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Development of a Novel Assay to Monitor Nuclear Pore Complex in Saccharomyces cerevisiae

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Development of a Novel Assay to Monitor Nuclear Pore Complex in *Saccharomyces cerevisiae*

By

Leslie Helen Stanton

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Abstract

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Eukaryotic cells can be distinguished from prokaryotic cells because they posses membrane bound organelles. The presence of organelles in cells allowed cellular processes to be isolated into compartments, thus allowing additional levels of regulation to be applied to simple cellular processes. One of these membrane bound organelles, the nucleus, functions to isolate the cell's DNA from the cytoplasm. Large aqueous pores span the nuclear envelope and determines which molecules can enter and exit the nucleus. These channels are called Nuclear Pore Complexes (NPCs). The NPC is made up of a central core that spans the NE, a nuclear basket structure, fibers that extend into the cytoplasm, and is composed of only ~33 different proteins called Nucleoporins. The NPC exhibits an eight-fold rotation symmetry around the plane of the NE, and nucleoporins are present in eight, or multiples of eight, copies. Although the structure and composition of the NPC is well characterized, the process in which NPCs are inserted into the intact NE of yeast is unknown.

In order to learn more about NPC assembly into the intact NE, I developed an assay to monitor the distribution of old and new nucleoporins in live *S. cerevisiae* cells. I used the photoconvertable fluorescent protein Dendra to examine a report that new NPC assembly occurs exclusively in daughter buds while old NPCs remain with mother cells. We examined two different Nups and observed new pore formation in both mother and daughter cells, additionally we determined that old pores are inherited by both mother and the daughter cells. We hypothesized that the differences in our observations from the previous report was due to differences experimental technique.

To begin to understand the early events of NPC assembly, we first determined which proteins interact with the essential transmembrane Nup Ndc1. We then constructed conditional mutants of different combinations of these proteins and determined that Nup59 is functionally redundant with the combined element of Pom34 and Pom152. We show that these conditional mutants are not viable and result in mislocalization of core and cytoplasmic Nups. We use the Dendra assay to show that depletion of these elements result in a reversible defect in NPC assembly.

Finally, we examine the role of Nup157 and Nup170 in NPC assembly. A *nup157*Δ strain conditionally expressing Nup170 results in mislocalization of core and

cytoplasmic reporter Nups, but not nuclear Nups. This strain also has a reduced number of NPCs by EM in non-permissive conditions. Cells depleted of Nup170 and Nup157 were examined with the Dendra assay and new protein accumulated in the cytoplasm suggesting a block in NPC assembly. This block is overcome by reintroduction of Nup170. The work presented here represents a new way to study NPC assembly and reveals a few early events in NPC assembly.

Karsten Weis

For Soni

You will always be loved,

you will never be forgotten.

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DNA	Deoxyribonucleic Acid
EM	Electron Microscopy
ER	Endoplasmic Reticulum
FLIP	Fluorescence Loss in Photobleaching
FRAP	Fluorescence Recovery After Photobleaching
kDa	Kilodalton
GAP	GTPase Activating Protein
GEF	GTP Exchange Factor
GFP	Green Fluorescent Protein
Kap	Karyopherin
MS	Mass Spectrometry
mRNA	messenger Ribonucleic Acid
RNP	Ribonucleoprotein
NE	Nuclear Envelope
NLS	Nuclear Localization Sequence
NPC	Nuclear Pore Complex
Nup	Nucleoporin
POM	Pore Membrane Proteins
SDS-PAGE	Sodium-Dodecylsulfate Polyacrylamide Gel Electrophoesis
SPB	Spindle Pole Body
UV	Ultraviolet

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Chapter 1

Introduction

Introduction

Eukaryotic cells are distinguishable from prokaryotic cells by the presence of membrane bound organelles. The evolution of organelles in cells is important because it allowed cellular processes to be isolated into compartments, thus allowing additional levels of regulation to be applied to simple cellular processes. One of these membrane bound organelles, the nucleus, functions to isolate the cell's DNA from the cytoplasm. In prokaryotes, proteins are translated as soon as an mRNA is transcribed from DNA, however in eukaryotes, the translational machinery resides in the cytoplasm, and the mRNA must be exported out of the nucleus to be translated. The membrane of the nucleus is made up of two lipid bilayers, the inner nuclear envelope and the outer nuclear envelope. The Nuclear Pore Complex (NPC) is a large channel that spans both layers of the nuclear envelope and determines which molecules can enter and exit the nucleus; this process is called nucleocytoplasmic transport.

Nucleocytoplasmic Transport

Transport through the NPC is dependent on the size of the molecule. Proteins that are smaller than 40 kDa can diffuse freely in and out of the nucleus, while proteins that are larger than 40 kDa are transported in or out of the nucleus with the aid of soluble transport factors called Karyopherins. Karyopherins have several functions: 1) they interact with Ran 2) they recognize cargo that needs to be imported or exported and 3) they interact with proteins that contain phenylalanine-glycine-rich repeats and line the central channel of the NPC. Fourteen Karyopherins have been identified in *S. cerevisiae* (reviewed in Ström, and Weis, 2001). There are two types of Karyopherins: Importins and Exportins. Importins are exclusively involved in protein import into the nucleus while Exportins are involved in nuclear export. Karyopherins recognize proteins for import or export based on the presence of import or export signal sequences.

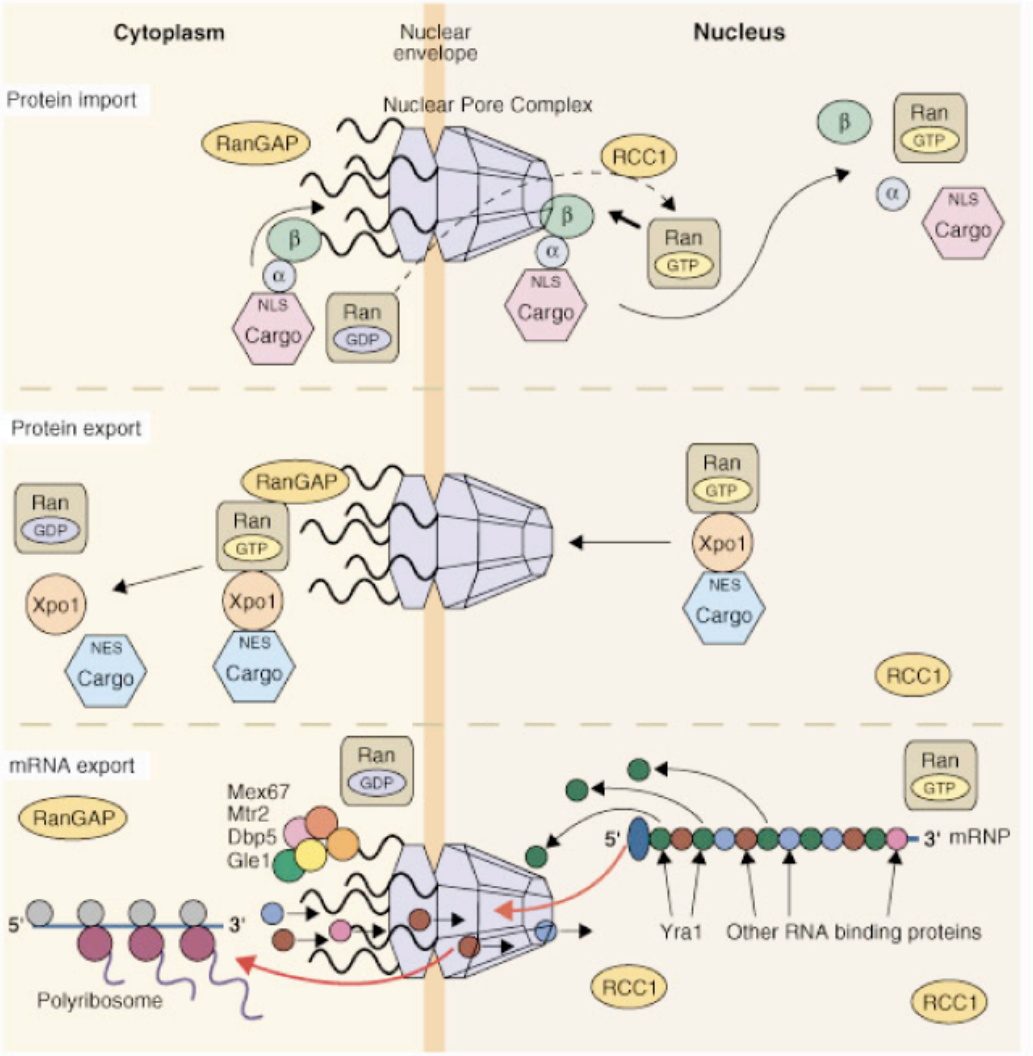
Ran is a small GTPase, which exists in two states, GTP bound or GDP bound. The nucleotide state of Ran is essential for determining whether karyopherins should bind or release their cargo. Exportins bind cargo in the nucleus with RanGTP; importins release cargo in the nucleus with RanGTP. The proteins RanGEF and RanGAP are responsible for changing the nucleotide-bound state of Ran. RanGEF is an exchange factor that replenishes RanGDP with RanGTP. RanGAP stimulates GTP hydrolysis. The localization of these proteins is responsible for determining the directionality of nucleocytoplasmic transport (Nachury, and Weis, 1999). RanGEF is located in the nucleus and RanGAP is located in the cytoplasm.

A protein which contains a nuclear localization sequence (NLS) in the cytoplasm is recognized and bound by Importin-β, and this complex moves through the NPC. Once the complex of Importin-β-cargo enters the nucleus, it binds to RanGTP which causes the cargo to be released from Importin-β (Fig 1.1A). Nuclear export begins in the nucleus; a tripartate complex is formed with cargo, exportin and RanGTP. The complex is exported from the nucleus, RanGTP is hydrolyzed to RanGDP, and the cargo is released (Fig 1.1B).

mRNA Export

mRNA export uses a different mechanism for export from the nucleus than proteins. Export is coupled to mRNA processing, and mRNAs must be properly capped,

Figure 1.1



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Nature Cell Biology 2, E55 - E58 (2000)

Figure 1.1 Nucleocytoplasmic Transport A) In protein import, cytoplasmic proteins containing NLSs are recognized by import receptor proteins (Importin α and Importin β), which move together through NPCs. Within the nucleus, the complex dissociates when it encounters the small GTPase, Ran, bound to GTP, and delivers its cargo to the nucleus. B) Protein export occurs through the cooperative association in the nucleus of RanGTP, cargo containing a NES and an export receptor. Once on the cytoplasmic side of the NPC, hydrolysis of GTP bound to Ran results in dissociation of the complex and delivery of the cargo to the cytoplasm. C) In mRNA export, RNA-binding proteins package the mRNA into an exportable configuration. The mRNP interacts with factors on the cytoplasmic fibrils of the NPC, where they mediate translocation through the pore and removal of shuttling RNA-binding proteins. Adapted by permission from Macmillan Publishers Ltd: Cole, C.N. 2000. mRNA export: the long and winding road. *Nat.Cell Biol.* 2:E55-8. *Nature Cell Biology* 2, E55 - E58 (2000).

spliced, and poly-adenylated before they can leave the nucleus. mRNAs are guided through the NPC by proteins, and the complex of mRNAs and associated proteins are called the messenger ribonucleoprotein particle (mRNP). The composition of the mRNP changes throughout mRNA export, but the exact composition of mRNPs is not known. The main mRNA exporter in yeast is a dimer composed of two proteins, Mex67 and Mtr2. Like karyopherins, Mex67 interacts with FG-repeat Nups to move the mRNA through the NPC. One idea about how translocation through the pore occurs is through the use of a Brownian ratchet, which strips Mex67/Mtr2 from the mRNA as it encounters the cytoplasmic filaments of the NPC (Lund, and Guthrie, 2005). Without Mex67/Mtr2, the mRNA can no longer interact with FG repeats in the channel of the NPC and is stuck in the cytoplasm. One important factor in this process is the nucleoporin Dbp5, which facilitates the removal of Mex67 from mRNA. From this point, Dbp5 may prevent the backsliding of mRNA back through the NPC and this process continues until the whole mRNA has exited the NPC (Fig 1.1C).

The Nuclear Pore Complex

The Nuclear Pore Complex (NPC) is an immense structure made of many proteins which spans the inner and outer nuclear envelope, and forms an aqueous channel through which all nucleocytoplasmic transport events occur. The NPC is one of the largest macromolecular assemblies in the eukaryotic cell. The vertebrate NPC is approximately 125MDa, while the yeast NPC is over 60 MDa (Reichelt et al., 1990, Rout, and Blobel, 1993). Electron microscopy studies of Xenopus NPCs show that the structure of the NPC is a cylinder that spans a channel in the nuclear envelope and exhibits eightfold rotational symmetry (Reichelt et al., 1990). This central core cylinder appears to be symmetrical, but asymmetry is visible on the nuclear and cytoplasmic sides of the NPC. Long fibers emanate from the central core structure into the nucleoplasm and meet in a ring-like structure, while filaments protrude from the core into the cytoplasm (Jarnik, and Aebi, 1991). These two structures are called the nuclear basket and cytoplasmic filaments, respectively (Fig 1.2A). The NPCs of Saccharomyces cerevisiae are similar in appearance to vertebrate NPCs (Kiseleva et al., 2004), but approximately 25% smaller, with a diameter of 95 nm compared to the vNPC which has a diameter of 110-120 nm (Goldberg et al., 1996). Yeast cells increase the number of NPCs present on the NE throughout interphase and reach approximately 200 pores before cell division cuts this number in half (Winey et al., 1997).

Even though the NPC is one of the largest macromolecular assemblies in the cell, proteomics studies have revealed that the NPC is composed of only ~33 different proteins. These proteins are called nucleoporins, or Nups. The 33 Nups were characterized by their presence in a highly enriched NPC fraction and analyzed by immunofluorescence for punctate fluorescent localization to the NE (Rout et al., 2000). These 33 Nups were also visualized by immunogold EM to determine their subcellular localization (Table I). Based on the immunogold EM, the localization of a Nup can be divided into 3 classes: nuclear Nups, which are only present on the nuclear side of the NPC, cytoplasmic Nups, which are only present on the cytoplasmic face of the NPC, and symmetrical Nups, which are present on both sides of the NPC. Several symmetrical Nups are not equally distributed on both sides or the NPC. These Nups

Table I

Yeast	Vertebrate	Localization	Essential in	FG Repeats
Nup1	Nup153	nuclear	no	yes
Nsp1	Nup62	symmetric	yes	yes
Nup2	Nup50	biased-nuc	no	yes
Nup42	NLP1/hCG1	cytoplasmic	no	yes
Nup49	Nup58/Nup45	symmetric	yes	yes
Nup53	Nup35	symmetric	no	no
Nup59	Nup35	symmetric	no	no
Nup57	Nup54	symmetric	yes	yes
Nup82	Nup88	cytoplasmic	yes	no
Nup84	Nup107	symmetric	no	no
Nup85	Nup75/Nup85	symmetric	no	no
Nic96	Nup93	symmetric	yes	no
Nup100	Nup98	biased-cyto	no	yes
Nup116	Nup98	biased-cyto	no	yes
Nup145N	Nup98	biased-nuc	yes	yes
Nup120	Nup160	symmetric	no	no
Nup133	Nup133	symmetric	no	no
Nup145C	Nup96	symmetric	yes	no
Nup157	Nup155	symmetric	no	no
Nup170	Nup155	symmetric	no	no
Nup159	Nup214/CAN	cytoplasmic	yes	yes
Nup188	Nup188	symmetric	no	no
Nup192	Nup205	symmetric	yes	no
Gle1	hGle1	biased-cyto	no	no
Gle2	Rae1/Gle2	symmetric	no	no
Seh1	Seh1	symmetric	no	no
Sec13	Sec13	symmetric	yes	no
Nup60		nuclear	no	yes
Ndc1	Ndc1	membrane	yes	no
Pom34	-------	membrane	no	no
Pom152	-------	membrane	no	no

(Suntharalingam, and Wente, 2003)

are referred to as biased. The relative abundance of individual Nups was also examined in this study, revealing that many Nups are present in multiples copies which varies between 8 and 32. The fact that Nups are present in multiples of 8 copies is consistent with the observed 8-fold rotational symmetry of the NPC.

Nups can also be subdivided by the role that they play in NPC architecture. Approximately two thirds of the Nups are involved in building the structure of the NPC and make up the structures of the central core, nuclear basket, or cytoplasmic fibers of the NPC (Fig 1.2B). A special class of these structural Nups are the transmembrane Nups, or pore membrane proteins (POMs). Despite the fact that the NPC is a large structure that spans the NE, there are only 3 proteins which are transmembrane proteins. The final class of Nups contain repeats of the amino acids phenylalanine (F) and glycine (G), and are called FG repeat Nups. These Nups interact with the nucleocytoplsmic transport machinery and line the inner channel of the NPC. The FG repeats Nups form a hydrophobic barrier which prevents large molecules (greater than 60 kDa) from entering or exiting the nucleus, unless they are bound to transport factors that facilitate movement through the barrier (Paine et al., 1975).

An interesting observation about the yeast NPC is that many Nups can be deleted individually without an effect on NPC function or architecture. Ten Nups are essential, and six nups have conditional phenotypes when deleted. The remaining Nups are not required for cell viability. Additionally, there are several instances of Nups that are highly similar and possibly arose from a whole genome duplication that *S. cerevisiae* underwent ~100 million years ago (Wolfe, and Shields, 1997). Many of the

Figure 1.2

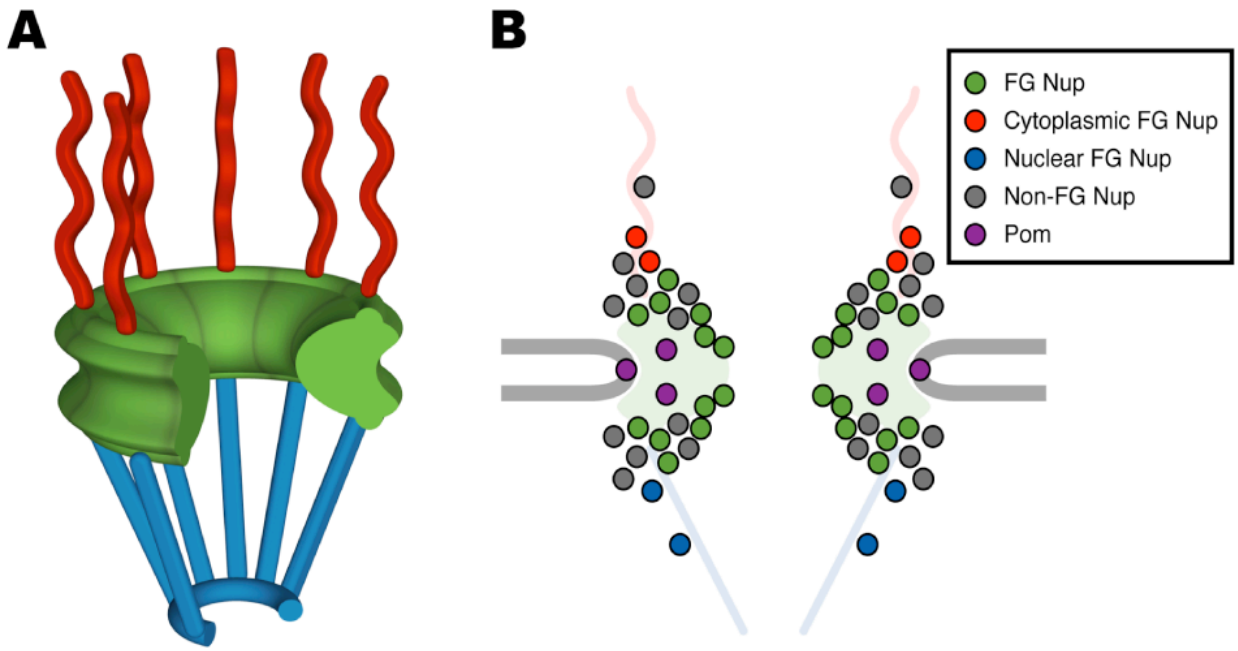

Figure 1.2 Schematic representation of NPC structure and composition A) The structure of the NPC is divided into 3 area: the central core which spans the NE (green), the nuclear basket (blue) and the cytoplasmic filaments (red). B) A representation of the location of different types of Nups in the NPC. Figure created by Bryan Zeitler.

non-essential yeast genes are synthetically lethal with other Nups, indicating that there is a high degree of functional redundancy within the NPC.

Although the overall structure of the NPC is well characterized, the structures of individual Nups and the positions which they occupy within the NPC is not well understood. Computational studies have predicted that Nups only contain 8 different fold types, and ~85% of the residues examined were either α-solenoid, FG repeats, or β-propeller structures (Devos et al., 2006). Additionally, transmembrane helixes, coiled coil, cadherin, Nup98, and RRM fold types were also predicted to be present in Nup structures. FG repeats present on a third of nups, are largely unstructured, and line the center of the pore where they interact with karyopherins. The proteins that make up the central core scaffold of the NPC are beta propellers and alpha solenoids domain for the most part. The particular combination of α-solenoids and β-propellers is a common motif in vesicle coats, another cellular structure that, like NPCs, gives curvature to membranes.

Structural studies of Nups have revealed that many of the aforementioned predictions about the structures of Nups were correct. Several structures predicted to contain β-propellers, including Nup133, Nup120, and Nup159 (Berke et al., 2004, Weirich et al., 2004, Leksa et al., 2009), were valid. Seh1 and Sec13 were structurally determined to have six blades instead of the normal seven, however, neighboring proteins provide the seventh blade in each case (Brohawn et al., 2008, Debler et al., 2008, Fath et al., 2007, Hsia et al., 2007). Studies of the α-solenoid group of proteins revealed different α-helical folds were present than the predicted α-solenoid. One striking discovery was the ACE1 domain, a 65kDa stretch consisting of 28 helices, which was present in Nic96, Nup84, Nup85, and Nup145C. Structural predictions of Nup84, Nup85, and Nup145C, all members of the Nup84 subcomplex, as combinations of α-solenoids and β-propellers had led to the idea that the Nup84 subcomplex was related to COPII vesicle coats and performed a similar function of bending membranes. The discovery of the ACE1 motif was exciting because it had only been detected in Sec31, a member of the COPII vesicle coat. The sequence of the ACE1 domain is not well conserved and it is difficult to determine other occurrences without structural data, but may be present in other Nups as well.

Another source of information about the location of particular nucleoporins within the NPC comes from biochemical purifications. In most cases, purification of one Nup results in the co-purification of several other Nups. These copurifying groups of Nups are called subcomplexes. Subcomplexes are thought the be the basic building blocks of NPCs. Different subcomplexes may have different roles at the pore; for example, the Ndc1 subcomplex (which consists of Ndc1, Pom152, and Pom34) bridges the NE and the central core of the pore and possibly represents the innermost layer of the NPC. The Nup157/Nup170 complex interacts with the Ndc1 subcomplex and potentially plays a role in making a hole in the NE. The Nup84 subcomplex (Nup84, Nup85, Nup120, Nup133, Seh1, Sec13), which forms a Y-shape and is structurally similar to the COPII vesicle coat, is thought to interact with Nic96 and Nup157 to help stabilize the curvature of the membrane without actually inserting into the lipid bilayer. In yeast, approximately two thirds of all known Nups associate with a subcomplex (Lutzmann et al., 2005). However, it has so far proven impossible to purify intact, function nuclear pore complexes. Previous research has taken advantage of the modular assembly of

nucleoporins to conduct low resolution structural analysis of one these smaller subunits, the Nup84 subcomplex, demonstrating that it adopts a Y-like structure (Lutzmann et al., 2002). Subcomplexes exclusively containing structural Nups have also been identified along with subcomplexes containing both structural and FG repeat Nups (Belgareh et al., 2001). Some Nups, such as Nic96, occur in more than one subcomplex, suggesting these nups may bridge the interaction between different NPC subunits.

In 2007, Alber et al (Alber et al., 2007a, Alber et al., 2007b) attempted to determine the position of every Nup in the yeast NPCs by using Immunogold EM, computational fold predictions, pulldowns, and analytical ultracentrifugation data. The results were translated into positional data which were used to construct a computational map of the NPC. It will be interesting to find out how accurate this computational map is as advances are made in NPC ultrastructural studies and nucleoporin crystallography.

NPC Assembly

While many individual nucleoporins have been well characterized, the process in which nucleoporins come together to form the NPC remains unknown. In general, it is thought to occur in a modular fashion, since most NPC components can be isolated as members of stable subcomplexes, but it is not known how much of the NPC is assembled before insertion onto the NE and whether subcomplexes are assembled before, during, or after being added to the pore. In eukaryotes, NPC assembly is thought to occur via at least two mechanisms. First, NPCs can be assembled post-mitotically as the nuclear envelope reforms around chromatin. Post-mitotic NPC assembly has been dissected in the Xenopus extract system and was shown to require the small GTPase Ran, which associates with chromatin (Walther et al., 2003b). Binding of ELYS to chromatin precedes binding of the vNup107-60 subcomplex (Franz et al., 2007). The vNup107-60 complex is required for the recruitment of other Nups (Harel et al., 2003, Boehmer et al., 2003, Walther et al., 2003a). In particular, the transmembrane Nup POM121 interacts with the cytoplasmic domain of the vNup107-160 complex to bring NDC1 to the site of NPC formation (Rasala et al., 2008).

Secondly, NPCs are also continuously assembled and inserted into the intact nuclear envelope during interphase. Many unicellular eukaryotes, such as *S. cerevisiae*, which do not undergo nuclear envelope breakdown during the mitosis, have to rely exclusively on this pathway for the de novo assembly of NPCs. However, despite its importance, the mechanism by which Nups are recruited and inserted into intact nuclear envelopes remains poorly understood. It is thought that interphase NPC assembly occurs in a stepwise fashion. In the first step, transmembrane proteins which are present on the inner NE and outer NE interact with some factor to make a channel in the NE. The second step involves the recruitment of structural Nups and the formation of the main structures of the NPC. In the final step, FG repeat Nups are recruited to the NPC and the structure becomes functionally competent.

There are still many events in do novo interphase NPC assembly that need to be discovered, but a few steps in NPC assembly have recently been characterized, and mark the beginning of the construction of an order of assembly. This recent evidence is consistent with a stepwise assembly hypothesis. Interphase NPC assembly in *S. cerevisiae* appears to take place on both the inner NE and the outer NE. The nuclear

transport machinery is required for proper NPC assembly, although interactions with chromatin have not yet been demonstrated. Interestingly, no ELYS homolog has been found in yeast, which might explain why interactions with chromatin have not been found. The transmembrane proteins Ndc1, Pom152, and Pom34, which together form the transmembrane subcomplex, interact with several structural Nups and are thought to be one of the earliest factors present at nascent pores. Interestingly, the loss of the transmembrane subcomplex's link to Nup59 in conditional mutants results in the destabilization of already formed pores (Onischenko et al., 2009). Evidence for the involvement in pore formation of proteins other than Nups is beginning to appear. The deletion of two ER membrane proteins, Rtn1, Yop1, results in nuclei with pre-pore like structure on both the inner and outer NE. Apq12 and Brr6 also have pore assembly defects. Therefore it is possible that the transmembrane proteins do not mediate pore formation by themselves, but some transient factor is required with the transmembrane proteins to puncture the channel.

Although the earliest step in NPC assembly is not well understood, the purported next step has recently been characterized. As mentioned above, when the transmembrane subcomplex loses its interaction with Nup59, it results in oversized NPCs and holes in the NE that lack proteins. It was not clear whether these were pores that were being formed that lacked a critical amount of transmembrane subcomplex and could not make complete pores, or if they were intact pores that had fallen apart. A major focus of my thesis was to address this functional question in NPC assembly.

An interesting intermediate between the post mitotic and interphase de novo assembly has recently been discovered. The fungus *Aspergillus nidulans* contains many Nup homologs to yeast NPCs, and during mitosis, many of these proteins exit the nuclear pore (Osmani et al., 2006). The resulting structure only contains the transmembrane proteins, the Nup84 subcomplex, Nup170, and Gle1. This minimal pore might be useful in future studies of *S. cerevisiae* NPC assembly and most likely represents an evolutionary intermediate between open and closed mitosis that these cells have adapted.

Purpose of this Study

Interphase NPC assembly is an important mechanism in which cells assemble NPCs, yet the process of NPC assembly into intact nuclear envelopes remains poorly understood. Yeast were chosen for this study because unlike metazoans, interphase NPC assembly is the only mechanism in which to assemble pores. In this study, we focus on the development of a method to examine NPC assembly and maintenance in live cells, and examine several different steps in NPC assembly.

In Chapter 2, we develop and optimize an assay to visually monitor two pools of a protein within the cell. We then use this assay to examine a report which states that new NPC assembly only occurs in daughter cells during mitosis and that old NPCs remain in the mother cell. Our findings suggest that new NPC assembly is not limited to daughter cells during mitosis, and that old NPCs are distributed between mother and daughter cells during mitosis.

Next, in Chapter 3, we characterize the interaction partners of the transmembrane Nup Ndc1, which we determined to be Pom152, Pom34, Nup53, and Nup59. Nup157 and Nup170 interact indirectly with Ndc1 through Nup53 and Nup59.

We examine the effects of perturbing the interacting of Ndc1 with these proteins in vivo and find that they are required for proper localization of Ndc1 and other reporter Nups. We show that depletion of Pom34 in *nup53*Δ *nup59*Δ cells results in the cytoplasmic accumulation of cytoplasmic Nup82, and that restoration of Pom34 results in the normal localization of Nup82. The depletion of Nup59 in *pom34*Δ *pom152*Δ cells results in nuclear accumulation of Nup133, and these cells cannot recover upon reintroduction of Nup59. We suggest that these two scenarios represent two different states of NPC assembly. Pom34 depleted *nup53*Δ*nup59*Δ cells still have Pom152 and Ndc1 at the NPC, and while Nup59 depleted *pom34*Δ *pom152*Δ cells have lost all contacts with the rest of the NPC and cannot recover. Therefore Nup59, Pom152, and Pom34 play an important role in NPC maintenance.

Finally, in Chapter 4, the role of Nup157 and Nup170 is examined in greater detail. Loss of both proteins results in fewer NPCs on the NE. Loss of these two proteins also results in the mislocalization of core and cytoplasmic Nups, but not nuclear Nups. We use a novel assay employing the photoconvertable protein Dendra to determine how these proteins mislocalize and find that old NPCs are stable, but new NPC assembly is halted. New NPC assembly resumes upon reintroduction of Nup170. All members of the Ndc1 subcomplex are present in these cells, and this phenotype represents a different stage in NPC assembly that the one we observed upon depletion of Nup59 in *pom34*Δ *pom152*Δ cells. Together, these results further our understanding of early events in NPC formation.

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Chapter 2

Characterization of the Inheritance of Old and New Nucleoporins in Mother and Daughter cells

Background

There are two mechanisms by which NPC assembly occurs in eukaryotes. In metazoans, the NPC is reassembled onto the NE as it reforms after mitosis. However, in both metazoans and yeast, NPCs are also inserted into the intact NE during interphase. Yeast undergo a closed mitosis, and assembly into the intact NE is the only mechanism by which NPC biogenesis is thought to occur. Very little is known about interphase NPC assembly. Assembly requires a series of steps and involves the recruitment of different subsets of Nups to the inner and outer side of the NE, but little is known about the steps themselves or the composition of structures that lead to pore formation. A basic model of pore formation steps is: 1) recruitment of reticulons and transmembrane nucleoporins (POMs) to the NE which might act as receptors for soluble Nups, 2) recruitment of soluble Nups to POMs, 3) fusion of the inner and outer NE to form a channel, and 4) recruitment of FG repeat Nups to form a transport competent pore. It is possible that the recruitment of soluble Nups (step 3) occurs before a hole is made in the NE (step 4). It is not known how much of the NPC is assembled on the ER membrane before being transported to the NE.

There is little knowledge about non-nucleoporin factors involved in NPC assembly, however it is known that the process of nuclear import is required. Several karyopherins (Kaps) and Ran have been implicated in NPC assembly, and alleles of Kaps and Ran display various NPC assembly defects in non-permissive conditions. Ran mutants accumulate small vesicles in the cytoplasm that contain Nups (Ryan et al., 2003). Kap95 mutants accumulate sheets of membrane in their cytoplasm. The core Nup53 has an NLS which is recognized by Kap121 and the deletion of this NLS results in partial mislocalization of Nup53 (Lusk et al., 2002). Additionally, many loss of function alleles and deletions of nucleoporins result in pore clustering defects, while some alleles and deletions of Nups result in pore assembly defects. Finally, several mutant and conditional alleles of non-Nup proteins have pore assembly defects, such as Brr6, Apq12, or double deletions of the reticulons *rtn1Δyop1Δ* (Scarcelli et al., 2007, Dawson et al., 2009, de Bruyn Kops, and Guthrie, 2001).

The unpredictable location of where new NPCs will form is one obstacle prohibiting the development of new technology to study pore formation. Luedeke *et al* reported restricted diffusion between mother and daughter cells of transmembrane ER proteins while soluble ER proteins could diffuse freely back and forth (Luedeke et al., 2005). Later work suggested that old NPCs remain with mother cells during cell division, while new NPC formation only occurs in daughter cells during cell division (Shcheprova et al., 2008). Armed with this new information about the location of pore formation, we set out to develop a way to study old and new pores using the photoconvertable fluorescent proteins Dendra.

Dendra is a monomeric photoactivatable fluorescent protein that was isolated from the coral *Dendronephthya* (Gurskaya et al., 2006). Dendra is a green fluorescent protein but can be converted to a red fluorescent protein with a short pulse of blue light (Figure 2.1A). Blue light causes the Dendra protein to undergo a proteolytic cleavage, therefore conversion is irreversible. Dendra photoconversion allows two pools of a protein to be observed over time in living cells. In order to examine the localization of new NPC synthesis in *S. cerevisiae*, we tagged both transmembrane and soluble reporter Nups with Dendra to examine the distribution of old and new NPCs in budding

cells. Surprisingly, we found that both mother and daughter nuclei receive old protein, and that newly formed NPC are not restricted to the daughter cell.

Results

Dendra enables tracking of old and new nucleoporins

To test whether Dendra would work in *S. cerevisiae*, we tagged Nup133, a core Nup, in wildtype cells with 3xDendra and followed its distribution before and after photoconversion. Before conversion, Nup133 displayed a punctate distribution around the rim of the nucleus in the green channel. There was no signal apparent in the red channel. We then exposed the cells to a short pulse of UV light and imaged again. No green signal was present after photoconversion, however, bright punctate nuclear rim staining was apparent in the red channel (Fig 2.1B). The cells were imaged again after 30 minutes, and faint, punctate signal could be detected in the green channel, indicating that new Nup133-3xDendra had been synthesized. Converted 3xDendra could still be detected in the red channel. Therefore, Dendra can be photoconverted in yeast, and photoconversion creates an old and new pool of protein that can be monitored.

Old and new Ndc1 is equally distributed in mother and daughter cells

Earlier FRAP studies of transmembrane ER proteins and the soluble Nup49 showed that the transmission of these proteins from mother to daughter bud was prohibited by a diffusion barrier, and new NPC formation was only observed in daughter cells. Because we were interested in specifically imaging sites of new NPC formation, we used our Ndc1-3xDendra strain to see if we could observe the formation of new NPCs in daughter cells. Overnight cultures of Ndc1-3xDendra were diluted back and grown until they reached log phase. These cells were spun down and resuspended in a smaller volume to concentrate them, and an aliquot was placed on an agarose pad containing media for imaging. Cells were placed on a stage heated to 30˚ and converted with a short pulse of blue light. An acquired image of the green channel had no signal but spherical punctate signal was present in the red channel, therefore conversion was complete (Fig 2.2). Cells were imaged in bright field over several hours to determine how many divisions the cells had undergone, and after two divisions images were acquired in the red and green channels. After two cell divisions, converted (red) protein can be visualized in both mother (purple arrowheads) and daughter cells (azure arrowheads), which is the opposite of what is observed in Shcheprova *et al* . *A*dditionally, new protein (green) is unambiguously present in both mother and daughter cells. We therefore concluded that old transmembrane Nups are not retained in the mother cell, and that new NPC assembly is not restricted to daughter cells.

Old and new Nup49 is equally distributed in mother and daughter cells

The original work which demonstrated that new NPC formation is restricted to daughter cells in Shcheprova *et al* was performed with Nup49, so we tagged Nup49 with 3xDendra to see if the inheritance of Nup49 differed from the POM Ndc1. Briefly, cells were grown to log phase, converted, imaged, and allowed to undergo two cell divisions. The cells were imaged again after the cell divisions were complete. We again observed the opposite results of Shcheprova *et al.* Both old and new Nup49 was present in mother and daughter cells, and we could not detect any asymmetry in the

division of these two pools of proteins (Figure 2.3). We therefore concluded that Ndc1 and Nup49 display similar inheritance patterns.

Figure 2.1

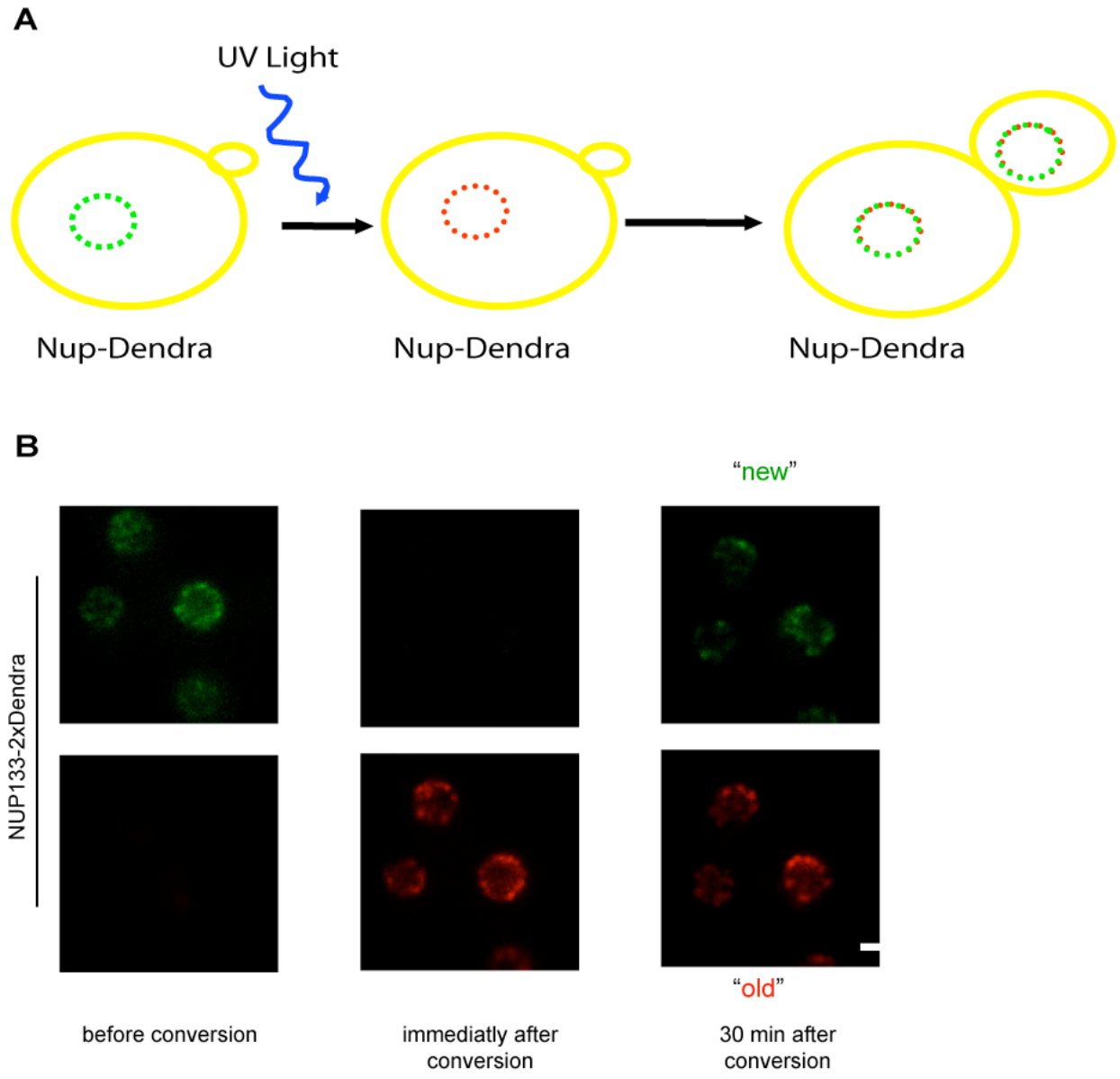

Figure 2.1 Dendra Photoconversion of nucleoporins A) A schematic diagram of Dendra conversion in *S. cerevisiae*. Dendra fluoresces green until exposed to UV light (left panel). Dendra fluoresces red after exposure to UV light (middle panel). New protein can be visualized after time has passed (right panel). B) Actual conversion in a yeast cell. Before conversion, Nup133-2xDendra is present in the green image but not in the red image (left panel). After conversion, Dendra is only present in the red channel (middle panel). After 30 minutes, green protein that was synthesized after conversion can be visualized while protein made before conversion is present in the red channel (right panel).

Figure 2.2

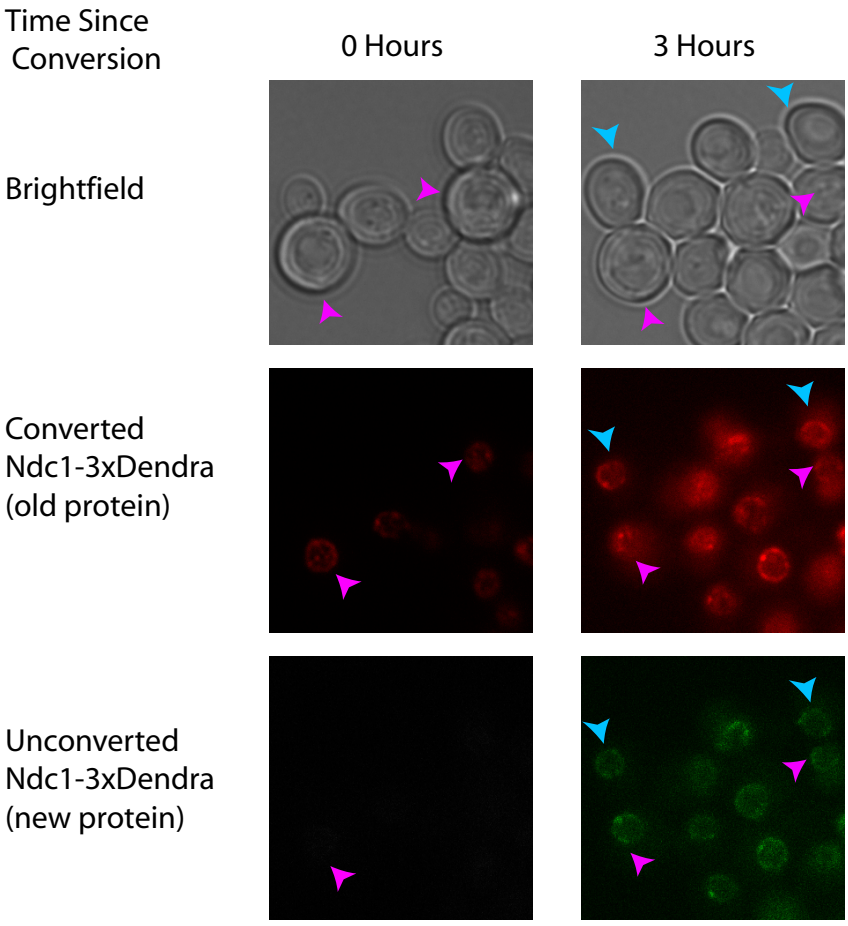

Figure 2.2 Transmembrane nucleoporins can diffuse to daughter cells during mitosis
Ndc1-3xDendra immobilized cells were exposed to UV light at time zero. Images were acquired at time zero and three hours post photoconversion. Purple arrows indicate mother cells, while azure arrows mark daughter cells.

Figure 2.3

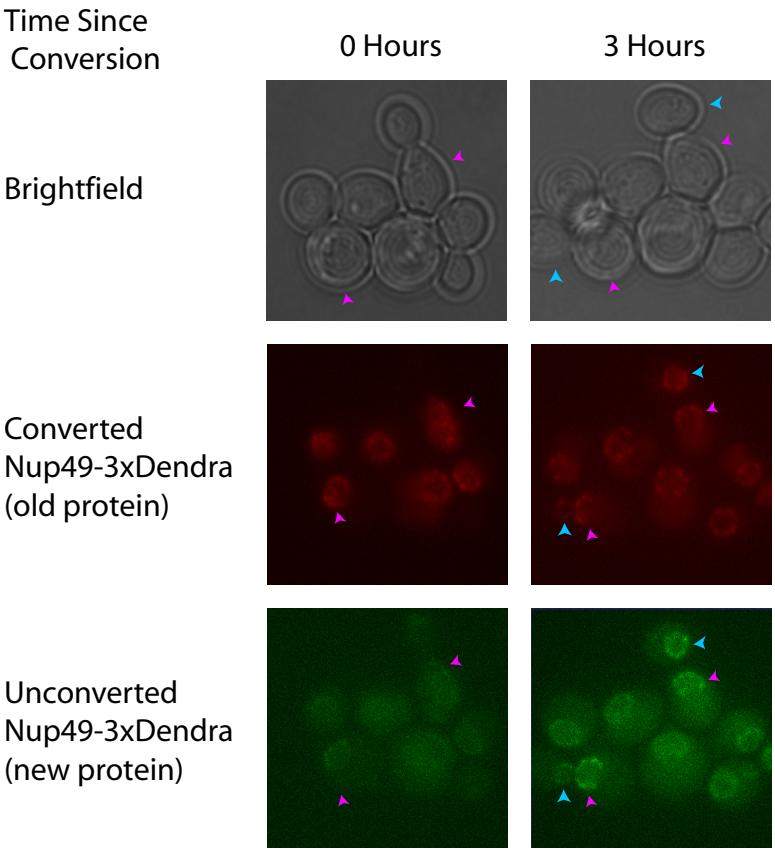

Figure 2.3 Nup49-3xDendra distribution is unaffected by the diffusion barrier Nup49-3xDendra immobilized cells were converted at time zero. Images were acquired directly after conversion and three hours after conversion. Mother cells are marked with purple arrowheads and daughter cells are marked with azure arrowheads.

Discussion

Dendra works as a visual pulse chase label in yeast

The study of NPC assembly has been limited by the use of a single color fluorophore for some time. Many strains with NPC assembly defects have been discovered, but we have been unable to determine the nature in which the phenotypes arise because of the lack of a visual pulse chase system in yeast. The FlAsH and ReAsh labeling system was used as an attempt for a double label system in yeast, but unfortunately, only one color worked in yeast (unpublished data, Madrid AS). Dendra was not available until recently but has marked a turning point in the study of NPC assembly. Our initial Dendra construct was very dim, and only two very long exposures could be taken before the fluorophore was photobleached, and did not prove to be very useful (data not shown). However, we were able to convert this construct with blue light. We constructed a trimer form of Dendra which was much brighter, required less lengthy exposure times and therefore yielded less photobleaching. It was with this

trimer of Dendra that long term time lapse experiments became possible. We successfully used 3xDendra to examine old and new proteins to determine the inheritance patterns of several Nups.

Different experimental techniques lead to different results

Previous results by Shcheprova *el al* reported that new NPC assembly only occurs in daughter cells, and that old NPCs are retained in mother cells, and that a diffusion barrier exists to prevent transmembrane ER proteins from moving from the mother into the daughter cell. In eukaryotes, the ER is continuous with the outer NE therefore it seems that transmembrane Nups of the NPC are also be affected by the diffusion barrier. We examined several different Nups with Dendra to observe this phenomenon. What we observed conflicted with Shcheprova *el al* in that both new and old NPCs are present in mother and daughter cells. We therefore attempted to determine the reasons for why our data is not in agreement.

The first and most obvious difference in our experiments is the technique used. Shcheprova *et al* use FLIP to demonstrate that continuous bleaching of the mother does not affect daughter NPCs. They also use FRAP to bleach the mother and daughter nuclei to show that mother cells do not assemble new pores over time, while daughter cells do assemble new pores. The longest FRAP experiment is 15 minutes which is a much shorter duration than in our Dendra experiments. It is possible that the longer time frame of Dendra experiments allows NPCs to overcome the diffusion barrier, which could account for our observations.

Another difference between these two sets of experiments is the timeframe in which the experiments begin. For Shcheprova *el al*, FRAP and FLIP is done on cells which have already begun to bud. In all cases, there are NPCs present in the nucleus of the budding cell, and it is possible that these NPCs are inherited from the mother cell. No experiments to address this fact are included in Shcheprova *el al*. Dendra experiments are performed on cells that have not yet begun to bud, and it is clear that old protein moves into the buds of cells as time progresses. Additionally, daughter cells of daughter cells whose mothers have undergone conversion have old (converted) protein present in their nuclei. As cell division proceeds during the course of Dendra experiments, the amount of old protein present in mother cells decreases by roughly half for ever division that it undergoes (data not shown). Previous reports show that the number of NPCs increase throughout the cell cycle (Winey et al., 1997); this number is halved during cell division, and the process of NPC production throughout interphase begins again. A similar scenario is observed in our Dendra experiment. All of these observations led us to conclude that old NPCs are distributed evenly in mother and daughter cells.

Materials and Methods

Yeast Strains and Plasmids Yeast media and strain construction were all performed according to established protocols. All plasmids used in this study are listed in Table II, and all yeast strains are listed in Table III.

Fluorescence microscopy

Cells were grown at 30°C in synthetic dextrose containing selection media supplemented with a twofold excess of adenine. Cells were grown overnight and

diluted back in fresh media; the cells grew for 3 hours (early log phase). Cells were concentrated and 2μL were placed on a 2% low melt agarose pad dissolved in synthetic media with a twofold excess of adenine. A heated stage was used to keep cells at 30˚ for the duration of the experiment. Cells were converted with 4x20ms DAPI exposures, then green and red images were acquired. After the amount of time specified for each experiment, red and green images were acquired. All images were acquired with a digital camera (CA742-98; Hamamatsu Photonics) controlled by the Metamorph software program (MDS Analytical Technologies). Images were processed using Photoshop (CS2; Adobe), and figures were assembled using Photoshop and Illustrator (CS2; Adobe).

Table II

Plasmid	Description	Reference
pKW1950	pFA6a-Dendra2-B-HisMX6	this work
pKW2208	pFA6a-3xDendra2-B-HisMX6	this work

Table III

Strain	Relevant Genotype	Reference
KWY1813	Ndc1-Dendra	this work
KWY2100	Ndc1-3xDendra	this work
KWY2062	Nup49-3xDendra	this work

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Chapter 3

The Role of the Ndc1 Interaction Network in Yeast Nuclear Pore Complex Maintenance and Assembly

Background

Despite the fact that the NPC is a large proteinaceous channel that spans the nuclear envelope (NE), there are only three transmembrane Nups within the NPC. These proteins are called POre Membrane proteins, or POMs, and are named Ndc1, Pom152 and Pom34. Pom152 and Pom34 are not essential and cells which lack both of these proteins have no discernible phenotypes (Miao et al., 2006). Ndc1 is the only yeast POM that is essential. Interestingly, Ndc1 is also present in another structure which spans the NE, the Spindle Pole Body (SPB) and it is not known whether the essential function of Ndc1 results from its role in the SPB or in the NPC. Several attempts have been made to isolate alleles of Ndc1, which only affect either SPB function or NPC function, but none have been successful. Therefore it seems likely that Ndc1 plays a similar functional role in both the SPB and the NPC.

Previous work from our lab (Madrid et al., 2006) has shown that Ndc1 is important for the correct localization of nucleoporins to the NE. To circumvent the problem of Ndc1's essential function, a conditional strain was constructed, which only expressed Ndc1 in the presence of galactose. Cells were grown for 24h in dextrose to deplete Ndc1 and the localization of central, nuclear, and cytoplasmic Nups were visualized. Approximately 60% of each reporter Nup mislocalized in the Gal-Ndc1 depletion strain. However, when Ndc1 was depleted in a strain which also lacked Pom152, the percentage of Nups which localized properly decreased sharply to almost 10%. The *pom152Δ GAL-NDC1* strain was examined by TEM and many abnormalities were observed, including increased pore diameter and pores which lacked protein. This suggests that Pom152 and Ndc1 have redundant roles in NPC function. However, the actual function that Ndc1 and Pom152 are involved in at the NE, for example whether they play a role in NPC assembly or maintenance, is not clear from Madrid *et al*. In an attempt to learn more about the function of the POMs, biochemical purifications were performed to determine if any proteins interacted with the POMs, and in vivo experiments were performed to determine whether the loss of the proteins that reside at the core of the NPC have an effect on further NPC assembly.

Results

Identification of Ndc1 interacting proteins

In order to determine if Ndc1 is present at the NPC in a complex or alone, we created a strain which expressed Ndc1 with a TAP tag (Puig et al., 2001). The Ndc1-TAP tag strain expressed Ndc1 from the endogenous locus and grows normally. Crude yeast extract underwent several centrifugation steps to obtain fraction that was highly enriched for membranes. The membrane fraction was subjected to the detergent Digitonin and then affinity purified. The resulting sample was analyzed by LCMS-MS and MALDI-TOF, and 6 proteins were found that specifically interacted with Ndc1 (Fig 3.1A). Five of these proteins are Nups, Nup170, Nup157, Pom152, Nup59, and Pom34, while the final protein is a component of the SPB, Mps3. Because Ndc1 is present in both the NPC and the SPB, the presence of Mps3 was not unexpected.

Ndc1 forms a subcomplex with Pom152 and Pom34

During the course of this research, a study was published which mapped the interactions of all of the NPC components (Alber et al., 2007b, Alber et al., 2007a). Our

Figure 3.1

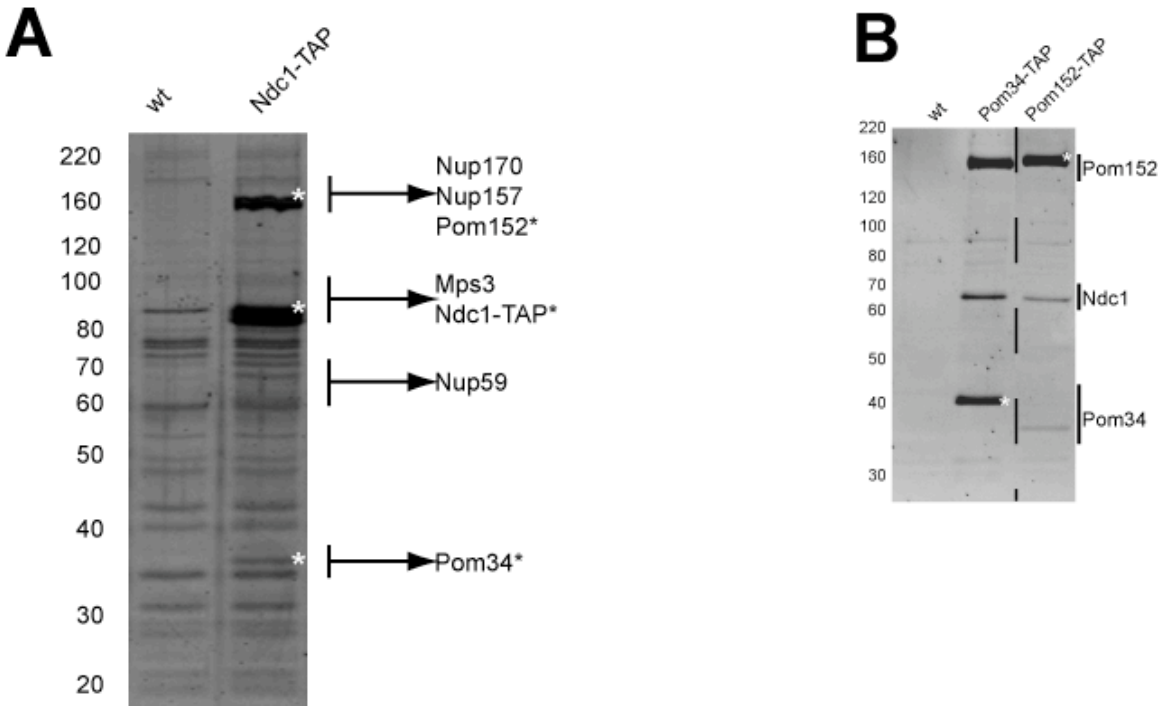

Figure 3.1 Identification of Ndc1-interacting proteins and characterization of the Ndc1 subcomplex. A) Affinity purification from yeast expressing TAP-tagged Ndc1 (*NDC1-TAP*) or untagged controls. Gel areas were excised and subjected to LC-MS/MS. Lines mark borders of areas subjected to MS analysis. B) Comparison of purifications performed from cells expressing TAP-tagged versions of Pom34 (*POM34-TAP*), Pom152 (*POM152-TAP*), or untagged controls.

data are consistent with these results, however, this study did not address whether the observed NPC protein interactions are direct or indirect. To further understand the interactions between Ndc1 and the other two POMs, we constructed Pom152 and Pom34 TAP-tagged strains and performed affinity purifications (Fig 3.1B). Purifications of Pom152 pull down Ndc1 and Pom34, and Pom152 and Ndc1 co-purify with Pom34 . None of the other proteins detected by mass spectrometry are present in detectable

Figure 3.2

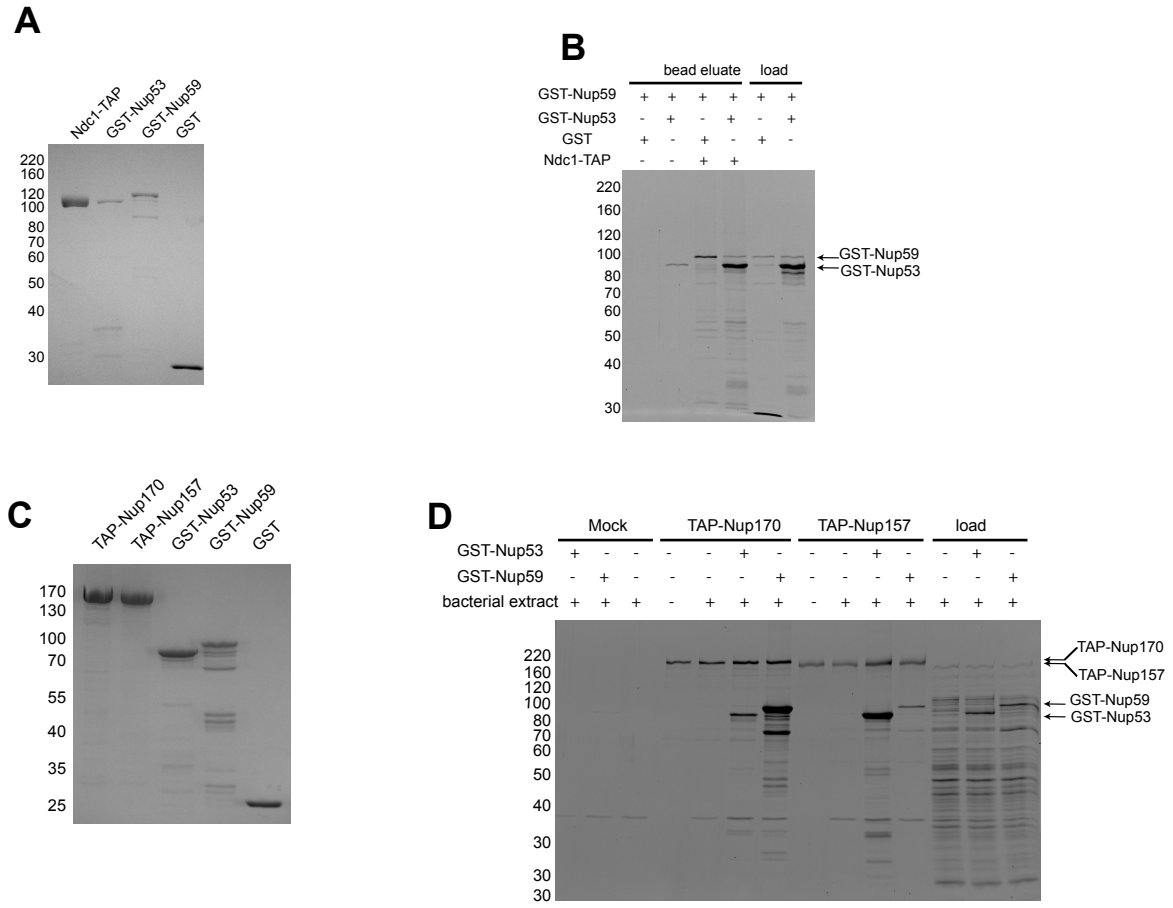

Figure 3.2 In vitro interactions of Ndc1 interacting proteins A) Purified proteins separated by SDS-PAGE and stained with Coomassie brilliant blue. B) SYPRO-Ruby–stained gel showing eluates from Ndc1-TAP beads after incubation with variants of Nup53 and Nup59. C) Coomassie brilliant blue–stained gel showing samples of purified proteins. D) SYPRO-Ruby–stained gel showing samples of the eluates from TAP-Nup170, TAP-Nup157, or TAP beads after incubation with different mixtures of *E. coli* extract, 6×His-MBP-Nup53, 6×His-MBP-Nup59, or 6×His-MBP. Migration rate references for all gel image panels are shown in kilodaltons.

amounts. This suggests that Pom152 and Pom34 directly interact with Ndc1. Therefore Ndc1, Pom152 and Pom34 form a complex with each other.

Nup53 and Nup59 interact directly with Ndc1

Because of their presence in the original TAP tag mass spectrometry experiment, Nup59, Nup170, and Nup157 were tested for direct interactions with Ndc1. Nup53, although absent in the TAP tag pull down, was also tested for interactions because of its similarity to Nup59. An in vitro approach was used to determine if interactions between Ndc1 and these protein could be detected. Ndc1 was purified from *S. cerevisiae* extracts using S-protein conjugated agarose beads, while all other proteins were purified from *E. coli* which were expressed as GST fusions (Fig 3.2A, C).

To determine if Nup53 and Nup59 directly interacted with Ndc1, purified Ndc1 S-Beads or S-Beads alone were mixed with either GST-Nup53 or GST-Nup59 fusion proteins. Bound proteins were eluted with high salt. Both GST-Nup53 and GST-Nup59 bound specifically to Ndc1 S-beads (Fig 3.2B). GST-Nup53 and GST-Nup59 did not bind to S-Beads alone, nor did GST alone bind to the Ndc1 S-beads, therefore both Nup53 and Nup59 interact directly with Ndc1.

Binding experiments with Ndc1 S-beads were with performed with Nup157 and Nup170 to determine if these proteins interacted directly with Ndc1, but no direct binding was detected (data not shown). This suggests that the interactions we observed for Ndc1 with Nup157 and Nup170 from the TAP tag pull downs are most likely indirect interactions mediated by a bridging factor. The obvious candidates for a bridging factor between Ndc1 and Nup157 and Nup170 are Nup53 and Nup59. Indeed, previous work documents interactions between Nup53/Nup59 and Nup170 (Marelli 1998). To test this possibility, TAP tagged versions of Nup157 and Nup170 were purified from *S. cerevisiae* and tested for their ability to bind to MBP fusions of Nup53 and Nup59 which had been purified from *E. coli* (Fig 3.2D). Interestingly, Nup170 bound to both MBP-Nup53 and MBP-Nup59, while Nup157 only bound to MBP-Nup53, suggesting that the interactions between these two highly identical pairs of proteins are different despite their homology.

Nups which interact with Ndc1 are required for viability

Our biochemical analysis yielded four proteins, which interact with Ndc1: Pom152, Pom34, Nup53, and Nup59. Single deletions of the four Ndc1 interacting proteins are viable, and the double deletion of Nup53 and Nup59 or Pom34 and Pom152 are also viable (Miao et al., 2006, Madrid et al., 2006, Marelli et al., 1998). By contrast, deletions of Nup53 with either Pom34 or Pom152 are unable to grow (Miao et al., 2006, Marelli et al., 1998). Furthermore, cells with deletions of Nup59 with either Pom 34 or Pom152 are are unable to grow. In order to examine the roles of the four proteins which directly interact with Ndc1, we constructed several combinations of deletion and depletion strains of the four proteins and examined their viability (Table 1). We found that Nup59 is essential for viability in the absence of Pom152 or Pom34 independently of whether Nup53 is present. When Nup53 and Nup59 are deleted, cells which express Pom152 or Pom34 are viable. However, upon depletion of Poms these cells can no longer grow. Therefore, Ndc1 interacting proteins can be divided into two functional elements: Nup59 alone, and Pom152 together with Pom34. The essential role of these functional elements is independent of Nup53, so Nup53 can be classified as functionally distinct from Nup59.

Table IV. cell viability phenotype in POM152, POM34, NUP53 and NUP59 deletion and/or conditional expression mutants

genotype	conditions	viability	reference
Nup53Δ	n/a	+	(Marelli et al., 1998)
Nup59Δ	n/a	+	(Marelli et al., 1998)
POM152Δ	n/a	+	(Marelli et al., 1998)
POM34Δ	n/a	+	(Miao et al., 2006)
NUP53Δ NUP59Δ	n/a	+	(Marelli et al., 1998)
NUP53Δ POM152Δ	n/a	+	(Marelli et al., 1998)
NUP53Δ POM34Δ	n/a	+	(Miao et al., 2006)
NUP59Δ POM152Δ	n/a	-	(Marelli et al., 1998)
NUP59Δ POM34Δ	n/a	-	(Miao et al., 2006)
POM152Δ POM34Δ	n/a	+	(Miao et al., 2006)
NUP53Δ POM152Δ POM34Δ	n/a	+	this study
pGAL1-3xHA-NUP59 POM152Δ POM34Δ	galactose	+	this study
pGAL1-3xHA-NUP59 POM152Δ POM34Δ	dextrose	-	this study
pMET3-3xHA-NUP59 NUP53Δ POM152Δ POM34Δ	- methionine	+	this study
pMET3-3xHA-NUP59 NUP53Δ POM152Δ POM34Δ	+ methionone	-	this study
pGAL1-3xHA-POM34 NUP53Δ NUP59Δ	galactose	+	this study
pGAL1-3xHA-POM34 NUP53Δ NUP59Δ	dextrose	-	this study
pGAL1-3xHA-POM152 NUP53Δ NUP59Δ	galactose	+	this study
pGAL1-3xHA-POM152 NUP53Δ NUP59Δ	dextrose	-	this study

Ndc1 interacting partners are required to localize Ndc1 to the NE

To determine whether deletion of any of the Ndc1 interacting proteins would disrupt Ndc1's localization at the NE, we constructed Ndc1-GFP labelled versions of the conditional mutants. The role of Nup53 and Nup59 on Ndc1's localization was tested first. We examined Ndc1-GFP in *pom152Δ pom34Δ nup53Δ* cells which expressed Nup59 under a Met promoter. When methionine is present in media, expression of Nup59 is repressed, but expression of Nup59 begins when methionine is removed from

Figure 3.3

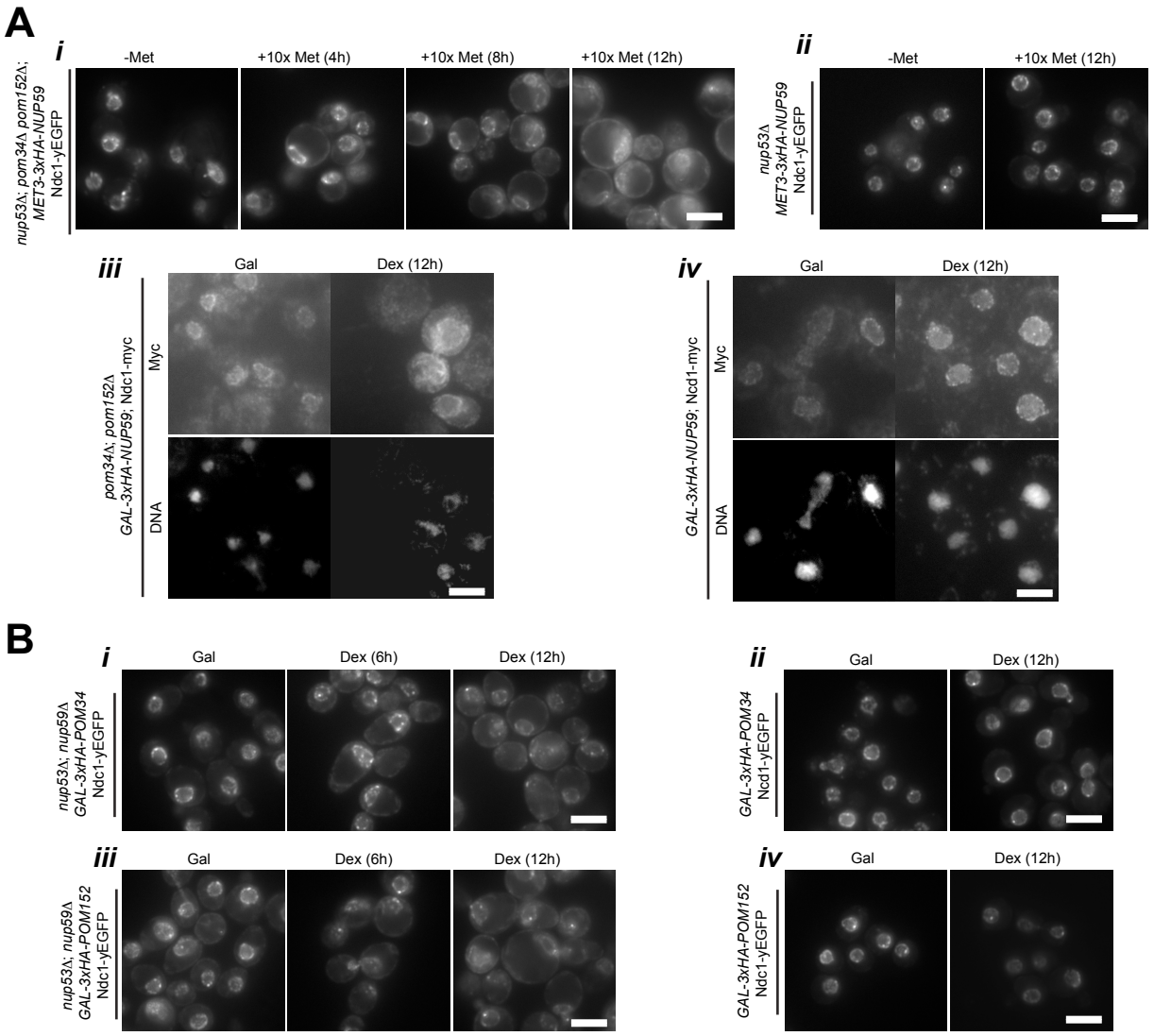

Figure 3.3 The role of Pom152, Pom34, Nup53, and Nup59 in targeting of Ndc1 to NE. (A) Localization of Ndc1 was compared in *nup53*Δ *pom34*Δ *pom152*Δ *MET3-3×HA-NUP59* (i), *nup53*Δ *MET3-3×HA-NUP59* (ii), *pom34*Δ *pom152*Δ *GAL-3×HA-NUP59* (iii), and *GAL-3×HA-NUP59* (iv) cells by fluorescence microscopy (via the yeast EGFP tag in i and ii) or by indirect immunofluorescence (via 13×myc tag in iii and iv) in permissive (galactose medium [gal] or medium lacking methionine [−met]) and nonpermissive conditions (dextrose-containing medium [dex] or medium containing 10× methionine [+10× met]). For immunofluorescence, cells were also stained with DAPI to label DNA. (B) Similar analysis of Ndc1–yeast EGFP localization in *nup53*Δ *nup59*Δ *GAL-3×HA-POM34* (i), *GAL-3×HA-POM34* (ii), *nup53*Δ *nup59*Δ *GAL-3×HA-POM152* (iii), and *GAL-3×HA-POM152* (iv). Bars, 5 μm.

the media. When Nup59 was expressed, Ndc1 localized correctly, but when Nup59 was depleted by addition of Met, Ndc1 mislocalized to the peripheral ER (Fig 3.3A*i*). This mislocalization of Ndc1 was also observed after depletion of Nup59 in *pom152Δ pom34Δ* cells (Fig 3.3A*iii*). We also examined cells in which Pom152 and Pom34 were present, but Nup59 was depleted (Fig 3.3A*iv*). Ndc1 mislocalization was never observed

in these Nup59 depleted cells, even in a *nup53Δ* background Fig (3.3A*ii*). Therefore, the two Poms together can compensate for the loss of Nup59.

To test whether the Poms played a role in the correct targeting of Ndc1, we constructed *nup53Δ nup59Δ* strains in which either Pom34 or Pom152 was placed under a GAL promoter. We again examined the localization of Ndc1 with GFP. When either Pom was expressed, Ndc1 mostly localized properly to the NE (Fig 3.3B*i* and *iii*), but some peripheral ER localization could be detected also. In both Pom34 and Pom152 depleted strains, Ndc1-GFP at the NE became more faint over time. Mislocalization of Ndc1 was not observed in *nup53Δ nup59Δ* cells which expressed Pom152 or Pom34. Therefore, both Nup59 and the Poms play an important role in recruiting Ndc1 to the NPC.

Ndc1 interacting proteins are required for proper localization of reporter Nups

Previous results have demonstrated that Ndc1 is required for proper NPC structure and function (Madrid et al., 2006). Because we observed mislocalization of Ndc1 when its interaction partners were removed, we wanted to investigate the consequences of removing Ndc1's interacting partners on other NPC components. We modified each depletion strain and introduced GFP-versions of various Nups. These Nups included cytoplasmic Nup82, central Nup188 and Nup133, and Nuclear Nup2. All strains were grown in permissive conditions, shifted to non-permissive conditions, grown until each protein was depleted, and cells were visualized by fluorescence microscopy in the time course of this experiment.

We first examined the effect of Nup59 depletion in the presence and absence of Nup53 in *pom34Δ pom152Δ* cells (Fig 3.4A). All of the reporters examined were properly localized in *pom34Δ pom152Δ* cells, which expressed Nup59. However, upon depletion of Nup59, Nup82, Nup133, and Nup188 are partially mislocalized into foci which appear to be localized on the NE. Nup2 also displays a mislocalization to foci in the NE but not all cells examined possess this phenotype.

In *pom34Δ pom152Δ nup53Δ* cells which conditionally express Nup59, reporter nups display a slight mislocalization in permissive conditions (Fig 3.4B). Depletion of Nup59 led to a more pronounced mislocalization of all four reporter Nups. Therefore, both Nup53 and Nup59 are essential for correct structural organization of the NPC in the absence of both Pom152 and Pom34, because the structural defects caused by the absence of both Nup59 and Nup53 become more pronounced compared to when only one of these proteins is deleted. The role of Nup53 and Nup59 is not entirely redundant, as only Nup59 is synthetically lethal in a *pom152Δ pom34Δ* strain.

We next examined the effect of depleting either Pom152 or Pom34 in *nup53Δ nup59Δ* cells. No major localization defects of the reporter Nups were observed in either strain in permissive conditions, except for Nup82-GFP, which mislocalized in GAL-3xHA-POM152 cells (Fig. 3.5B). However, this phenotype was not observed in GAL-3xHA-POM34 cells (Fig 3.5A); GAL promoters cause a high level of expression and this phenotype is potentially a consequence of the high level of Pom152 that is expressed in these cells. Upon depletion of either Pom34 or Pom152, *nup53Δ nup59Δ* cells completely mislocalized cytoplasmic Nup82 and partially mislocalized central Nup133 and Nup188. However, the nuclear Nup2 localized properly. The depletion of

Pom152 or Pom34 in *nup53Δ nup59Δ* cells yields similar phenotypes, indicating that

Figure 3.4

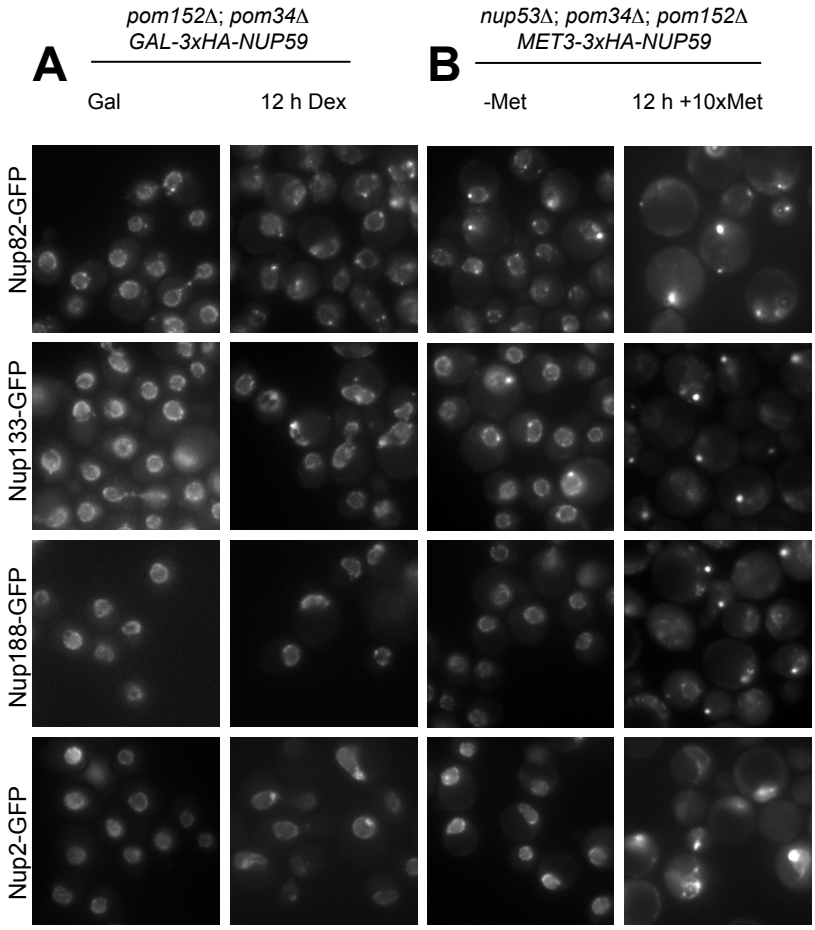

Figure 3.4 Roles of Nup53 and Nup59 in NPC organization and function. (A and B) *pom152*Δ *pom34*Δ *GAL-3×HA-NUP59* (A) or *pom152*Δ *pom34*Δ *nup53*Δ *MET3-3×HA-NUP59* (B) cells expressing GFP-tagged Nup82, Nup133, Nup188, or Nup2. Gal, galactose medium; dex, dextrose-containing medium; −met, medium lacking methionine; +10× met, medium containing 10× methionine. Bar, 5 µm.

these proteins function in a similar way in the structural organization of the NPC. This is consistent with our biochemical data, which showed that the integrity Ndc1 subcomplex

depends on both Pom34 and Pom152.

Figure 3.5

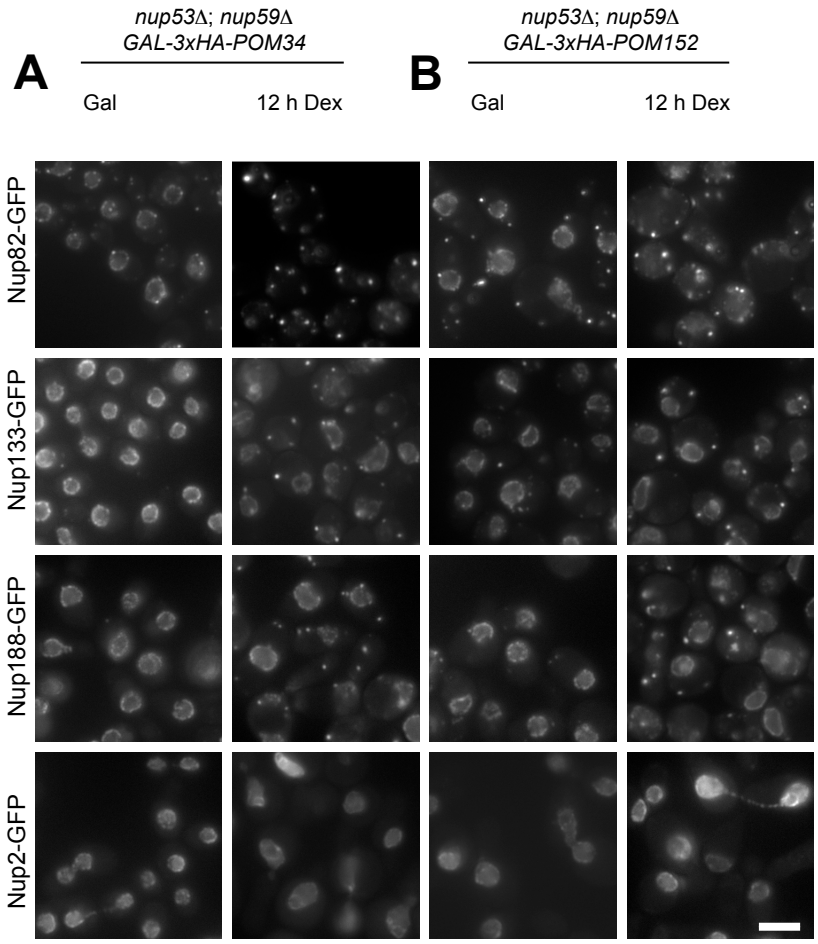

Figure 3.5 Roles of Pom152 and Pom34 in NPC organization and function. (A and B) Fluorescence analysis as described in Fig. 3.4 in *nup53*Δ *nup59*Δ cells conditionally expressing Pom34 (A) or Pom152 (B). Gal, galactose medium; dex, dextrose-containing medium. Bar, 5 μm.

Ndc1 interacting proteins are required for correct ultrastructural morphology of NPCs

To examine if NPC morphology was affected in the conditional mutant strains, transmission EM analysis was performed in permissive and non-permissive conditions. As expected, GAL-NUP59 *pom152Δ pom34Δ* cells grown in permissive conditions showed no defect (Fig 3.6A*i*). However, once Nup59 was depleted, *pom152Δ pom34*

Figure 3.6

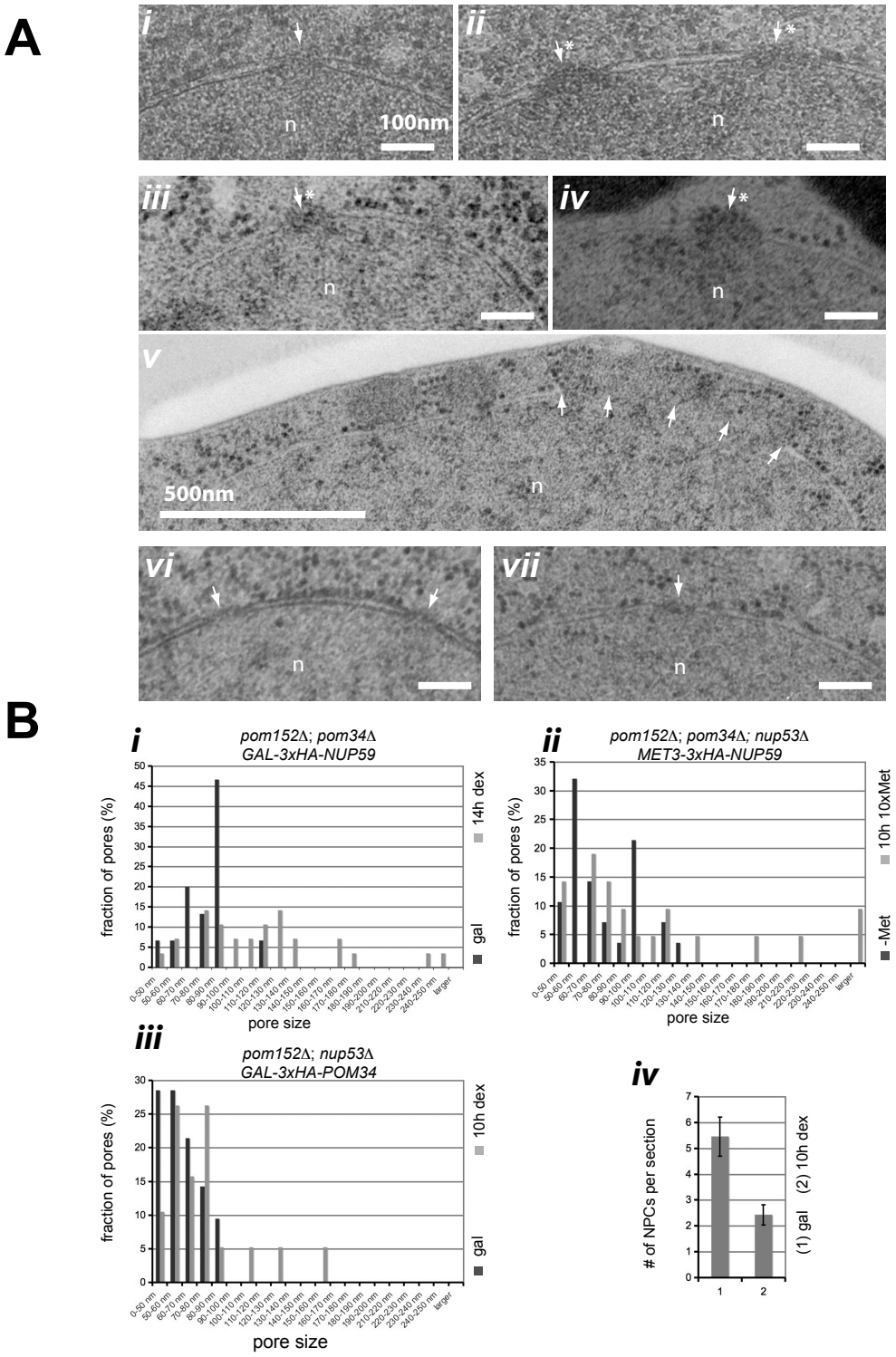

Figure 3.6 Transmission EM analysis of NPC morphology in conditional mutants affecting Ndc1-interacting nucleoporins. A) NPCs (arrowheads) in *pom152*Δ *pom34*Δ *GAL-3×HA-NUP59* cells in permissive conditions (i) did not display morphological abnormalities. (ii) After incubation in nonpermissive conditions, a large fraction of dilated pores often displaying fuzzy electron-dense material could be detected (asterisks). (iii) A similar phenotype was observed in *pom152*Δ *pom34*Δ *nup53*Δ *MET3-3×HA-*

NUP59 cells in permissive conditions. After incubation in nonpermissive conditions, these cells displayed not only dilated pores (iv) but also large openings in NE (v, arrows). In *nup53*Δ *nup59*Δ *GAL-3×HA-POM34* cells, no obvious NPC size abnormalities could be detected both in permissive (vi) and nonpermissive conditions (vii). n, nucleus. Bars: (i–iv, vi, and vii) 100 nm; (v) 500 nm. (B, i–iii) Comparison of pore size distributions in *pom152*Δ *pom34*Δ *GAL-3×HA-NUP59* cells (i), *pom152*Δ *pom34*Δ *nup53*Δ *MET3-3×HA-NUP59* cells (ii), and *nup53*Δ *nup59*Δ *GAL-3×HA-POM34* cells (iii) incubated in permissive and nonpermissive conditions. (iv) Comparison of pore numbers per section in *nup53*Δ *nup59*Δ *GAL-3×HA-POM34* in either permissive or nonpermissive conditions. Gal, galactose medium; dex, dextrose-containing medium; −met, medium lacking methionine; 10× met, medium containing 10× methionine. Error bars indicate SEM.

cells contained many NPCs with increased pore diameter (Fig 3.6A*ii*). This pore dilation phenotype was also observed in MET-3xHA-NUP59 *pom152Δ pom34 nup53Δ* cells in both permissive and non permissive conditions (Fig 3.6A*iii,iv,v*). In addition to increased pore diameter, large openings in the NE which lacked protein-dense material were observed in *pom152Δ pom34 nup53Δ* after Nup59 depletion. This is similar to the phenotype, which is observed by the depletion of GAL-NDC1 in *pom152Δ* cells (Mardrid 2006). We also analyzed *nup53Δ nup59Δ* cells which expressed Pom34 under a GAL promoter. There was no detectable pore defect in either permissive or non-permissive conditions in these cells. However, in non-permissive conditions, the number of NPCs observed was significantly reduced from the number of NPCs observed in these cells in permissive conditions (Fig 3.6B). From these results, we conclude that the removal of Ndc1 interacting proteins results in ultrastructural NPC abnormalities and reduced numbers of NPCs.

The decrease in NPC density in Pom34 depleted *nup53Δ nup59Δ* cells is due to a pore assembly defect

Depletion of Pom34 in *nup53Δ nup59Δ* cells results in mislocalization of Ndc1 and other reporter Nups, however no obvious defect was observed for these cells in the NPCs by EM. We presume that these NPCs are stable, and the reduced number is caused by an pore assembly defect. Depletion takes many hours, and cells continue to divide during this time. Thus, no new pores are made after Pom34 is depleted, but as cell division continues, old existing pores are diluted between budding mothers and daughter. The results obtained thus far point to the conclusion that the loss of Pom34 in *nup53Δ nup59Δ* cells inhibits new pore assembly, and the reduced number of pores that are observed by EM could be explained by this fact.

To determine whether the mislocalized protein in GAL-POM34 *nup53Δ nup59Δ* cells represents newly synthesized protein that has not been incorporated into the NE or whether it is protein which was previously part of NPCs that have fallen apart, we used the fluorescent protein Dendra. Dendra is a fluorescent protein which undergoes an irreversible conversion from green to red when exposed to UV light (Gurskaya et al., 2006). Any Dendra that is synthesized after this pulse of UV light will be green. This effectively divides the Dendra protein within a cell into two pools, red Dendra which represents pre-pulse protein, and green Dendra, which is protein made after the pulse of UV. We used this approach to convert Dendra during the depletion of particular Ndc1 interacting partners to monitor the fate of old and new reporter Nups throughout the course of depletion.

To determine the nature of mislocalization of the reporter Nups, we used the cytoplasmic Nup82 because it displays the most prominent mislocalization in GAL-3xHA-POM34 *nup53Δ nup59Δ* cells. Like Nup82-GFP, Nup82-2xDendra localizes properly in GAL-3xHA-POM34 *nup53Δ nup59Δ* cells in permissive conditions. Conversion of Nup82 Dendra at the zero time point of a 10 hour experiment was not useful because the red protein is divided equally between mother and daughter cells therefore the red signal is too dilute to visualize at the end of the experiment. A time course was used to determine a more appropriate time in which to begin the experiment (Fig 3.7A). Cells were visualized every two hours. Nup82-Dendra appeared normal four hours after depletion began, but was mislocalized 6 hours into the time course. Therefore, we began our Dendra experiment four hours post depletion. An additional 6 hour chase period was included to allow complete depletion of Pom34, so the total depletion time was ten hours. GAL-3xHA-POM34 *nup53Δ nup59Δ* cells were converted by a UV pulse at four hours post depletion, and visualized again after 6 additional hours. After the total of ten hours incubation period, newly synthesized Nup82-Dendra was present in cytoplasmic foci, while old Nup82-Dendra was still present in the nuclear envelope (Fig 3.7B). From this result, we can conclude that Pom34 is required to incorporate newly synthesized Nup82 into NPCs, which suggests that these cells possess a NPC assembly defect.

Because GAL-3xHA-POM34 *nup53Δnup59Δ* cells in non-permissive conditions accumulate Nup82-Dendra in their cytoplasm, we hypothesized that this newly synthesized protein might represent an assembly intermediate. To test this hypothesis, we grew GAL-3xHA-POM34 *nup53Δnup59Δ* in dextrose to deplete Pom34, converted the Nup82 Dendra, and switched these converted cells to galactose containing media. The cells were allowed to grow for 3 hours in the presence of Pom34 before being imaged again (Fig 3.7C). We observed that the red, mislocalized protein that was

Figure 3.7

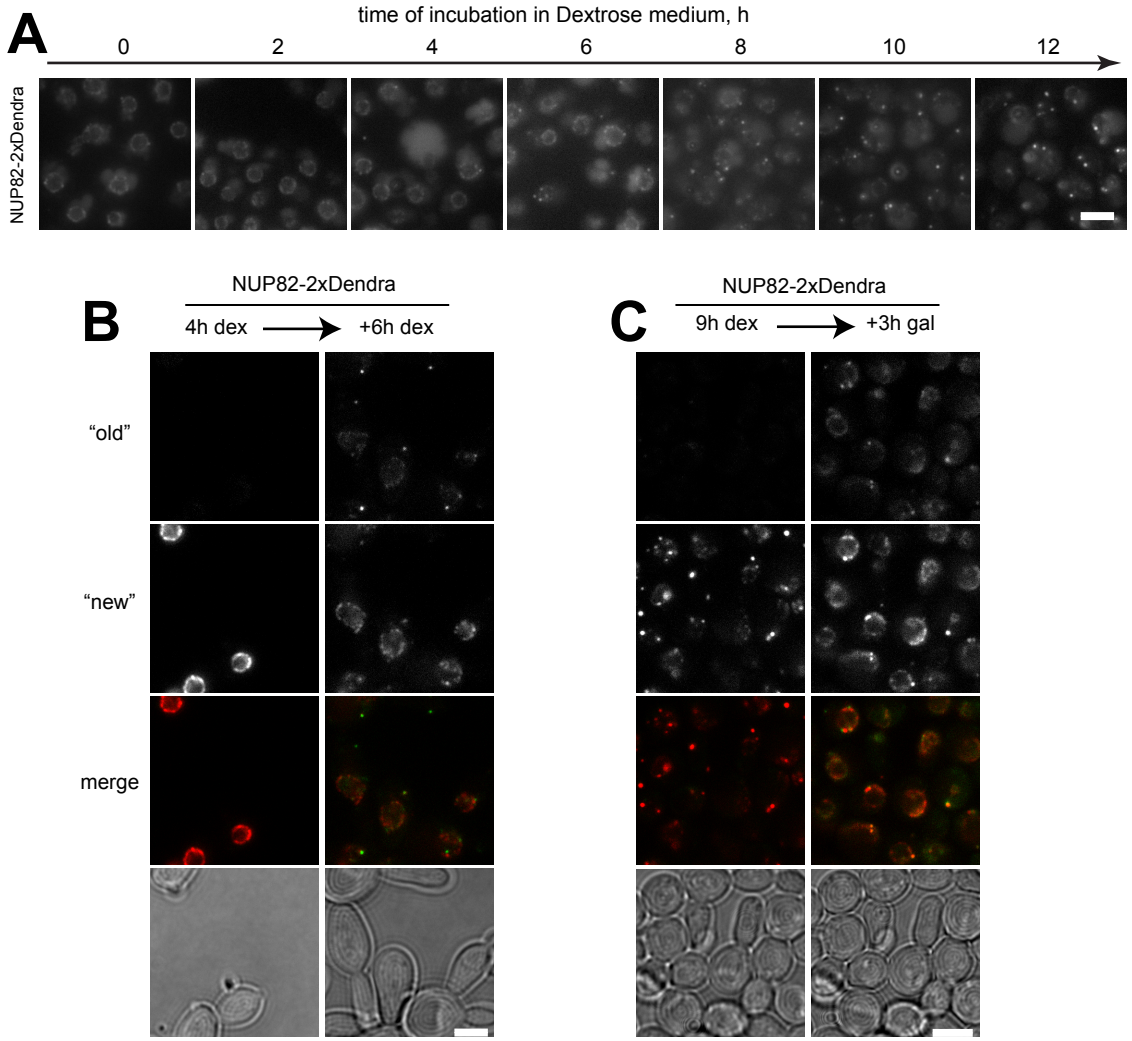

Figure 3.7 Dendra assay to analyze distribution of nucleoporins in *nup53Δ nup59Δ GAL-3×HA-POM34* cells. A) Time course to visualize the Nup82-Dendra distribution in *nup53*Δ *nup59*Δ *GAL-3×HA-POM34* cells after repression of *POM34* expression without photoconversion. B) Distribution of newly made and preexisting Nup82-Dendra in *nup53*Δ *nup59*Δ *GAL-3×HA-POM34* cells. Cells were incubated for 4 h in nonpermissive conditions, photoconverted (left), and the same field of cells was reimaged after an additional 6-h incubation period (right). C) Cells were incubated in dextrose medium (dex) for 9 h to induce Nup82 mislocalization, placed in galactose medium (gal), and photoconverted (left). After a 3-h incubation, the same cells were imaged again to detect the distribution of preexisting and newly synthesized Nup82-Dendra. Bars, 5 µm.

present directly after conversion had all been incorporated into the NE. In addition, all of the newly synthesized Nup82 was properly localized in the NE. Therefore, the assembly block that occurs as a consequence of depleting pom34 in 3 hours cells is reversible.

The cause of enlarged NPCs in pom34Δ pom152Δ GALNUP59 cells is unclear

When viewed by EM in non-permissive conditions, GAL-3xHA-NUP59 *pom34Δpom152Δ* cells have abnormally large NPCs and holes in the NE that lack proteins. This EM Data suggests that the phenotypes of GAL-3xHA-NUP59 *pom34Δpom152Δ* in non-permissive conditions is the result of a NPC stability defect as opposed to a pore assembly defect. To determine if the reported Nup mislocalization phenotype of GAL-3xHA-NUP59 *pom34Δ pom152Δ* cells is caused by either an assembly or maintenance defect, we performed Dendra experiments on these cells. GAL-3xHA-NUP59 *pom34Δ pom152Δ* cells were grown in dextrose for 6 hours, converted before mislocalization was detectable and imaged again after 6 hours for a total depletion time of 12 hours (Fig 3.8). Upon loss of Nup59, we observed mislocalization of old protein into bright foci on the nuclear envelopes. Newly synthesized protein in these cells also accumulated in these foci, for the most part. Therefore, these cells display a pore maintenance defect.

To test whether these cells could recover from the depletion of Nup59, we performed an experiment in which we depleted Nup59 from the *pom34Δ pom152Δ* cells before conversion. Nup133-2xDendra was present in foci on the nuclear envelope at the time of conversion, and the cells were switched to permissive conditions for 5 hours. Despite the reintroduction of Nup59, these cells failed to recover (data not shown). Therefore, the NPC maintenance defects that these cells display are irreversible.

Figure 3.8

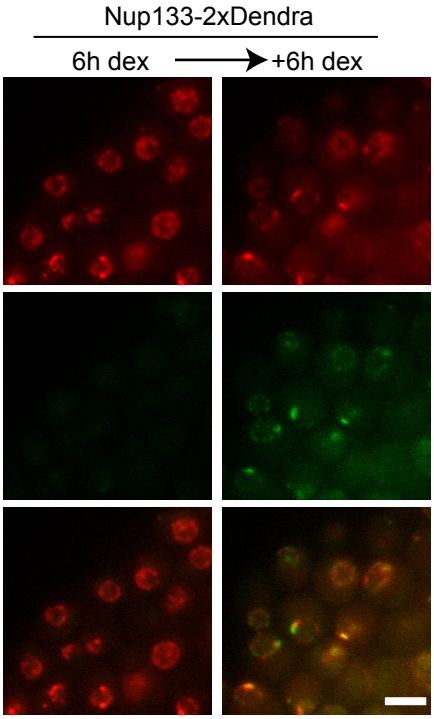

Figure 3.8 Dendra assay to analyze distribution of nucleoporins in *pom34Δ pom152Δ GAL-3×HA-NUP59* cells. Distribution of newly made and preexisting Nup133-Dendra in *pom34Δ pom152Δ GAL-3×HA-NUP59* cells. Cells were incubated for 6 h in nonpermissive conditions, photoconverted (left), and the same field of cells was reimaged after an additional 6-h incubation period (right).

Discussion

Characterization of the biochemical interactions of Ndc1

At the time this study was initiated, very little was known about the proteins that interact with Ndc1. We found that Ndc1 interacts with six different nucleoporins, Pom152, Pom34, Nup53, Nup59, Nup157, and Nup170. Ndc1 directly interacts with Pom152, Pom34, Nup53 and Nup59, while its interactions with Nup157 and Nup157 are indirect. We determined that Ndc1 forms a stable subcomplex with the other two transmembrane proteins, Pom152 and Pom34. The integrity of the Ndc1 subcomplex is compromised in cells which lack either Pom152 or Pom34. Additionally, Pom152 binds directly to Ndc1, but in the absence of Pom34, the binding of Pom152 to Ndc1 is weakened.

Our experiments showed that Ndc1 interacts directly with both Nup53 and Nup59, but that these two proteins compete for binding to Ndc1. Both Nup53 and Nup59 contain a amphipathic alpha helix domain at their C-Terminus, and our data suggests that binding to Ndc1 is dependent on this alpha helix for both proteins (data not shown). This result is consistent with previous reports in which overexpression of Nup53 leads to overproliferation of intranuclear membranes; both Ndc1 and Pom152 were localized to the membrane overproliferations (Marelli et al., 2001). Direct interactions of Nup157 and Nup170 with Ndc1 could not be confirmed by in vitro binding experiments, despite the fact that the two proteins were present in the pulldown of Ndc1. However, we did observe direct binding of both Nup157 and Nup170 with Nup53 and Nup170 with Nup59 suggesting that the interaction between Ndc1 and Nup157/Nup170 is bridged by Nup53 and Nup59. vNup53, the vertebrate homolog of Nup53/Nup59, was recently shown to interact with both vNdc1 and Nup155 (the vertebrate homolog of yNup157/Nup170) (Hawryluk-Gara et al., 2008). Therefore, the interactions between Ndc1, Nup157/Nup170, and Nup53/Nup59 seem to be conserved. It is also important to note that all of the proteins which we found to directly interact with Ndc1 are either transmembrane proteins themselves, or contain some kind of facet which helps them associate with membranes. Therefore we propose that Ndc1 and its four directly interacting proteins form the most internal layer of the NPC, and that Nup170 and Nup157 form the next layer of the NPC, with which other core nucleoporins interact to make up the structural scaffold of the NPC.

Ndc1 binding proteins can be subdivided into two functional groups

Because the NPC is a complex channel which spans two lipid bilayers, it is not surprising that the deletion of one protein has no effect on the cell. However, deletion of several members of the NPC can cause disruptions in its complex structure. In this study, we characterize several functional groups, or proteins that can act together or alone to achieve the same function. The proteins that interact with Ndc1 can be divided into two functional groups. The first group contains Pom34 and Pom152, and is synthetically lethal with the second group, which consists of Nup59 alone. Both functional groups are important for the correct targeting of Ndc1 to the nuclear envelope, therefore it is easy to imagine why the cells cannot recover from a situation in which Pom34, Pom152, and Nup59 are deleted. Additionally, these two functional elements mediate interactions between Ndc1 and nucleoporins which occupy the more peripheral layers of the NPC. We showed that both Nup157 and Nup170 interact with

Nup59. Nup157 and Nup170 are require together for viability, and both are important for proper NPC assembly (Makio et al., 2009). Pom152 has also been shown to interact with Nup170 (Makio et al., 2009) and another essential NPC component, Nic96 (Nehrbass et al., 1996). Therefore it is likely that the two functional groups of Ndc1 interacting proteins play an important role at the core of the NPC by mediating interactions between Ndc1 and the rest of the NPC.

The role of Nup53 is more difficult to classify. Both Nup53 and Nup59 interact with Ndc1 in vitro. The absence of both Nup53 and Nup59 in a pom152 pom34Δ background leads to more severe mislocalization phenotypes than either Nup53 or Nup59 alone in the same background. However, when Nup53 and the two Poms together are deleted, the cells have no phenotype and Nup53 cannot compensate for the loss of Nup59 in *pom34 pom152Δ* cells. Nup53 is not sufficient to restore localization of Ndc1 when Nup59 is depleted, and the combination of the GAL-3xHA-NUP59 *pom34Δpom152Δ* is not viable when grown on dextrose. Additionally, we were unable to detect Nup53 in the pulldown of Ndc1. Therefore, even though Nup53 and Nup59 are quite very similar in sequence, they have have diverged and have distinct roles in the NPC. The role of Nup53 remains unclear.

Ndc1 interacting Nups and their role in NPC biosynthesis and maintenance

Depletion of the Ndc1 interacting proteins gives rise to many defects, including mislocalization of Ndc1, mislocalization of reporter nucleoporins, ultrastructural defects, and loss of viability. The degree of severity of these defects seemed to depend on the particular combinations of Ndc1 interacting proteins deleted, but based on our data we can make some general statements about the four Ndc1 interacting proteins. The four Ndc1 interacting proteins are required to connect Ndc1 to the NPC, to keep Ndc1 at the nuclear envelope, and to maintain the structural character of the NPC. Interestingly, our depletion mutants could be divided into two distinct classes of phenotypes. The first of these classes are cells in which Nup53 and Nup59 are deleted, and either Pom152 or Pom34 is present with a conditional promoter. The second class contains Nup59 under a conditional promoter, and Nup53, and both Poms, or just the Poms are deleted (*nup53Δ pom152 Δpom34Δ* or *pom152 Δpom34Δ*).

Depletion of either Pom34 or Pom152 (GAL-3xHA-POM34 *nup53Δ nup59Δ* or GAL-3xHA-POM152 *nup53Δ nup59Δ*) in the first class of mutants resulted in the mislocalization of a subset of nucleoporins into cytoplasmic foci. Additionally, NPCs in this strain in non permissive conditions contained fewer pores than cells in permissive conditions, but no ultrastructural defects were observed. To characterize the way in which the nucleoporins mislocalized in these cells, we labelled Nup82 with Dendra. Pom34 was depleted for 6 hours, and the old, converted Nup82-Dendra remained at the nuclear envelope. However, the newly synthesized Nup82 Dendra was present in cytoplasmic foci. Therefore, the depletion of Pom34 in a *nup53Δ nup59Δ* background does not affect old pores, however, new NPC assembly is blocked. This conclusion is consistent with our EM results which determined that these cells have fewer pores in non-permissive conditions. Because these cells continue to divide during the time in which Pom34 is being depleted, the old pores are diluted between mother and daughter cells. The proteins that will form new pores are sequestered from the nuclear envelope in the cytoplasmic foci. We also found this block in new NPC assembly to be reversible.

Within 3 hours of Pom34 re-introduction, converted Nup82-Dendra had been transferred to the NE and any new protein that was made was also present at the NE. Therefore, this phenotype is reversible, and is probably only lethal because too few NPCs remain per cell after many divisions. A similar defect in NPC biosynthesis is observed in cells which lack both Nup157 and Nup170, and this phenotype is also reversible when one of the two proteins is re-introduced to cells (next chapter). Both of these conditional situations (GAL-3xHA-POM34 *nup53Δ nup59Δ* and MET-NUP170 *nup157Δ*) result in the uncoupling of Ndc1 from Nup157 and Nup170, therefore the interaction of Ndc1 with Nup157 and Nup170 is important for new NPC biosynthesis.

Depletion of Nup59 in *pom34Δpom152Δ* cells resulted in a set of phenotypes that were distinct from the GAL-3xHA-POM34 *nup53Δnup59Δ* cells. Mutants in the previous class still have a partially intact Ndc1 subcomplex, but depletion of Nup59 in *pom34Δpom152Δ* cells completely dissolves the interactions between Ndc1 and Nup157/Nup170. GAL-3xHA-NUP59 in *pom34Δpom152Δ* cells were unable to properly localize many reporter Nups. Ultrastructural analysis showed that the NEs in these cells contained NPCs with increased diameter, and large holes in the NE that lacked proteinaceous material. These two defects were also observed in cells, which lack Ndc1 (Madrid et al., 2006). To learn about how mislocalization occurred in these cells, Nup133-Dendra was tagged. Newly synthesized protein localized to the NE, but instead of displaying a punctate distribution, most of the protein accumulated in a cap-like structure on one side of the nucleus. Old protein, which was normally distributed in permissive conditions, was also present in the cap structures. This mislocalization of old protein indicates that these cells have an NPC maintenance defect, which is quite distinct from the previous class of mutants. However, we could not determine whether these cells have an assembly defect, because the new protein localized to to the location of the old protein and was perhaps properly incorporated. Interestingly, *pom34Δpom152Δ* cells cannot recover from depletion of Nup59. Based on the data presented in this study, we suggest that the complete disruption of the NDC1 subcomplex and subsequent loss of Nup157/Nup170 leads to NPC instability in these cells, which subsequently leads to disintegration of existing pores and large holes in the NE.

Materials and Methods

Yeast Strains and Plasmids Yeast media and strain constructions were done according to established protocols. All plasmids used in this study are listed in Table V and yeast strains used in this study are listed in Table VI.

Identification of Ndc1-interacting proteins Pull-downs were performed using a modification of the protocol described in (Carvalho et al., 2006) Proteins were visualized with GelCode staining reagent (Bio-Rad Laboratories), and the desired areas differing in protein content were excised from the gel and identified by matrix-assisted laser desorption/ionization time of flight MS and LC-MS/MS.

Fluorescence microscopy Cells were grown at 30°C in permissive synthetic selection media (containing galactose or containing dextrose and lacking methionine) or in the nonpermissive synthetic selection media (containing dextrose or containing dextrose and 0.2 mg/ml methionine) with twofold excess of adenine. For all live imaging

experiments except for color Dendra assays, cells were concentrated by centrifugation, mounted under a cover slide in culture media, and imaged directly. All Images were acquired with a digital camera (CA742-98; Hamamatsu Photonics) controlled by the Metamorph software program (MDS Analytical Technologies). Images were processed using Photoshop (CS2; Adobe), and figures were assembled using Photoshop and Illustrator (CS2; Adobe). For depletion Dendra experiments, cells were grown overnight in the permissive media essentially as described in the previous paragraph and diluted into the nonpermissive media. Cells were collected for Dendra microscopy at the times specified. Cells were washed with fresh medium and concentrated during the final wash step. 2 μl cells was placed on a 2% low melt agarose pad prepared using respective medium. Dendra was converted by 4 × 20–ms pulses of UV light in DAPI channel followed by 8 s YFP exposure to bleach remaining signal. Slides were incubated at 30°C between time points. For recovery experiments, cells were grown in nonpermissive conditions for the times stated. Cells were washed three times with 500 μl of the permissive media, and 2 μl was placed on an agarose pad prepared using the permissive media. Cells were imaged immediately, incubated at 30°C for the time stated, and imaged again. Dendra was converted with one 20-ms UV exposure followed by a 10-s YFP exposure to bleach remaining signal. After the chase period, the same field of cells was imaged again in rhodamine and YFP channels.

Ultrastructural analysis The EM analysis of yeast mutants was performed essentially as described in (Madrid et al., 2006).

Table V

Plasmid	Description	Source
pKW2194	pFA6a-(link7xHIS-Tev-S)-hisMX6	this study
pKW2188	pRS306-NDC1(C)-(S-TEV-ZZ)	this study
pKW2240	pRS306-pGAL-NDC1-llink-73HIS-TEV-S	this study
pKW2336	pGEX-2TK-Nup53	(Patel, and Rexach, 2007)
pKW2337	pGEX-2TK-Nup59	(Patel, and Rexach, 2007)
pKW2349	pFA6a-pMmet3-33HA-HisMX6	this study
pKW2352	pFA6a-NDC1-yEGFP-CaURA3	this study
PKW2340	pFA6a-NDC1(TEV)-133myc-CaURA3	this study

Plasmid	Description	Source
pKW2424	pFA6a-Nup82(C)-yEGFP-CaURA3	this study
pKW2423	pFA6a-Nup133(C)-Yegfp-CaURA3	this study
pKW1547	pRS316-Nup2-GFP	this study
pKW2333	pFA6a-Nup82-23Dendra-CaURA3	this study
pKW2329	pFA6a-Nup133(C)-23Dendra-CaURA3	this study
pKW2207	pFA6a-23Dendra-HISMX6	this study
pKW2447	pRS314-RAD5	this study

Table VI

Strain	Relevant Genotype	Reference
KWY165	wildtype	this study
KWY1936	NDC1-TAP	this study
KWY1932	POM152-TAP	this study
KWY1986	POM34-TAP	this study
KWY2028	GAL-NDC1-TAP	this study
KWY2377	GAL-TAP-Nup157	this study
KWY2378	GAL-TAP-Nup170	this study
KWY2089	pom34Δ pom152Δ GAL-3×HA-NUP59	this study
KWY2228	nup53Δ pom34Δ pom152Δ	this study
KWY2285	nup53Δ pom34Δ pom152Δ MET3-3×HA-NUP59	this study
KWY2237	nup53Δ nup59Δ GAL-3×HA-POM34	this study
KWY2238	nup53Δ nup59Δ GAL-3×HA-POM152	this study
KWY2390	nup53Δ pom34Δ pom152Δ MET3-3×HA-NUP59 NDC1-yEGFP	this study

Strain	Relevant Genotype	Reference
KWY2072	pom34Δ pom152Δ GAL-3×HA-NUP59 NDC1-yEGFP	this study
KWY2239	pom34Δ pom152Δ GAL-3×HA-NUP59 NDC1-myc	this study
KWY2354	nup53Δ nup59Δ GAL-3×HA-POM34 NDC1-yEGFP	this study
KWY2355	nup53Δ nup59Δ GAL-3×HA-POM152 NDC1-yEGFP	this study
KWY2434	pom34Δ pom152Δ GAL-3×HA-NUP59 NUP82-yEGFP	this study
KWY2128	pom34Δ pom152Δ GAL-3×HA-NUP59 NUP133-yEGFP	this study
KWY2134	pom34Δ pom152Δ GAL-3×HA-NUP59 NUP188-yEGFP	this study
KWY2438	pom34Δ pom152Δ GAL-3×HA-NUP59 NUP2-yEGFP	this study
KWY2433	nup53Δ pom34Δ pom152Δ MET3-3×HA-NUP59 NUP82-yEGFP	this study
KWY2410	nup53Δ pom34Δ pom152Δ MET3-3×HA-NUP59 NUP188-yEGFP	this study
KWY2441	nup53Δ pom34Δ pom152Δ MET3-3×HA-NUP59 NUP2-yEGFP	this study
KWY2435	nup53Δ nup59Δ GAL-3×HA-POM34 NUP82-yEGFP	this study
KWY2431	nup53Δ nup59Δ GAL-3×HA-POM34 NUP133-yEGFP	this study
KWY2408	nup53Δ nup59Δ GAL-3×HA-POM34 NUP188-yEGFP	this study
KWY2439	nup53Δ nup59Δ GAL-3×HA-POM34 NUP2-GFP	this study
KWY2436	nup53Δ nup59Δ GAL-3×HA-POM152 NUP82-yEGFP	this study
KWY2432	nup53Δ nup59Δ GAL-3×HA-POM152 NUP133-yEGFP	this study
KWY2409	nup53Δ nup59Δ GAL-3×HA-POM152 NUP188-yEGFP	this study
KWY2440	NUP2-GFP	this study

Strain	Relevant Genotype	Reference
KWY1947	NUP133-2×Dendra	this study
KWY2503	nup53Δ nup59Δ GAL-3×HA-POM34 NUP82-2×Dendra RAD5	this study
KWY2406	nup53Δ pom34Δ pom152Δ MET3-3×HA-NUP59 NUP82-2×Dendra	this study

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Chapter 4

The Nucleoporins Nup170 and Nup157 are Essential for Nuclear Pore Complex Assembly

Background

Nup170 and Nup157 are two nucleoporins that were identified in a synthetic lethal screen with the transmembrane Nup, Pom152 (Aitchison et al., 1995). Nup170 and Nup157 display a high degree of sequence similarity, and these paralogs seem to have similar but not identical roles in the NPC. Both Nup170 and Nup157 are homologous to the mammalian gene Nup155. Interestingly, vNup155 can complement the synthetic lethality of *nup170Δ pom152Δ cells* (Aitchison et al., 1995). Early reports hypothesized that the role of Nup170 and Nup157 was similar but spatially distinct, meaning that one was present on the cytoplasmic face of the pore and the other on the nuclear side of the pore, but Immunogold EM showed that each protein is present on both sides of the pore (Rout et al., 2000). Deletion of either Nup170 or Nup157 does not affect the cell's viability, but the deletion of both genes results in cell death.

Both Nup157 and Nup170 are predicted to contain N-terminal ß-propeller and alpha-solenoid structures which mimic clathrin, a membrane coatomer protein which gives curvature vesicles. Although there is no direct evidence that Nup157 and Nup170 interact with membranes, both proteins interact directly with proteins that interact with transmembrane nucleoporins, and potentially stabilize membrane curvature without directly interacting with lipids. Nup157 has been shown to bind in vitro to the Nup84 subcomplex, a protein subcomplex that is another potential membrane interactor. Both Nup157 and Nup170 have been biochemically purified with nucleoporins that are located near the central channel of the NPC, and are potentially important components in the core structure of the NPC.

In an attempt to learn more about the role that Nup170 and Nup157 play in NPC assembly, a *nup157Δ* strain was constructed which expressed Nup170 under a conditional promoter. Cells that lack both of these highly similar proteins have fewer NPCs than cells in permissive conditions. Additionally, these cells mislocalize some but not all of the nucleoporins examined. Finally, *nup157Δ* Nup170 depleted cells display a reversible NPC assembly block. Therefore, we conclude that together Nup157 and Nup170 are required for an early step in the process of NPC assembly.

Results

Depletion of Nup170 in *nup157Δ* cells results in fewer NPCs on the NE

In order to examine the roles of the redundantly functioning proteins Nup170 and Nup157, a strain was constructed which expressed Nup170 under a repressible MET promoter with Nup157 deleted. These cells express Nup170 when grown in media that lacks methionine, but when methionine is present, expression of Nup170 is stopped. Nup170 can barely be detected after 4 hours in media containing methionine (data not shown). Strains which express MET-NUP170 and Nup157 are viable when grown on media containing Methionine, but MET-NUP170 *nup157Δ* cells are not viable when grown on media containing methionine.

To learn more about the morphology of cells which lack both Nup170 and Nup157, transmission EM was performed on MET-NUP170 *nup157Δ* cells in permissive and non-permissive conditions. Wild type cells contained many NPCs with normal morphology. The NPCs of *nup157Δ* cells in which Nup170 had been depleted for 8 hours contained pores with normal morphology, however, the cells contained far fewer NPCs on the NE than in permissive conditions (Fig 4.1A). The number of pores per

micrometer of linear distance along the NE was calculated for many sections in both permissive and non-permissive conditions. The number of pores decreased steadily throughout the depletion timecourse; at 8 hours of depletion, the number of pores in MET-NUP170 *nup157Δ* cells was 3 times fewer that the number of NPCs in MET-NUP170 cells (Fig 4.1B). By 16 hours of depletion, nuclei appeared abnormal and contained invaginations and projections of the NE. These changes were possibly brought about by the decreased level of NPCs.

Figure 4.1

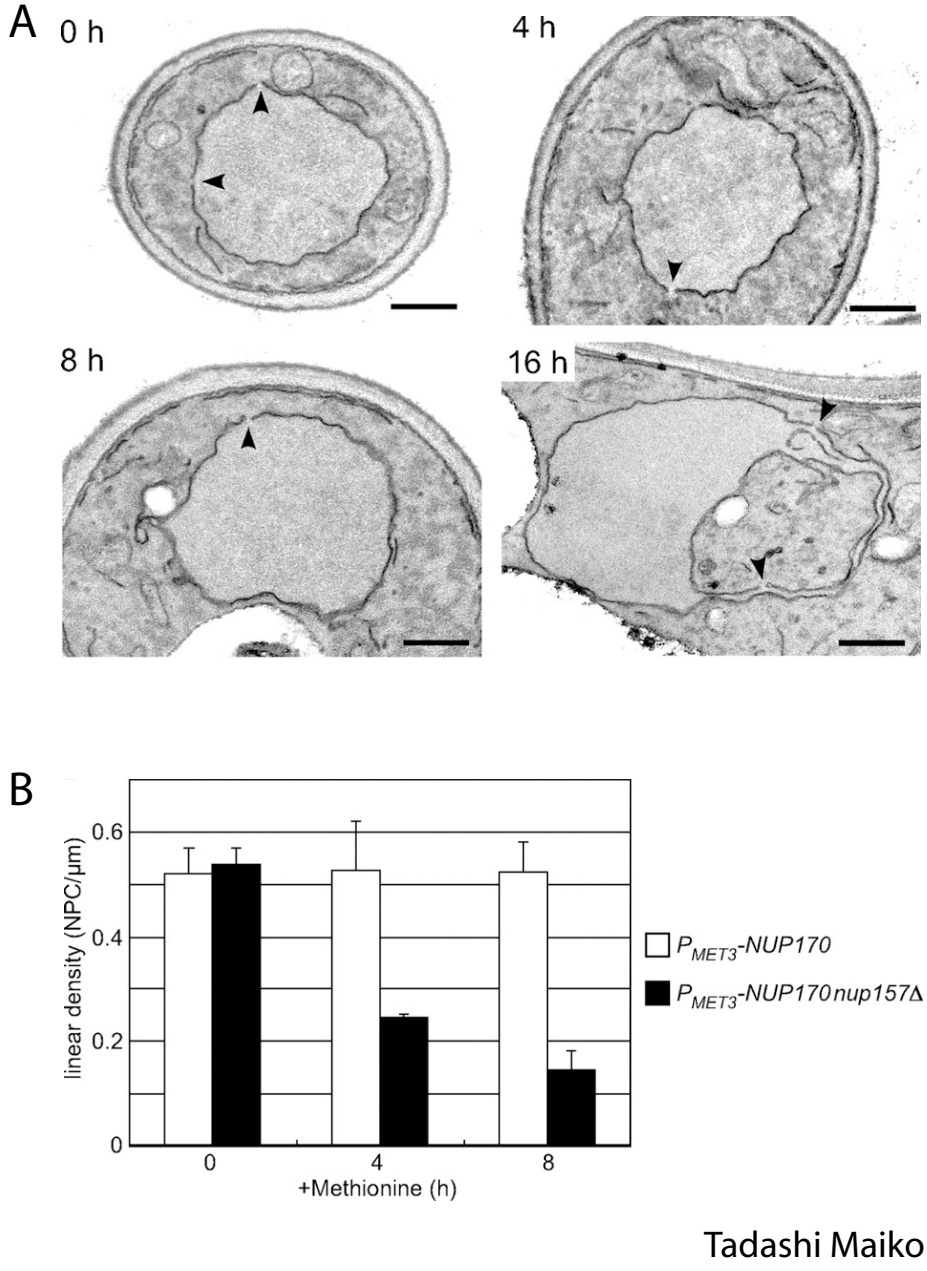

Figure 4.1 Depletion of Nup170p in Nup157Δ cells leads to a progressive decrease in NPC density
TMY1126 was grown in media lacking methionine. Methionine was added to repress NUP170 expression

and cells were processed for examination by TEM. A) Typical TEM images of TMY1126 at the indicated times after methionine addition. Arrowheads indicate gaps in the NE of a size consistent with NPCs. By 16 h, the NE becomes distorted, with this section showing a large invagination of the cytoplasm. Bars, 0.5 µm. (B) The number of NPCs was counted in each cell section and divided by the length of the NE to calculate a linear density of NPCs. A minimum of 30 cells was examined in each experiment, and standard deviations were estimated from three independent experiments. White bar, TMY1098; shaded bar, TMY1126.

A subset of reporter Nups mislocalize upon the loss of Nup170 and Nup157

It is likely that the reduction of NPCs observed by EM in cells which lack Nup157 and Nup170 is caused either by a block in the assembly of new NPCs, or by destabilizing newly forming and existing pores. To learn more about the effect of the loss of Nup157 and Nup170 on other nucleoporins, several nucleoporins were labelled with GFP and their distribution was examined in the presence and absence of Nup157 and Nup170. GFP labelled nucleoporins that were improperly localized in permissive conditions were excluded. The distribution of each nucleoporin was visualized at 0h and 6h after depletion of Nup170 in *nup157Δ* cells. A wide variety of nucleoporins was chosen, including transmembrane nups, nuclear nups, cytoplasmic nups, and core nups from several different subcomplexes (Fig 4.2). After 6 hours of depletion, the nucleoporins that displayed the most prominent mislocalization were Nup82-GFP and Nup159-GFP. Interestingly, both of these nucleoporins are located on the cytoplasmic face of the pore. These two proteins seemed to accumulate in foci scattered

throughout the cytoplasm, and occasionally adjacent to the NE. Multiple foci could be observed in each cell. Boldly contrasting the cytoplasmic nucleoporins were the nuclear nucleoporins. Four nuclear Nups were observed (Nup1-GFP, Nup2-GFP, Nup60-GFP, and Mlp1-GFP), and after 6 hours of depletion of Nup170 in *nup157Δ* cells, no mislocalization was detected. The transmembrane nucleoporin Pom152 was also properly localized at the NE. Lastly, nucleoporins that are located in the central channel displayed a variety of phenotypes. Members of the Nup84 subcomplex (Nup84-GFP, Nup85-GFP, and Nup133-GFP), Gle1-GFP, Nup100-GFP, and transmembrane Nup

Ndc1-GFP were still present at the NE, although some cytoplasmic foci could be

Figure 4.2

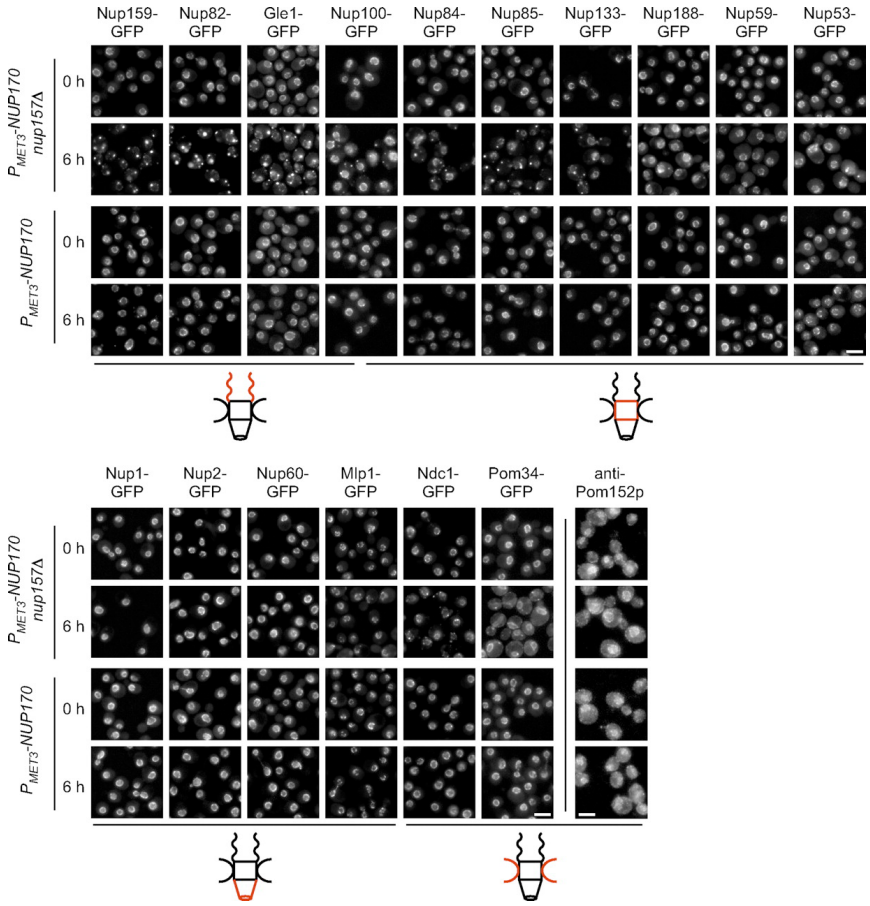

Figure 4.2 The loss of Nup170p and Nup157p has distinct effects on the localization of Nucleoporins A) Various nucleoporins were tagged with GFP in the TMY1098 and TMY1126 strains, and the fusion proteins were examined at the indicated times after repression of *NUP170* expression using an epifluorescence microscope. Pom152p localization was analyzed by indirect immunofluorescence using an anti-Pom152p antibody (mAb118C3). Diagrams adjacent to the images depict the general localization of each nup within the NPC (red). Nup100p is present on both faces of the NPC but is biased to the cytoplasm. Bars, 5 μm.

observed.

Nucleoporins that contained even fewer cytoplasmic foci were Pom34-GFP, Nup188-GFP, Nup59-GFP, and Nup53-GFP. The NE signal of these was more pronounced than the previous class of Nups above, but not quite as normal as the nuclear Nups.

A reversible NPC assembly defect occurs upon loss of Nup157 and Nup170

To determine the origin of the mislocalized reporter nucleoporins, two MET-NUP170 *nup157Δ* Dendra strains were constructed. Nup82 was chosen to be tagged with Dendra because of its early, widespread mislocalization phenotype after Nup170 depletion. Nup60 was also chosen because of its nearly normal localization pattern after depletion of Nup170. An Ndc1-3xDendra strain was constructed but

Ndc1-3xDendra did not properly localized in permissive conditions and therefore could not be examined. MET-NUP170 *nup157Δ* cells were switched to media containing methionine, immediately converted with a pulse of UV light, and imaged. For both Nup82-3xDendra and Nup60-3xDendra, the red exposure showed a normal, punctate NPC distribution while no signal was present in the green post-conversion exposure, so all of the Dendra had been effectively converted. Nup170 was depleted for 6 hours before the next set of images were acquired. After this six hour time period was over, the red signal from Nup82-3xDendra in MET-NUP170 *nup157Δ* cells was noticeably more dim. In the 6 hour depletion period, the cells had undergone at least one division, so the decrease in red signal can be attributed to this fact (Fig 4.3A). The old Nup82-3xDendra was still properly distributed around the perimeter of the NE and therefore displayed a normal distribution. The green Nup82-3xDendra was absent from the NE, and was instead present in bright cytoplasmic foci. Therefore it appears that the newly synthesized Nup82 protein in cells which lack Nup157 and Nup170 fails to be properly incorporated into the NE.

In MET-NUP170 *nup157Δ* cells the Nup60-3xDendra also displayed typical punctate NE localization at the beginning of the depletion. After 6 hours of Nup170

Figure 4.3

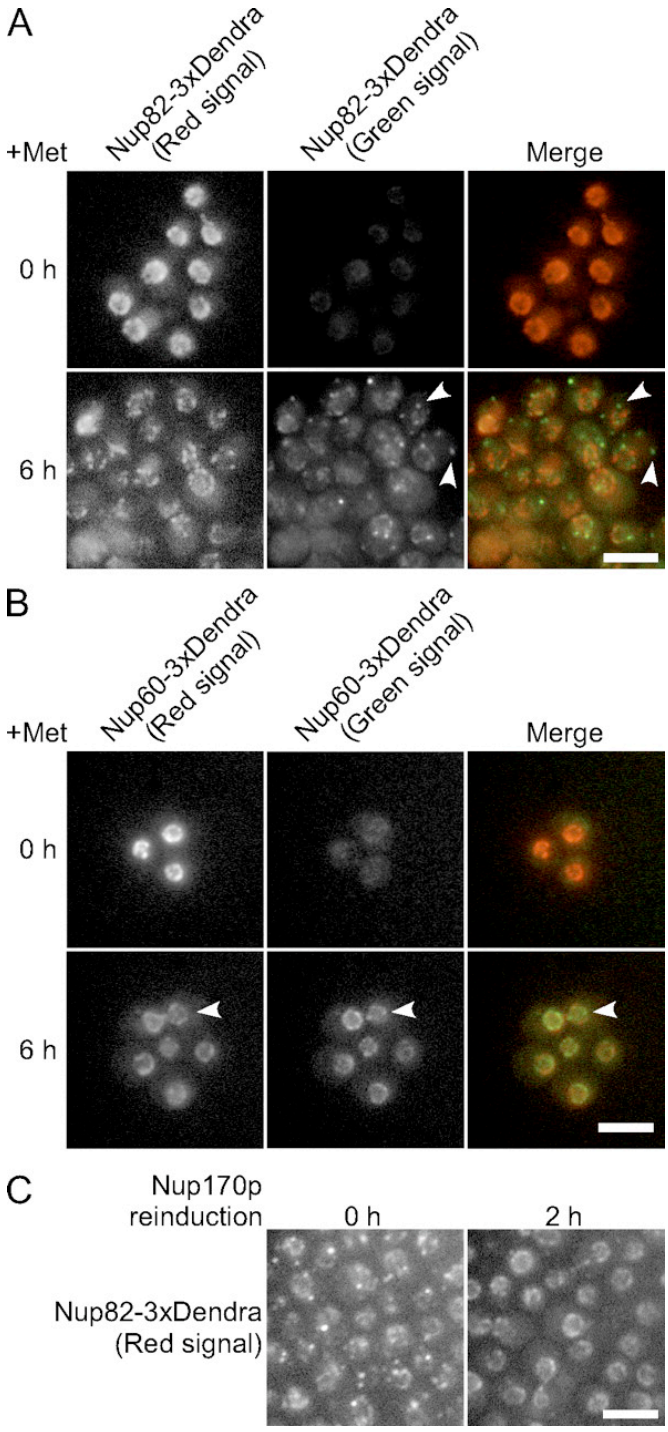

Figure 4.3 Nup170p and Nup157p are required for the incorporation of newly synthesized nups into NPCs. The strains KWY2216 (*NUP82-Dendra* P_{MET3}*-NUP170 nup157Δ*; A) and KWY2215 (*NUP60-Dendra* P_{MET3}*-NUP170 nup157Δ*; B) were transferred to media containing methionine to repress *NUP170* expression. At the same time, UV light was applied to the cells to induce photoconversion of Dendra (green to red). Cells were imaged at 0 and 6 h after photoconversion. Nup82-Dendra produced after photoconversion (green) appears at foci generally distinct from existing NPCs (A, arrowheads). NE-associated Nup60-Dendra-green foci that were distinct from Nup60-Dendra-red were also detected (B,

arrowheads). (C) KWY2216 cells were switched to media containing methionine; then, at 5 h after *NUP170* repression, UV light was applied to the cells to induce photoconversion of Dendra to the red fluorophore. Cells were transferred to the media lacking methionine for reinduction of *NUP170*. At 0 and 2 h after induction, images of the Nup82-Dendra (red) were captured using an epifluorescence microscope. Bars, 5 µm.

depletion, old Nup60-3xDendra was still present at the NE, but more faint than at time zero, because like in the previous experiment with Nup82-3xDendra, the cells divide between the beginning and the end of the experiment (Fig 4.3B). However, after 6 hours, new Nup60-3xDendra was also present at the NE. No mislocalization of the new protein was observed. Interestingly, the old and new Nup60-3xDendra foci do not seem to overlap, however, these images were not acquired at the same instant, and because these cells are alive, not fixed, it is possible that the difference is because the nucleus has moved slightly between the acquisition of the two images.

After it was established that newly synthesized Nup82-3xDendra accumulated in cytoplasmic foci, an experiment was performed to determine whether the Nup82-3xDendra foci could return to the NE after reintroduction of Nup170 (Fig 4.3C). Cells were grown in media with methionine for 6 hours, then switched to media with no methionine and converted with UV light. The converted Nup82-Dendra was present in cytoplasmic foci. Three hours after the reintroduction of Nup170, old Nup82-3xDendra had been reincorporated onto the NE. All of the new Nup82-3xDendra that had been synthesized in this time period was also present at the NE. Therefore it seems that cells can recover from the block in NPC assembly that is caused by loss of Nup170 in *nup157Δ* cells.

Discussion

The process of nuclear pore complex assembly into intact nuclear envelopes across both the inner and outer nuclear envelop is complicated and requires many factors. The role of two of the proteins that are involved in this process, the structural nucleoporins Nup157 and Nup170, were examined in this chapter. The results presented here suggest that Nup157 and Nup170 act as a functionally redundant group, as either one member must be present for proper NPC assembly to occur. The assembly of new NPCs comes to a halt upon the loss of both members of this functional group. Cells which contain one member, for example, Nup157, have no problems with NPC assembly. Interestingly, EM analysis shows that the loss of both Nup157 and Nup170 does not have any effects on the structure of existing NPCs. In addition, old and new reporter nucleoporin was followed throughout the depletion of Nup170 in *nup157Δ* cells and the old protein which was part of pre-existing pores was not mislocalized for both Nup82 and Nup60. These results are consistent with the conclusion that old pores are not affected by the loss of Nup157 and Nup170. Therefore it is unlikely that these two proteins play a role in NPC maintenance, but are involved in the assembly of new NPCs.

Results obtained by EM analysis showed that the number of NPCs decreased as the depletion of Nup170 in *nup157Δ* cells progressed. Additionally, a subset of GFP-labelled reporter nucleoporins accumulated in cytoplasmic foci. The nucleoporins in these cytoplasmic foci rapidly accumulate at the NE upon reinitiation of Nup170 expression. These results led to the conclusion that the loss of both Nup170 and

Nup157 leads to a block in assembly of new NPCs. Several other nucleoporin mutants (*nic96-1* and *nup192-15*) display a decrease in NPC number in non-permissive conditions but have not yet been tested to determine whether this phenotype is a result of an assembly block or a pore maintenance defect (Winey et al., 1997). Based on the similarity of these alleles to the MET-NUP170 *nup157Δ* depletion strain, it is likely that these alleles also display an assembly defect. It will be interesting to learn the outcome of these experiments.

An interesting feature of the MET-NUP170 *nup157Δ* strain is the proper localization of nuclear nucleoporins in non-permissive conditions. The loss of Nup170 and Nup157 does not seem to prevent nuclear nucleoporins from being imported and associating with the NE. Dendra experiments performed with the nuclear Nup60 showed that new protein localizes to the inner NE. EM analysis of MET-NUP170 *nup157Δ* cells revealed nuclei which contain structures on the inner NE that resemble NPCs but do not span the NE. Therefore the functional group of Nup157 and Nup170 do not seem to be required for assembly of the inner NE portion of the NPC. However, Nup157 and Nup170 seem to function in the addition of many core and cytoplasmic Nups onto the outer NE. Therefore is seems likely that Nup157 and Nup170 are important in the fabrication of the core channel structure of the NPC, since without these two proteins, NPC like structures accumulate on the inside of the NE. It is possible that these protein play a direct role in the reaction that punctures a hole in the NE and connects the two sides of the assembling NPC, as both proteins have been predicted to contain lipid binding domains, and MET-NUP170 *nup157Δ* accumulate NPC like structures on the inner NE (data not shown). Also, Nup157 has been shown to interact with the Nup84 subcomplex and Nup59, which is also predicted to interact with lipids. From these experiments it is not clear which protein is important in the process which punctures the NE, but it is potentially Nup157, Nup170, or one of the many proteins which interact with Nup157 and Nup170. It is also possible that all of these proteins are required together to make a hole in the membrane.

The process of NPC assembly into intact nuclear envelopes is not simple, and progress made by this study, though interesting, still does not address the most initial events that must occur to bring together the inner and outer nuclear envelopes. It is likely that this step is not the first step in NPC assembly since nuclear Nups are shuttled into the correct position in the absence of Nup170 and Nup157. It is interesting to note that the deletion of some non-nucleoporins, in particular Apq12 and Brr6, cause cells to develop NPC assembly phenotypes. Both proteins reside on the NE and their functions are unknown (Scarcelli et al., 2007, de Bruyn Kops, and Guthrie, 2001). Though progress was made in this study, there is still a lot that needs to be understood about the process of NPC assembly.

Materials and Methods

Yeast Strains and Plasmids Yeast media and strain construction were performed according to established protocols. All plasmids used in this study are listed in Table VII and yeast strains are listed in Table VIII. The plasmid used for the P_{MET3}-*NUP170* integration, pTM1046, was constructed by the replacement of the BglII–PacI region of pFA6a-kanMX6-PGAL1-3HA(Longtine et al., 1998) with a BglII–PacI fragment containing a promoter region of the *MET3* gene, which was made by PCR using

BY4742 genomic DNA as a template and the primers 5′-GCGAGATCTTTTAGTACTAACAGAGAC-3′ and 5′-GCGTTAATTAAAGACATGTTAATTATACTTTATTC-3′.
For repression of *MET3-NUP170*, cells were grown in the supplemented minimal media lacking methionine to a mid-logarithmic growth phase. Methionine (from the 20-mg/ml stock) was then added into the media to a final concentration of 200 µg/ml and incubated at 30°C for the indicated times.

Fluorescence Microscopy Yeast strains expressing various GFP and mRFP fusion proteins were taken from cultures at various time points after methionine addition. The locations of GFP and mRFP fusion proteins in live cells were visualized using either an epifluorescence or confocal microscope. Epifluorescence images were obtained with either a microscope (BX50; Olympus) using a UPlanS-Apochromat 100×/1.40 NA oil objective lens (Olympus) and a charge-coupled device (CCD) camera (AxioCam HRm; Carl Zeiss, Inc.) controlled by AxioVision software (Carl Zeiss, Inc.), or with a microscope (IX80; Olympus) using a UPlanS-Apochromat 100×/1.40 NA oil objective lens (Olympus) with a CCD camera (CoolSNAP HQ; Photometrics) controlled by InVivo software (Media Cybernetics).

Photoconversion of Dendra Fusion proteins We performed two sets of photoconversion experiments using Dendra fusion proteins. In the first set, we examined the localization of Dendra fusion proteins after loss of Nup170p and Nup157p. Cells were grown in media without methionine, then switched to a 2% low-melt agarose pad containing minimal medium and 200 µg/ml methionine and converted (4x20ms DAPI). Cells were imaged, then allowed to grow 6 h at 30°C. Cells were imaged after the incubation. The second set of experiments examined the localization change of Dendra fusion proteins after derepression of *NUP170*. A P_{MET3}*-NUP170 nup157Δ* yeast strain expressing *NUP82-3xDendra* or *NUP60-3xDendra* was grown in media lacking methionine. Methionine was then added, and the cells were incubated for 5 h to induce cytoplasmic foci of Nup82-3xDendra or Nup60-3xDendra. The cells were washed with media lacking methionine and then placed on an agarose pad (2% low-melt agarose containing the supplemented minimal medium lacking methionine) to allow Nup170p production. Nup82-3xDendra or Nup60-3xDendra was photoconverted by 4 × 20-ms pulses of UV light, and the Dendra-red signals were imaged just after photoconversion. Cells were allowed to grow on the agarose pad for 2 h at 30°C, and images of the Dendra-red signal were captured.

Table VII

Plasmid	Description	Source
pTM1046	*P_{MET3}-NUP170*	this study

Table VIII

Strain	Relevant Genotype	Source
TMY1098	nup170::KanMX6-PMET3-HA3-NUP170	this study
TMY1126	nup157D::URA3 nup170::KanMX6-PMET3-HA3-NUP170	this study
KWY2215	NUP82-Dendra-HIS3MX6 nup157D::URA3 nup170::KanMX6-PMET3-HA3-NUP170	this study
KWY2216	NUP60-Dendra-HIS3MX6 nup157D::URA3 nup170::KanMX6-P-HA3-NUP170	this study

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