspect that some of the variability observed during this analysis could result from inheritance of different amounts of protein and/or mRNA from the parent heterokaryon during spore formation. Future analysis will be aimed at testing this hypothesis. We will also define more comprehensively the nuclear defects caused by these deletions using live cell imaging to follow microtubules and DNA to more clearly define the mitotic defects caused by these deletions.

Live Cell Imaging of Endogenously Tagged Nups During Mitosis

We previously demonstrated that at least five *A. nidulans* Nups are dispersed from the NPC at mitosis, whereas three remain (De Souza *et al.,* 2004). To more fully define the magnitude of the mitotic modification of the NPC we set out to endogenously C-terminally GFP/chRFP tag the predicted Nups as well as orthologues of Rcc1 and Ran. With the exception of An-Seh1, An-Cdc31 and An-Ran all target genes were modified with fluorescent protein tags to generate viable strains. Although we were able to tag An-*cdc31* and An-*ran*, the tagged versions only survived in heterokaryons and spores derived from the primary transformants could not grow on selective media to form colonies. Spores from these heterokaryons that contained nuclei with the tagging cassette were GFP positive, whereas the spores having untransformed nuclei were not. This proves GFP-tagged An-Cdc31 and An-Ran are nonfunctional proteins. In most genetic systems the fact that a tagged essential protein is nonfunctional can only be inferred from the observation that the tagged strain cannot be obtained. In *A. nidulan*s, as explained here, direct evidence can be obtained that a tagged protein is synthesized but is nonfunctional. For An-Ran the problem with GFP tagging is likely the size of GFP because we have recently incorporated an affinity peptide of 25 amino acids at its C-terminus, which is functional. It is currently unclear why we were unable to endogenously tag the nonessential An-*seh1* gene.

Tagged proteins were visualized both in the original and out-crossed strains. Data are presented for out-crossed strains, but there was no observable difference in the location or behavior of any of the tagged proteins with or without An-Ku70. One advantage of studying the kinetics of mitotic processes in *A. nidulans* is that mitosis typically proceeds in a semisynchronous manner within a hyphal segment. Therefore, by observing several cell compartments in which multiple nuclei undergo semisynchronous mitoses, it is possible to follow all stages of mitosis by gathering images at 1.5–2-min intervals. Multiple cells for each tagged protein have been followed through mitosis with reproducible kinetics and behavior. Typical examples are shown in Figure 5 and a representative movie file for each is provided.

With the exception of An-Trm1-GFP, most tagged proteins (Table 1) that have been shown to locate around the nuclear periphery in *S. cerevisiae* and other organisms, either at the NPC or NE also locate to the nuclear periphery of *A. nidulans*. In *S. cerevisiae TRM1* encodes two proteins because of differences in initiation methionine usage (Ellis *et al.,*

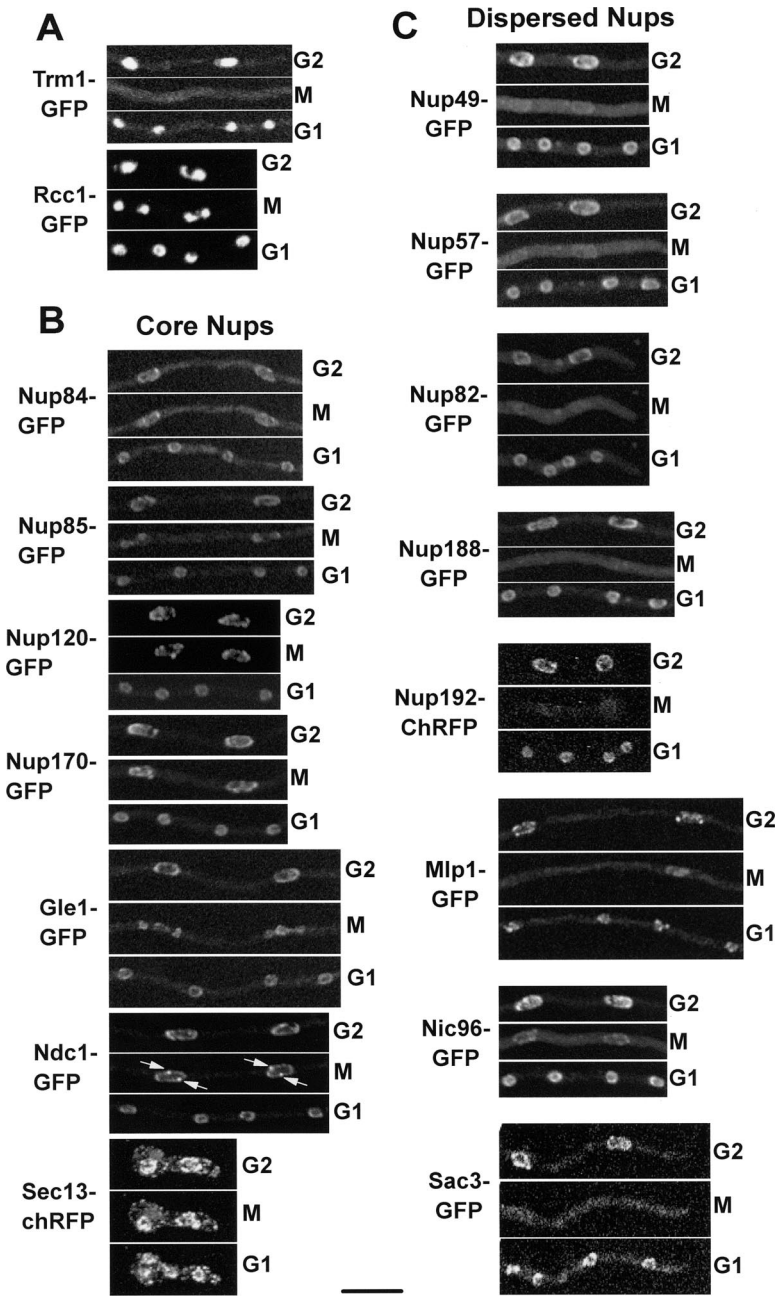

Figure 5. Cell cycle distribution of endogenously tagged Nups during mitosis. (A–C) For each endogenously tagged strain, images are shown from time-course microscopy during mitosis. Cells containing two nuclei, which divide to generate four nuclei, are shown. The images are maximum intensity projections from multiple Z-sections. Proteins are tagged either with GFP or with the mCherry variant of mRFP (Shaner *et al.*, 2004), indicated as chRFP. For each tagged strain, cells are shown in late G2 before mitosis (G2), at midmitosis just before nuclear division (M), and in G1 when nuclear division has been completed (G1). For each set of images the original movie file is presented as a supplementary file (An-Trm1-GFP Video 1, An-Rcc1-GFP Video 2, An-Nup84-GFP Video 3, An-Nup85-GFP Video 4, An-Nup120-GFP Video 5, An-Nup170-GFP Video 6, An-Gle1-GFP Video 7, An-Ndc1-GFP Video 8, An-Sec13-chRFP Video 9, An-Nup49-GFP Video 11, An-Nup57-GFP Video 12, An-Nup82-GFP Video 13, An-Nup188-GFP Video 14, An-Nup192-chRFP Video 15, An-Mlp1-GFP Video 16, An-Nic96-GFP Video 17, and An-Sac3-chRFP Video 18). Scale bar, ~5 μm.

1987), which locate to mitochondria and the inner nuclear membrane. However, An-Trm1-GFP was located within nuclei and was not concentrated at either the NE or mitochondria. What is more, during mitosis An-Trm1-GFP dispersed from nuclei then relocated back to nuclei upon completion of mitosis. This indicates that An-Trm1-GFP is not permanently associated with any nuclear structure such as the NE (Figure 5A and Supplemental Movie 1). Yeast Trm1p locates to the NE at least in part due to N-acetylation mediated by the N-terminal acetyltransferase C complex (NatC; Polevoda and Sherman, 2001; Murthi and Hopper, 2005). Perhaps the nuclear localization of An-Trm1 indicates it is not N-acetylated and, consistent with this possibility, the N-terminus of An-Trm1 does not conform to the NatC substrate specificity consensus.

We also followed An-RCC1-GFP during the cell cycle and did not detect any mitotic dispersal of this protein which, as in all studied cell types (Nemergut *et al.*, 2001), remained located with chromatin during interphase and throughout mitosis (Figure 5A and Supplemental Movie 2).

With one noticeable exception described below, during mitosis the tagged Nups either remained at the nuclear periphery (Table 1 and Figure 5B) or were dispersed throughout the cell (Table 1 and Figure 5C). The Nups observed to remain at the nuclear periphery are considered core Nups and include An-Nup84-GFP (Supplemental Movie 3), An-Nup85-GFP (Supplemental Movie 4), An-Nup120-GFP (Supplemental Movie 5), An-Nup170-GFP (Supplemental Movie 6), An-Gle1-GFP (Supplemental Movie 7), An-Ndc1-GFP (Supplemental Movie 8), and An-Sec13-chRFP (Supplemental Movie 9). This adds an

additional seven members to the previously described three core Nups consisting of An-Nup96-GFP, An-Nup133-GFP, and An-Pom152 (De Souza *et al.,* 2004).

None of the seven core Nups studied here displayed a particular location during mitosis except An-Ndc1-GFP, which routinely located around the nuclear periphery and to two foci at the nuclear periphery during part of mitosis (arrowed in mitotic nuclei, Figure 5B, Ndc1-GFP; see Supplementary Movie 8). Given that *S. cerevisiae* Ndc1 locates to both the NPC and the SPB (Chial *et al.,* 1998; Lau *et al.,* 2004) and that the *S. pombe* orthologue Cut11 locates to the NPC and transiently with the SPB during mitosis (West *et al.,* 1998), it is likely that the transient dots of An-Ndc1-GFP represent mitotic specific concentration at the SPB.

Observation of An-Sec13-chRFP indicates that a portion of this protein locates to the nuclear periphery, likely at the NPC, because Sec13p has been identified as a component of the Nup84/Nup107 NPC subcomplex (Siniossoglou *et al.,* 1996; Fontoura *et al.,* 1999; Enninga *et al.,* 2003; Loiodice *et al.,* 2004), and mass spectroscopy analysis of this complex in *A. nidulans* also detects An-Sec13 (Liu and Osmani, unpublished results). During mitosis, the An-Sec13-chRFP at the NPC is not dispersed, similar to other members of the Nup84 subcomplex (Figure 5 and Supplemental Movie 9). In addition to locating at the NPC, significant amounts of An-Sec13-chRFP was observed in dot-like structures at the cell periphery, particularly in the vicinity of the cell tip, but also throughout the cytoplasm. Interestingly, in germlings having 1–16 nuclei, it was relatively easy to discern nuclei with An-Sec13-chRFP at their periphery. However, longer germlings and hyphal apical cell compartments had a buildup of An-Sec13-chRFP toward the cell tip (Supplemental Figure 1 and Supplemental Movie 10, which shows confocal slice images through An-Sec13-chRFP–expressing cells) making observation of the nuclear periphery signal in projected stacked images more difficult.

In addition to the previous five Nups identified that disperse from the NPC during *A. nidulans* mitosis we have identified an additional eight (Table 1, Figure 5C) that display similar mitotic dispersal, including An-Nup49-GFP (Supplemental Movie 11), An-Nup57-GFP (Supplemental Movie 12), An-Nup82-GFP (Supplemental Movie 13), An-Nup188-GFP (Supplemental Movie 14), An-Nup192-chRFP (Supplemental Movie 15), An-Mlp1-GFP (Supplemental Movie 16), An-Nic96-GFP (Supplemental Movie 17), and An-Sac3-GFP (Supplemental Movie 18). Based on the observation that a core group of 10 Nups remain at the nuclear periphery, whereas 14 others are dispersed during mitosis, it is clear that the structure of the NPC of *A. nidulans* is drastically different between interphase and mitosis.

An-Nup2-GFP Relocates from the NPC to Chromatin during Mitosis

An-Nup2 was endogenously C-terminally tagged with GFP. Importantly, the An-Nup2-GFP strains were viable, underwent asexual and sexual development normally, and displayed no obvious phenotypes. Given that An-*nup2* is essential, this indicates the GFP-tagged version of An-Nup2 is functional.

An-Nup2-GFP dispersed from the NPC during mitosis. However, rather than dispersing throughout the cell, An-Nup2-GFP remained inside nuclei throughout mitosis. During mitotic exit An-Nup2-GFP rapidly returned back to the NPC as cells entered G1 and stayed at the NPC throughout interphase until the next mitosis (Figure 6A and Supplemental Movie 19). This dynamic location can be seen clearly in Supplementary Movie 19 and is reflected in the pixel profile through nuclei progressing into and through mitosis (Figure 6A). During interphase two peaks in the pixel profile, representing each side of the nucleus, can be distinguished, whereas at metaphase these peaks collapse to a single peak. During the transition from metaphase to telophase the single pixel profile peak is split into two because of the mitotic segregation of sister chromatids to which An-Nup-GFP is apparently associated. As nuclear division is completed and two transport competent nuclei are regenerated, An-Nup2-GFP again localizes to the nuclear periphery, as revealed in the pixel profile of each G1 nucleus.

Because the mitotic location of An-Nup2-GFP was reminiscent of proteins we have imaged that are located on *A. nidulans* chromatin, such as histone H1-mRFP and Rcc1-GFP, cells expressing An-Nup2-GFP were fixed and counterstained with DAPI to reveal the location of nuclear DNA. During interphase the An-Nup2-GFP signal is found surrounding the nuclear DNA as expected of a protein associated with the NPC (Figure 6B, interphase). During mitosis nuclear DNA undergoes condensation and mitotic nuclei can be distinguished based on their higher degree of DNA compaction. In all mitotic cells observed, rather than being located around the DNA, An-Nup2-GFP colocalized with compacted mitotic chromatin (Figure 6B, mitosis).

A strain was generated in which An-Nup2 was labeled with GFP and histone H1 with mRFP to follow by live cell imaging the mitotic location of An-Nup2-GFP with respect to nuclear chromatin. In Figure 6C a representative example of the location of An-Nup2-GFP and H1-mRFP are shown, along with a merged image and the pixel profiles of each signal. A supplementary movie (Supplementary Movie 20) is also provided. During G2, as expected, An-Nup2-GFP surrounds the nuclear DNA, and the green pixel profile of An-Nup2-GFP clearly locates outside the red profile representing H1-mRFP. This situation is dramatically changed during mitosis, with the two signals largely overlapping as seen in the merged image (Figure 6C, Merge) and by-the-pixel profiles for each signal. As mitosis is completed the An-Nup2-GFP signal again locates around the segregated chromatin as two daughter nuclei are generated during entry into G1. The data demonstrate that An-Nup2-GFP displays a distinctive location during mitosis in close proximity to condensed mitotic chromatin.

It has been well documented that several Nups, noticeably members of the Nup107 complex, locate to kinetochores specifically during open mitosis. However, only a fraction of these Nups locate to kinetochores. In contrast, the vast majority, if not all, An-Nup2 locates to chromatin, but we have been unable to detect any concentration of An-Nup2 to a particular chromosome domain. We therefore think that location of all An-Nup2 to chromatin is a phenomenon different from the previously reported location of a fraction of Nup107 subcomplex components to kinetochores (Belgareh *et al.,* 2001; Loiodice *et al.,* 2004). No function has been ascribed to the Nup107 complex components at kinetochores, but another NPC protein targeted to kinetochores, Nup358, plays a role in kinetochore assembly and function (Salina *et al.,* 2003; Joseph *et al.,* 2004).

DISCUSSION

To understand the molecular mechanisms by which the NPC of *A. nidulans* is disassembled and reassembled during mitosis it is necessary to define which Nups remain at the NPC and which disperse. To this end we have completed a systematic analysis of the components of the NPC by deleting and tagging most *A. nidulans* Nups and some nuclear transport genes.

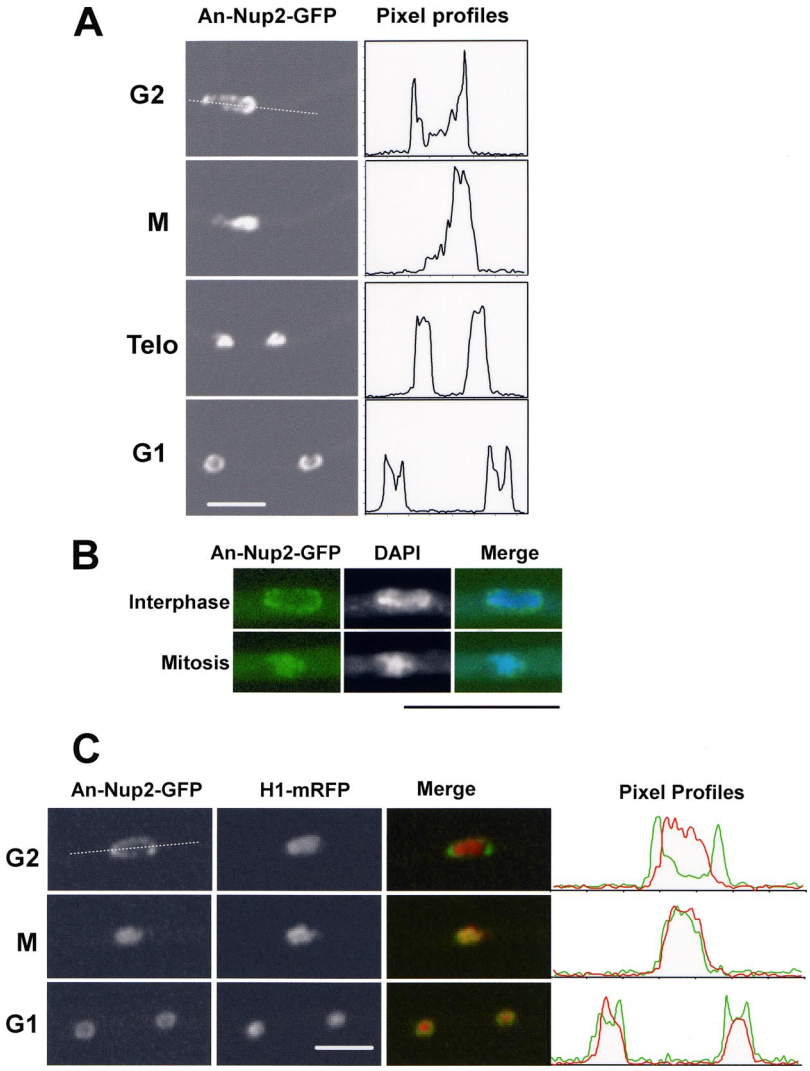

Figure 6. An-Nup2-GFP relocates from the NPC to DNA during mitosis. (A) The GFP signal for An-Nup2-GFP is shown during mitosis along with the pixel profile through the dividing nucleus. The micrographs are maximum intensity projections from multiple *Z*-sections from time-lapse confocal microscopy. The movie file is presented as a supplementary file (Supplementary Video 19). (B) The An-Nup2-GFP signal is shown in fixed interphase and mitotic cells. The location of DNA is shown by DAPI staining, and the merge of the two signals is shown. (C) The An-Nup2-GFP and histone H1-mRFP signals are shown as maximum intensity projections from live cell imaging in G2, mitosis (M), and G1 as indicated. A merged image is shown as are the pixel profiles for the An-Nup2-GFP (green) and histone H1-mRFP (red) signals through the dividing nucleus. The corresponding movie file is presented as a supplementary file (Supplementary Video 20). Scale bars, ~5 μm.

Identification of the Nucleoporins of A. nidulans

For the majority of *S. cerevisiae* Nups we were able to identify related proteins in *A. nidulans* with the exception of Nup1p, Nup53p, Nup59p, Nup60p, and Pom34p, as also determined by Mans *et al.* (2004). However, proteins with similarity to Nup53p and Nup59p, which are paralogs, have been detected in higher eukaryotes (Marelli *et al.*, 1998), and human Nup35 is considered an orthologue of Nup53p (Cronshaw *et al.*, 2002). The similarity between yeast Nup53p/Nup59p and human Nup35 lies mainly in the MPPN (mitotic phosphoprotein N′ end) domain, which is conspicuous by its absence in filamentous fungi. It is unclear why filamentous fungi lack a class of Nup found in many cell types. Because Nup53p binds to Nup170p (Marelli *et al.*, 1998), further insight will perhaps be forthcoming when the binding partners of An-Nup170 are defined.

Mutation of the Ran GTPase Cycle Genes

As in all systems, components of the RanGTPase cycle and importinβ1 are essential in *A. nidulans*. As expected, An-Rcc1 is located to nuclear chromatin and could generate a RanGTP gradient across the nuclear membrane by promoting RanGTP production within nuclei. However, as An-RanGAP gains access to nuclei during *A. nidulans* mitosis (De Souza *et al.*, 2004) the RanGTP gradient across the NE is likely lost at this stage of the cell cycle. Instead, because An-Rcc1 remains in the vicinity of mitotic chromatin, the RanGTP concentration is likely to be high around *A. nidulans* chromatin during mitosis. This proposed mitotic shift in the RanGTP gradient of *A. nidulans* is known to occur during open mitoses (Carazo-Salas *et al.*, 1999; Nemergut *et al.*, 2001; Li *et al.*, 2003; Li and Zheng, 2004; Kalab *et al.*, 2006), which helps promote mitosis (Hetzer *et al.*, 2002; Weis, 2003; Harel and Forbes, 2004). Therefore, An-Ran, An-Rcc1, An-RanGAP, and An-importinβ1 could play essential roles in both nuclear transport and mitotic regulation.

Deletion Analysis of Nonessential NPC Proteins

As found in *S. cerevisiae*, several Nup deletions in *A. nidulans* cause temperature sensitivity or cold sensitivity. Three of the mutations causing temperature sensitivity are members of the Nup84 subcomplex, a major structural component of the NPC (Siniossoglou *et al.*, 1996, 2000; Allen *et al.*, 2002; Lutzmann *et al.*, 2002, 2005) conserved in *S. pombe* (Bai *et al.*, 2004) and higher eukaryotes (termed the Nup107 complex; Fontoura *et al.*, 2001; Belgareh *et al.*, 2001; Vasu *et al.*, 2001).

The Nup84 complex consists of Nup84p, Nup85p, Nup120p, Nup133p, Nup145Cp, Seh1p, and Sec13p. Five of the seven

components of this subcomplex are not essential in *A. nidulans* or *S. cerevisiae,* including *NUP84*, *NUP85*, *NUP120*, *NUP133,* and *SEH1*. Similarly, in *S. pombe* and *C. elegans NUP120*, *NUP133,* and *SEH1* are nonessential genes (Table 1). During *A. nidulans* mitosis the Nup84 complex helps make a core NPC structure, and it is therefore somewhat surprising that over half of the core Nups we have identified are not essential for growth, including both of the predicted membrane-associated Nups. These would be expected to help anchor the NPC within the NE. These data verify there is considerable redundancy in the function of the core NPC proteins in systems undergoing open, closed, or partially open mitoses.

Regarding the membrane associated Nups, it was widely believed that Ndc1 and its orthologues were essential in all systems including *S. cerevisiae* (Chial *et al.,* 1998; Lau *et al.,* 2004; Madrid *et al.,* 2006) *S. pombe* (West *et al.,* 1998), and higher eukaryotes (Mansfeld *et al.,* 2006; Stavru *et al.,* 2006a). However, recent findings suggest no single membrane NPC protein is universally essential (Stavru *et al.,* 2006a, 2006b) because *C. elegans* has no absolute requirement for Ndc1 and protozoa lack Ndc1. Importantly, we have been unable to define phenotypes associated with either the ΔAn-*ndc1* or ΔAn-*pom152* alleles. This is particularly surprising for An-*ndc1* as its orthologues are involved in NPC function, SPB duplication, and spindle formation (Chial *et al.,* 1998; West *et al.,* 1998; Lau *et al.,* 2004) because Ndc1p is required for insertion of the NPC and SPB into the NE (Lau *et al.,* 2004). Because An-Ndc1 is not essential, it is unlikely to be required for insertion of NPCs or SPBs into the NE.

Deletion Analysis of the Essential Nucleoporins

Deletion of several essential Nups did not cause a tight growth-arrest phenotype, but instead cells grew for a period of time and the cell cycle continued leading to structural nuclear defects. This caused a variable phenotype with cells containing inconsistent numbers of nuclei with uneven amounts of DNA. There is therefore surprisingly no negative feedback system to prevent cells from progressing through the cell cycle when an essential component of the NPC is absent.

Because nearly all the essential Nups locate to the NPC, it is reasonable to conclude that their essential function involves the NPC. However, as An-*nup2* locates to the NPC during interphase but to chromatin during mitosis, it is possible that it has an essential function at the NPC and/or at chromatin during mitosis (see below). Similarly An-Sec13 exists in two locations. Some locates in the cytoplasm, likely in a complex with An-Sec31, to play a role in protein secretion as part of the COPII vesicle coat (Bickford *et al.,* 2004), and some resides at the NPC as part of the Nup84 subcomplex. In this regard it is notable that deletion of An-*sec13* causes a tight block in spore germination, which perhaps reflects defects in both NPC function and protein secretion.

Mitotic Location of NPC Components

During mitosis a large number of the NPC proteins do not locate at the NPC but instead disperse throughout the cell (Figure 7). Based on comparison to other systems, most of the *A. nidulans* dispersed Nups are predicted to be peripherally located within the NPC structure, including *all* of the seven identified FG-repeat Nups. In fact virtually all of the Nups predicted to locate at the NPC periphery, including components of the cytoplasmic fibrils and nuclear basket, are collectively dispersed from the NPC at mitosis. Of the 26 Nups studied to date, impressively, 14 disperse from the NPC during mitosis (Figure 7). It is therefore clear that the minimal mitotic NPC of *A. nidulans* is unlikely to be capable of mediating regulated nuclear transport, consistent with our previous work (De Souza *et al.,* 2004).

During mitosis the core Nups likely generate a structure that spans the NE to provide a conduit between the cytoplasm and nucleoplasm and act as a scaffold to which dispersed Nups return during mitotic exit (Figure 7B). This core structure contains the two predicted membrane-spanning Nups (orthologues of Ndc1 and Pom152) plus all members of the Nup84 subcomplex. Thus, although no data exist to confirm the location of *A. nidulans* Nups within the architecture of the NPC, our mitotic NPC data (Figure 7B) correlate well with previous work defining the Nup84 complex, along with membrane Nups, as being the structural core of the NPC (Tran and Wente, 2006).

Although most of the peripheral Nups disperse from the NPC during mitosis there was one notable exception. An-Gle1 does *not* disperse but remains at the NPC throughout the cell cycle. This suggests that An-Gle1 could be part of the core NPC in *A. nidulans*, at least during mitosis (Figure 7B). In *S. cerevisiae*, Gle1p is located on the cytoplasmic side of the NPC with Nup42p, Nup82p, Nup100p, Nup159p, and Nsp1. There Gle1p binds to the DEAD box helicase Dbp5p as does Nup159p. These interactions are thought to anchor Dbp5 at the cytoplasmic side of the NPC where this helicase functions to aid transport of mRNPs through the NPC (Snay-Hodge *et al.,* 1998; Tseng *et al.,* 1998; Hodge *et al.,* 1999; Strahm *et al.,* 1999; Kendirgi *et al.,* 2005). Recent work has shown that Gle1p activates the RNA-dependent ATPase activity of Dbp5 to facilitate mRNA export (Alcazar-Roman *et al.,* 2006; Cole and Scarcelli, 2006; Weirich *et al.,* 2006). Remarkably, all of the orthologues of known Gle1p interacting Nups are disassembled from the NPC during *A. nidulans* mitosis, although An-Gle1 remains (Figure 7). One potential mitotic NPC binding partner for An-Gle1 is suggested from work on human Gle1, which binds to Nup155 (Rayala *et al.,* 2004; Kendirgi *et al.,* 2005). Nup155 is a homologue of *S. cerevisiae* Nup170p and, perhaps importantly, *A. nidulans* An-Nup170 is the only other non-Nup84 subcomplex, non–membrane-associated Nup to remain at the NPC during mitosis. We suggest An-Gle1 could play novel functions during mitosis, and it is interesting to note that RNA has recently been proposed to play a role in spindle formation (Blower *et al.,* 2005).

An-Nup2 Locates to Mitotic Chromatin

An-Nup2 is released from the NPC during mitosis and locates to DNA (Figure 7). As cells progress out of mitosis, An-Nup2 returns to the NPC. This suggests An-Nup2 may fulfill two functions, one at the NPC during interphase and another at chromatin during mitosis. In *S. cerevisiae* the primary function of Nup2p is to facilitate nuclear transport by accelerating the rate of disassembly of importin α/β-cargo complexes at the nuclear side of the NPC (Gilchrist *et al.,* 2002; Matsuura *et al.,* 2003). If An-Nup2 has a similar capacity, it could facilitate release of importinα/β cargoes during nuclear transport. However, as An-Nup locates exclusively to chromatin during mitosis, it could perhaps accelerate disassembly of importin α/β-cargo complexes in the vicinity of chromatin and so help orchestrate mitosis. In addition, because An-Nup2 contains a C-terminal Ran-binding domain, which may stabilize RanGTP, An-Nup2 located at chromatin could help concentrate RanGTP around DNA and further help promote mitosis. It is possible that An-Nup2 may help to coordinate disassembly of the NPC with mitotic specific events. For instance, if An-Nup2 were required for chromatin condensation, its location to the NPC would ensure condensation could not occur during inter-

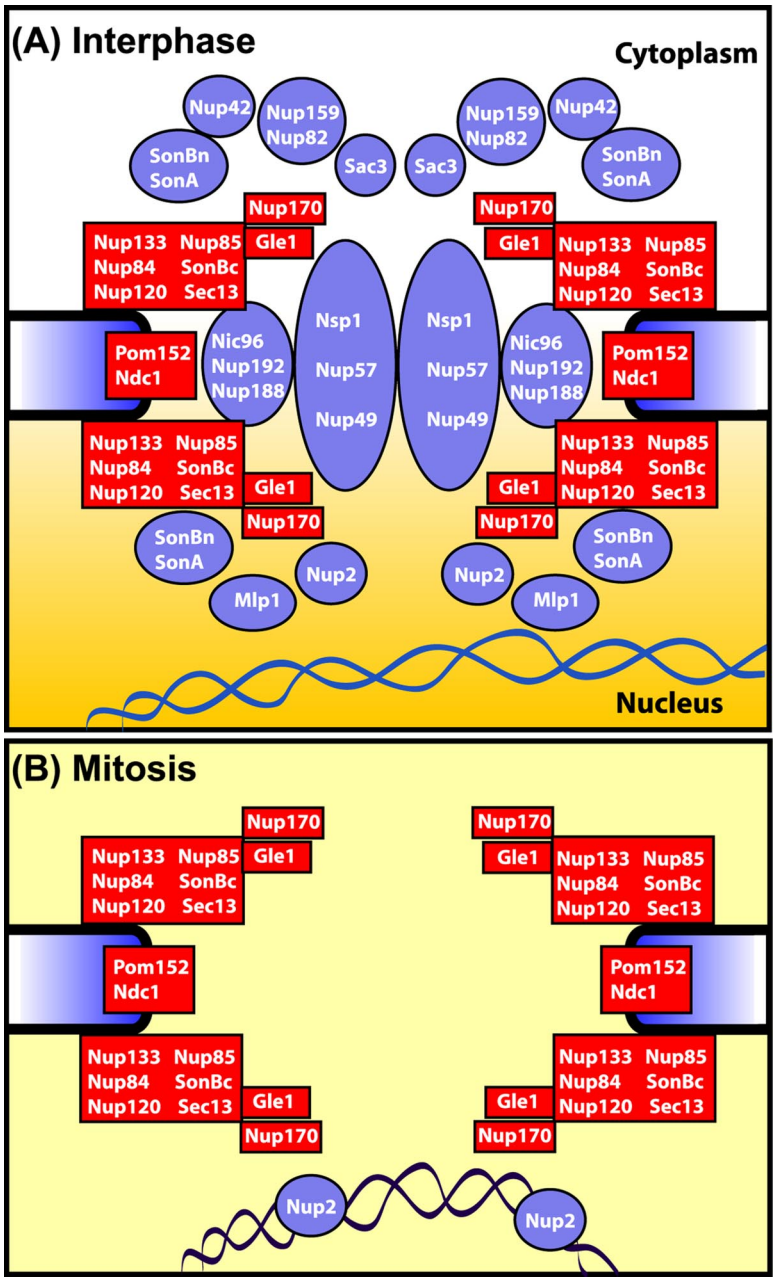

Figure 7. Mitotic changes in the composition of the *A. nidulans* NPC. (A) Depicted are the predicted location of *A. nidulans* Nups during interphase and (B) during mitosis. It is noteworthy that all the Nups we have found to disperse from the NPC (in blue) are known to be peripheral Nups, whereas the core Nups (in red) consist of the Nup84 subcomplex, membrane Nups and An-Gle1 and An-Nup170. All of these are considered core Nups in other systems with the exception of An-Gle1, which is a peripheral Nup involved in RNA export. An-Nup2 is unique as it locates from the NPC to chromatin during mitosis. The model is based on data from the current study and De Souza *et al.* (2004). The figure is modeled after Powers and Dasso (2004).

phase until An-Nup2 were released from the NPC. Furthermore, functional NPCs may not then be formed until An-Nup2 were released from chromatin and located back at the NPC to promote nuclear transport in G1. This would be an effective mechanism to coordinate NPC opening and closing with mitotic entry and exit.

CONCLUSIONS

The findings reported here provide an essential framework (Figure 7) from which to further define the molecular mechanisms of mitotic regulation of the NPC. The work provides numerous insights into the dynamic nature of the NPC structure during cell cycle progression. Of particular interest is the mitotic location of An-Nup2 to chromatin suggesting this essential protein has roles both at the NPC in interphase and around DNA during mitosis, perhaps to coordinate NPC function with mitosis. The findings also focus attention on mitotic An-Gle1. This peripheral RNA export Nup remains at the NPC during mitosis, whereas all other putative peripheral Nups are dispersed. This indicates that An-Gle1 could also serve a novel role during mitosis. Because *A. nidulans* mitosis is an evolutionary intermediary between open and closed mitoses, study of the NPC in this species should provide continuing insights into how the NPC is disassembled and reassembled during mitosis.

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