UNIVERSITY OF CALIFORNIA, SAN DIEGO

Cell Cycle Dependent Differences in Nuclear Pore Complex Assembly

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by

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ABSTRACT OF THE DISSERTATION

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by

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In metazoa, nuclear pore complexes (NPCs) assemble from disassembled precursors into a reforming nuclear envelope (NE) at the end of mitosis, and into growing intact NEs during interphase. Whether there are differences in the mechanism of NPC assembly in these two scenarios is a long standing question in the field that has not been addressed. Experiments described in this dissertation, show that ELYS, a nucleoporin critical for the recruitment of the essential Nup107/160 complex to chromatin, is crucial for

NPC assembly at the end of mitosis, but is not required in interphase. Conversely, the transmembrane nucleoporin POM121 is critical for the incorporation of the Nup107/160 complex into new assembly sites specifically during interphase and plays a role in fusing the two leaflets of the NE. We show that in contrast to post-mitosis, where the Nup107/160 complex is targeted to chromatin via ELYS, during interphase this NPC sub-complex assembles at sites of forming pores. These results indicate that, in organisms with open mitosis, NPCs assemble by two distinct mechanisms to accommodate cell cycle-dependent differences in NE topology.

Chapter 1: Introduction

1.1 The Nuclear Pore Complex: Architecture

Initial electron microscopy studies of the nuclear envelope, from as early as 1950, revealed fenestrations in the membrane, which have been named nuclear pore complexes. NPCs are large multiprotein assemblages (60-120 MDa), embedded in and traversing both leaflets of the NE, exhibiting eightfold radial symmetry about a central axis. Biochemical studies, extensive analysis by electron and fluorescence microscopy (Jarnik and Aebi 1991; Yang, Rout et al. 1998; Stoffler, Feja et al. 2003), and more recent computer modeling (Alber, Dokudovskaya et al. 2007; Alber, Dokudovskaya et al. 2007) have revealed an intriguing structure with several layers of architectural complexity.

1.1.1 The NPC assembles from nucleoporins

Each NPC is assembled from ~30 different proteins, nucleoporins (Nups), which are present in multiple copies due to the eightfold symmetry of the pore, amounting to a total of ~500 polypeptides (Figure 1). Based on their positions within the pore, residence times at the pore and structural features, Nups can be classified into three general categories; (i) scaffold Nups, which mainly consist of the multiprotein Nup107/160 and Nup93/205 subcomplexes

(Debler, Ma et al. 2008), (ii) peripheral Nups and (iii) the transmembrane Nups. While the NPC scaffold is thought to provide structural integrity to the highly curved pore membrane and the NPC itself, the peripheral Nups, many of which contain phenylalanine-glycine (FG)-repeats, are responsible for establishing the permeability barrier (D'Angelo, Raices et al. 2009) and mediating nuclear trafficking (Weis 2002). These proteins extend from the membrane-embedded scaffold either into the pore channel, such as the Nup62 complex, as filaments into the cytoplasm, like Nup88 and Nup214 or the nucleoplasm such as Nup153 and Nup98 (Alber et al., 2007b; Beck et al., 2004; Brohawn et al., 2009). In mammalian cells, three transmembrane Nups have been identified: POM121, gp210, and NDC1 (Chial et al., 1998; Hallberg et al., 1993; Mansfeld et al., 2006; Stavru et al., 2006b). As POM121 and NDC1 have been shown to interact with both the Nup107/160 and Nup93/205 subcomplexes (Mitchell, Wozniak personal communication), these Nups are thought to directly anchor the NPC to the NE.

The Nup107/160 complex has been shown to be an early and essential player in NPC formation both *in vitro* and *in vivo* (Harel et al., 2003; Walther et al., 2003a). In vertebrates it consists of nine polypeptides (Nup160, Nup133, Nup107, Nup96, Nup85, Nup43, Nup37, Seh1 and Sec13), which assemble into a Y-shaped complex (Lutzmann, Kunze et al. 2002). Of particular interest, its members are primarily composed of β-propellers and α-solenoids (Brohawn, Partridge et al. 2009), a protein fold composition shared exclusively

with other membrane coating protein complexes including clathrin coats and the COPII coatomer of the ER/Golgi (Alber et al., 2007; Brohawn et al., 2008; Devos et al., 2004). Furthermore, several of the scaffold Nups in both yeasts and vertebrates possess an ALPS-like motif shown to target curved membranes *in vitro* (Drin, Casella et al. 2007).

NPC assembly appears to be a stepwise process. *In vitro* studies using *Xenopus* egg extracts, revealed NPC assembly during NE reformation is initiated by the recruitment of the Nup107/160 complex (Belgareh et al., 2001; Harel et al., 2003; Walther et al., 2003b) to chromatin; a step mediated by the DNA-binding Nup ELYS/Mel28 (Franz et al., 2007; Gillespie et al., 2007; Rasala et al., 2006). This is followed by recruitment of ER membranes, containing the transmembrane Nups POM121 and NDC1, and subsequent incorporation of Nup155 and Nup53 (Antonin, Ellenberg et al. 2008). These results have been recapitulated in imaging studies of post-mitotic cells in culture and to some extent in interphase cells (Dultz, Zanin et al. 2008), and are discussed further in the following sections.

1.1.2 The nuclear envelope and transmembrane nucleoporins

The nuclear envelope is a double lipid bilayer membrane system delineating the cytoplasm from the nucleus and the two leaflets of the NE are called the inner and outer nuclear membranes (INM/ONM). The outer membrane is contiguous with the rough endoplasmic reticulum, with the perinuclear space (PNS) being a continuation of the ER lumen. Even so, both

the outer and inner membranes are unique membrane systems with specific integral membrane compositions. During open mitosis the NE and its associated membrane proteins retract into the mitotic ER (Ellenberg, Siggia et al. 1997; Daigle, Beaudouin et al. 2001; Beaudouin, Gerlich et al. 2002; Anderson and Hetzer 2007) to allow access of the cytoskeleton to the mitotic chromosomes.

Several nuclear envelope specific proteins have been identified and some have been shown to interact with chromatin and/or the nuclear lamina; these proteins are collectively termed NETS (Schirmer, Florens et al. 2005). Interestingly over 50% of these proteins have domains with high pI values indicating possible DNA binding and a small subset of NETs have been shown to directly bind DNA *in vitro* (Ulbert, Platani et al. 2006). Further, studies from our lab have shown that in telophase the NE reforms from via connections of ER tubules to chromatin and that the coordinated and collective effort of chromatin binding INM proteins is required for this process (Anderson and Hetzer 2007; Anderson, Vargas et al. 2009).

In mammalian cells, three transmembrane nucleoporins have been characterized: POM121, gp210, and NDC1 (Hallberg, Wozniak et al. 1993; Chial, Rout et al. 1998; Mansfeld, Guttinger et al. 2006; Stavru, Hulsmann et al. 2006). In yeast there are also three transmembrane Nups (NDC1p, POM152p and POM34p), however only NDC1 is conserved with metazoa (Tcheperegine, Marelli et al. 1999; Madrid, Mancuso et al. 2006; Miao, Ryan et

al. 2006). Transmembrane nucleoporins are thought to be crucial for interphase NPC biogenesis by potentially mediating fusion of the inner and outer nuclear membranes, and recruiting and anchoring soluble NPC components to the nuclear membrane (Suntharalingam, and Wente, 2003; Antonin et al., 2008).

RNAi-mediated silencing of POM121 alone or in combination with gp210 had no effect on cell viability but caused somewhat variable phenotypes with respect to the distribution of other nucleoporins (Antonin et al., 2005; Stavru et al., 2006b). RNAi depletion of NDC1, the only evolutionarily conserved transmembrane nucleoporin, caused more severe phenotypes and affected NE localization of multiple nucleoporins (Mansfeld et al., 2006; Stavru et al., 2006a). Interestingly, the knockout of NDC1 in C. elegans also affected nucleoporin distribution, yet NDC1 is not absolutely essential for viability (Stavru et al., 2006a). Together these studies suggest that the role of the three transmembrane nucleoporins in NPC biogenesis and maintenance might be redundant.

1.1.3 NPC breakdown

Nuclear pore complexes have been shown to be stable and immobile in an interphase context as well as in terminally differentiated cells (Daigle, Beaudouin et al. 2001; D'Angelo, Raices et al. 2009), however, each time a eukaryotic cell divides, the NE and transmembrane Nups become part of the mitotic ER and NPCs are disassembled into subcomplexes. Studies in starfish

oocytes have shown the combined disassembly of NPCs and nuclear envelope breakdown (NEBD) occurs in two phases (Lenart, Rabut et al. 2003). Phase one is characterized by the progressive and rapid loss of peripheral and FG repeat Nups (Nup98, Nup153 and Nup214) resulting in the loss of the permeability barrier, allowing molecules with diameters of up to 40nm to freely diffuse in and out of the nucleus. The second phase of this process is a more dramatic fenestration of the NE allowing passive diffusion of molecules of at least 500 kD. Interestingly, in spite of the large disruptions in the NE, in these oocytes the underlying structural lamina and the outline of the NE remain somewhat intact.

Live imaging in mammalian cells shows NPC disassembly during prophase to occur within a similar time frame (~5-6 minutes) and that the loss of Nups from the NPC, while beginning with a loss of Nup98 as previously described, is a somewhat synchronous event. These events are likely triggered by extensive phosphorylation of the Nups (Glavy, Krutchinsky et al. 2007) along with lamins and integral membrane proteins of the NE (Foisner and Gerace 1993). In this example of mammalian cells, the lamina also disassembles and the NE is absorbed into the mitotic ER.

The other scenario of NPC breakdown occurs during apoptosis, signaled in part by a breach of nuclear integrity. To this end several peripheral Nups have been shown to be caspase targets (Ferrando-May, Cordes et al.

2001; Patre, Tabbert et al. 2006). It is interesting that nuclear function can be disrupted so quickly by targeting this subset of proteins.

1.1.4 NPC assembly and contextual differences within the cell cycle

Despite our detailed knowledge of the protein composition of NPCs, relatively little is known about the pathways that lead to formation of functional NPCs. In organisms with an open mitosis, NPCs assemble at two different cell cycle phases. The first phase occurs at the end of mitosis and involves the recruitment of ER membranes and disassembled NPC components to chromatin (Walther, Alves et al. 2003; Anderson and Hetzer 2007; Dultz, Zanin et al. 2008). *In vitro* studies of NPC assembly during NE reformation using egg extracts revealed that nuclear pore formation is initiated by the recruitment of the Nup107-160 complex (Belgareh, Rabut et al. 2001) (Harel, Orjalo et al. 2003; Walther, Alves et al. 2003) to chromatin, via a DNA binding AT-hook motif in the Nup ELYS/Mel28 (Rasala, Orjalo et al. 2006; Franz, Walczak et al. 2007; Gillespie, Khoudoli et al. 2007). This is followed by arrival of the transmembrane Nups POM121 and NDC1, and subsequent incorporation of Nup155 (Nup157/Nup170 in yeast) and Nup53 (Nup53/Nup59 in yeast) ultimately leading to formation of complete and functional NPCs (Antonin, Ellenberg et al. 2008).

Experiments using live imaging of fluorescent Nups in post-mitotic HeLa cells also showed an ordered recruitment of nuclear pore proteins to chromatin. In agreement with the *in vitro* data, of the proteins in their study,

Nup133 was first to be recruited, followed by POM121 and then peripheral Nups (Dultz, Zanin et al. 2008).

The second scenario of NPC assembly occurs during interphase and requires the targeting and insertion of newly synthesized nucleoporins to an intact NE. Little is known about the mechanism of NPC biogenesis in interphase, and it is unclear how NPCs are inserted into the two lipid bilayers of an intact NE and if this process is distinct from post-mitotic assembly. We do know that NPCs are created *de novo* and that the Nup107/160 sub-complex is required from both sides of the NE indicating that the mechanism of assembly in this context requires coordination across the two leaflets of the NE (D'Angelo, Anderson et al. 2006).

1.2 The Nuclear Pore Complex: Function

1.2.1 Nucleo-cytoplasmic transport and the permeability barrier

Eukaryotic cells are defined by the compartmentalization of the nucleus from the cytoplasm by the nuclear envelope. NPCs allow passive diffusion of small molecules and are the sole sites of the highly regulated active transport of substrates larger than 60 KD between these two compartments. Active transport is driven by specific transport signals (NLS and NES), importins, exportins and the RanGTPase system (Gorlich and Kutay 1999). While mechanistic details are still controversial it is believed that import and export adaptor proteins, which bind to NLS and NES signal sequences on transport

substrates, interact with the disordered FG repeats extending into the NPC central channel to navigate through the pore. Studies in yeast have shown that the phenylalanine-glycine (FG) repeat motifs of several Nups extending into the NPC channel cooperatively contribute to form the permeability barrier (Patel, Belmont et al. 2007). Dramatic *in vitro* studies using purified FG repeat domains from Nsp1 (yeast homolog of Nup62), as well as GLFG repeats show that these motifs are able to form a physical gel that remarkably reproduces key characteristics of the NPC permeability barrier. Namely, NLS/importin mediated cargo entry; the cargo receptor Importin β was able to enter these gels >1000x faster than a non-transport related protein molecule of similar size (Frey, Richter et al. 2006; Frey and Gorlich 2007; Frey and Gorlich 2009).

Of particular interest is the recent implication of the classical NLS/karyopherin transport pathway in the movement of integral membrane proteins from the ER/ONM to the INM (King, Lusk et al. 2006). How these membrane proteins physically cross the pore membrane without disrupting the structure of the NPC and soluble transport is still unclear.

1.2.2 Cell cycle specific roles for nucleoporins

Evidence for specific roles of Nups during mitosis, when Nups are triggered for disassembly by phosphorylation (Glavy, Krutchinsky et al. 2007), comes in part from differential localization of nucleoporin subcomplexes. While the transmembrane Nups are absorbed by the ER and several soluble Nups become diffusely localized in the mitotic cytoplasm, the Nup107/160

complex localizes to the kinetochore (Loiodice, Alves et al. 2004), and RNAi mediated knockdown of ELYS leads to both spindle defects and chromosome bridges (Rasala, Orjalo et al. 2006). Additionally, Seh1 of the Nup107 subcomplex is required for proper localization of Aurora B at centromeres and proper kinetochore-microtubule attachment (Platani, Santarella-Mellwig et al. 2009). The peripheral Nup358 also relocates to mitotic kinetochores and is required for their maturation, chromosome congression and proper spindle assembly (Joseph, Liu et al. 2004; Joseph and Dasso 2008). Conversely, the metaphase checkpoint proteins Mad1 and Mad2, transiently present at kinetochores in mitosis, have been shown to localize to yeast NPCs in interphase via the Nup53p complex (Iouk, Kerscher et al. 2002).

In a broader context, nuclear pore proteins appear intimately linked to cell cycle timing with Nup153 and Nup358 being implicated in nuclear envelope breakdown (Prunuske, Liu et al. 2006) and cellular levels of Nup96 influencing cell cycle progression (Chakraborty, Wang et al. 2008). Further, in *Drosophila*, Megator, a Tpr homolog, forms a complex with the spindle assembly checkpoint protein Mad2 which associates with and promotes elongation of the mitotic spindle and proper chromosome movement (Lince-Faria, Maffini et al. 2009).

1.2.3 Nucleoporins as regulators of transcription and development

In addition to their role as transport channels, NPCs have been implicated in chromatin organization and gene regulation (Akhtar and Gasser,

2007; Capelson and Hetzer, 2009). Originally, a relationship between nuclear pores and active genes was implied by high-resolution images of mammalian nuclei showing distinct non-random association of de-condensed chromatin with nuclear pores. The ‘gene gating hypothesis’ (Blobel 1985), proposed that nuclear pores are capable of specifically interacting with active genes to promote co-regulation of transcription with mRNA export. This idea was supported by studies in yeast showing that Nups associate with promoters of active genes (Schmid, Arib et al. 2006) and that the expression of inducible genes is increased by interactions with nuclear pores (Taddei, Van Houwe et al. 2006). Furthermore, a genome-wide chromatin immunoprecipitation (ChIP) analysis in *S. cerevisiae* demonstrated that a subset of Nups occupy regions of highly transcribed genes (Casolari et al., 2004). As further evidence for the role of the NPC in regulation of active chromatin, nucleoporins have been found to participate in X-chromosome transcriptional hyper-activation in dosage compensation of *Drosophila melanogaster* (Mendjan et al., 2006).

Nups have also been shown to function as chromatin boundaries in *S. cerevisiae* (Dilworth et al., 2005; Ishii et al., 2002). Boundary activity involves protection from nearby activating or repressive signals (Capelson and Corces 2004) and constitutes another possible function for NPCs in the organization of discrete chromatin domains.

In contrast to studies in yeast, the only genome-wide study of Nup binding to chromatin in animal cells revealed a correlation between the binding

sites of Nups and regions enriched in repressive histone modifications (Brown, Kennedy et al. 2008), which exhibited characteristics of sequences known to associate with the nuclear periphery in human cells (Guelen et al., 2008). The differences between the yeast and mammalian data suggest that the genome-binding pattern of the NPC may be more diverse or complex in metazoa.

Many peripheral Nups have been shown to be mobile and to move dynamically on and off the pore (Rabut, Doye et al. 2004) and there is experimental support for the presence of intranuclear Nups in mammalian cells (Enninga et al., 2003; Griffis et al., 2002). Our lab has recently provided evidence that a subset of Nups bind to distinct regions of the *Drosophila* genome in both polytenized and diploid cells and that these interactions occur at off-pore locations. Most significantly, these experiments show that NPC proteins play an essential role in the induction of transcription during *Drosophila* development, suggesting a direct function for Nups in the regulation of gene expression in metazoa (Capelson, Liang et al.).

1.2.4 NPCs, the nuclear envelope and the cytoskeleton

As described in section 1.2.2, nuclear pore proteins have been implicated in mitosis specific roles related to proper spindle alignment, chromosome orientation and segregation. Interestingly, recent studies show Nups and NE specific proteins also interact, albeit indirectly, with the interphase cytoskeleton. Both yeast and vertebrates contain ONM specific membrane proteins which contain short ~50 amino acid KASH (Klarsicht, Anc-

1, Syne homology) domains facing the perinuclear space (PNS) (Mosley-Bishop, Li et al. 1999; Apel, Lewis et al. 2000). Termed nesprins in mammals, members of this protein family have been shown to interact with microtubules, actin and the cytolinker plectin (Zhang, Ragnauth et al. 2002; Wilhelmsen, Litjens et al. 2005; King, Drivas et al. 2008). This class of proteins has been shown to interact within the PNS via their KASH domains with INM specific proteins called Suns. In turn, the Sun proteins have been shown to interact with both lamins and NPCs; together these INM/PNS/ONM spanning interactions are called LINC complexes (Padmakumar, Libotte et al. 2005; Crisp, Liu et al. 2006; Haque, Lloyd et al. 2006). It is easy to imagine a number of cellular properties and functions that could rely on connections and "cross-talk" between the nuclear interior and exterior. While much remains to be elucidated, these results begin to shed light on a complex and essential communication network involving structural components of the nucleus, NPCs, NE transmembrane proteins and cytoskeletal elements.

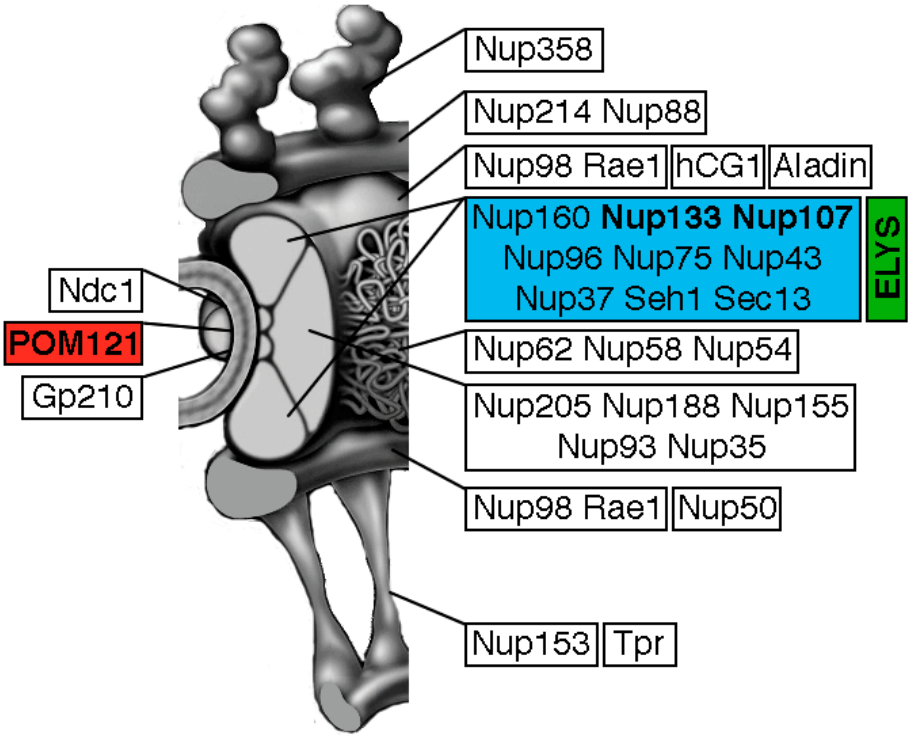

Figure 1: Schematic of the Nuclear Pore Complex

The Nuclear Pore Complex is composed of approximately 30 proteins called nucleoporins which exist as subcomplexes.

Chapter 2: Mechanistic differences in NPC assembly during the cell cycle

2.1 Summary

In metazoa, nuclear pore complexes assemble at the end of mitosis, when the nuclear envelope reforms and during interphase, as cell nuclei grow in size. It is unclear if the NPC assembly pathways during different cell cycle stages involve distinct molecular steps. Here, we show that the RNAi-mediated knockdown of ELYS, a nucleoporin required for the recruitment of the essential Nup107/160 complex to chromatin, blocks NPC assembly at the end of mitosis, but is dispensable for their formation in interphase. Conversely, the transmembrane nucleoporin POM121 and a functional NLS sequence is critical for the incorporation of the Nup107/160 complex into new assembly sites specifically in interphase.

2.2 Introduction

The two scenarios of NPC assembly, post-mitotic and interphase, occur in strikingly different physical contexts. In the first situation NPCs are reforming concomitantly with the NE with all NPC components having free access to chromatin and both sides of the reforming NE. Most studies of NPC assembly order and mechanism have recapitulated this context either in cell culture or cell free assays, such as *in vitro* nuclear assembly in *Xenopus* egg

extracts. Very few studies have targeted NPC assembly during interphase. Nonetheless the order of recruitment and general necessity of several Nups has been determined. Eight units of the Nup107/160 sub-complex comprise each of two rings, which form the central core of the NPC. This sub-complex has been shown to be required for NPC assembly both in cell culture and *in vitro* assays and also to be one of the first components of the NPC recruited to forming pores (Harel, Orjalo et al. 2003; Walther, Alves et al. 2003; Dultz, Zanin et al. 2008). Further, it has been shown to be required, during interphase, from both sides of the NE which makes intuitive sense considering the double ring structure of the NPC and that fusion of the inner and outer nuclear membranes is likely a coordinated event (D'Angelo, Anderson et al. 2006). Recently ELYS/Mel28 was identified as an additional member of this complex and has been shown to target the complex to chromatin via an AT hook motif during anaphase/telophase (Rasala, Orjalo et al. 2006; Franz, Walczak et al. 2007; Rasala, Ramos et al. 2008).

The transmembrane Nups have also been popular candidates for early involvement in pore assembly due to its being embedded in and connected to the nuclear envelope. POM121 was the first transmembane pore protein described and thus has been the subject of more extensive study (Hallberg, Wozniak et al. 1993). While there is agreement on its early involvement in post-mitotic NPC and NE formation and there are studies linking it to the Nup107 complex via ELYS (Antonin, Franz et al. 2005; Rasala, Ramos et al.

2008), in cell experiments have resulted in a range of conclusions some calling it essential and some calling it dispensable (Stavru, Nautrup-Pedersen et al. 2006). In this chapter we present a series of experiments providing evidence for mechanistic differences in NPC assembly pertaining to these two contexts of the cell cycle and the proteins described above.

2.3 Results

2.3.1 The nucleoporins ELYS and POM121 have non-redundant functions in NPC assembly

Studying potential differences between NPC assembly pathways at the end of mitosis and during interphase is complicated by the fact that a subset of nucleoporins are extremely long-lived, making it difficult to efficiently deplete these proteins by RNA interference (RNAi) (Rabut, Doye et al. 2004). A good example is the essential, multimeric Nup107/160 complex (Figure 1), which does not turn over once incorporated into the NE (D'Angelo et al., 2009; Rabut et al., 2004). To improve the extent of Nup107/160 complex depletion, we repeatedly transfected U2OS cells with either control siRNA oligos or oligos specific for the complex member Nup107 every other day for a total of 12 days. Cells were stained at two day time points with mAb414, an antibody that recognizes the four NPC components Nup358, Nup214, Nup153 and Nup62 (Davis and Blobel 1987) (Figure 2A). While the overall NPC number in control cells remained constant during the course of the experiment, the knockdown

of Nup107 resulted in strong reduction of NPC density. To obtain quantitative data, we generated maximum projections from confocal z-sections encompassing whole nuclei and measured the total fluorescence intensity of individual nuclei. We validated this method by quantifying total NPCs in wild-type cells synchronized in G1 and G2 and found it to accurately reflect the doubling of NPC number in interphase (Maul, Price et al. 1971)(Figure S1). Using this approach we detected a sharp decrease of nuclear pore numbers to ~ 30% in Nup107 depleted cells (Figure 2B). Consistent with the observed reduction in total NPC number, the protein levels of Nup107 were reduced to < 25% as determined by Western blotting (Figure 3). These results show that repeated RNAi treatments over prolonged periods of time allow the efficient depletion of scaffold nucleoporins and confirm that the Nup107/160 complex is required for NPC assembly.

We used this experimental strategy of repeated RNAi treatments to test if other NPC components implicated in early steps of pore assembly behave in a similar manner. We first focused on POM121, a transmembrane nucleoporin previously shown to be required for NE formation *in vitro* (Antonin, Franz et al. 2005). In cells treated with siRNA oligos against POM121 over the same period of 12 days the number of pores was also reduced (Figure 2). However, quantitative immunofluorescence analysis of mAb414 signal revealed that the total pore number plateaued at ~ 50-60% of control levels (Figure 2B); a striking contrast to the more dramatic pore reduction in the Nup107

knockdown. Consistent with the observed reduction in NPC number, the endogenous Nup107 protein concentration was also reduced to ~ 60% in POM121 depleted cells. Remarkably, while total pore numbers and Nup107 levels remained at 50-60% of control levels, after 12 days protein levels of POM121 were reduced to < 8% (Figure 3A, 3B), suggesting that this protein may not be essential for nuclear pore formation.

To further test this possibility, we stained cells with mAb414 and α-POM121 antibodies. Surprisingly, we found that a majority of NPCs in POM121 RNAi treated cells did not contain detectable POM121 signal (Figure 4, middle panels), indicating that cells are able to form nuclear pores in a POM121 independent manner although the total numbers are reduced. In contrast, virtually all NPCs that were visible in Nup107 knockdown cells contain both the remaining Nup107 and mAb414 reactive Nups (Figure 4 left panels).

Another NPC component that has been shown to be critical for NPC assembly is ELYS (Franz et al., 2007; Rasala et al., 2008). We found that knockdown of ELYS exhibited a similar phenotype to that of POM121: reducing total NPC numbers but still allowing the formation of ELYS negative NPCs (Figure 4 right panels). To determine if these POM121 or ELYS negative NPCs were fully formed pores or perhaps NPC intermediates we performed immunofluorescence staining of nuclear surfaces in cells treated with siRNA oligos for either POM121, ELYS or Nup107, with a wide array of

Nup antibodies representing different structural components of the NPC (Figure 5). We found that in each of our knockdown conditions, all of the Nups we stained for were present, and confirmed that NPCs were able to form, albeit in lower numbers, without POM121 or ELYS.

Additionally we performed transmission electron microscopy (TEM) and measured the approximate diameter of NPCs under these conditions. We found an expected decrease in NPC density but no differences in the shape or size of the NPCs as could be determined at this resolution (Figure 6).

The finding that NPC formation can occur in the absence of either POM121 or ELYS, yet their knockdown reduces total NPC numbers, raised the possibility of these two Nups having redundant or non-overlapping functions. The double knockdown of both proteins had an additive inhibitory effect on NPC assembly (Figure 7), suggesting that POM121 and ELYS play distinct roles in pore assembly.

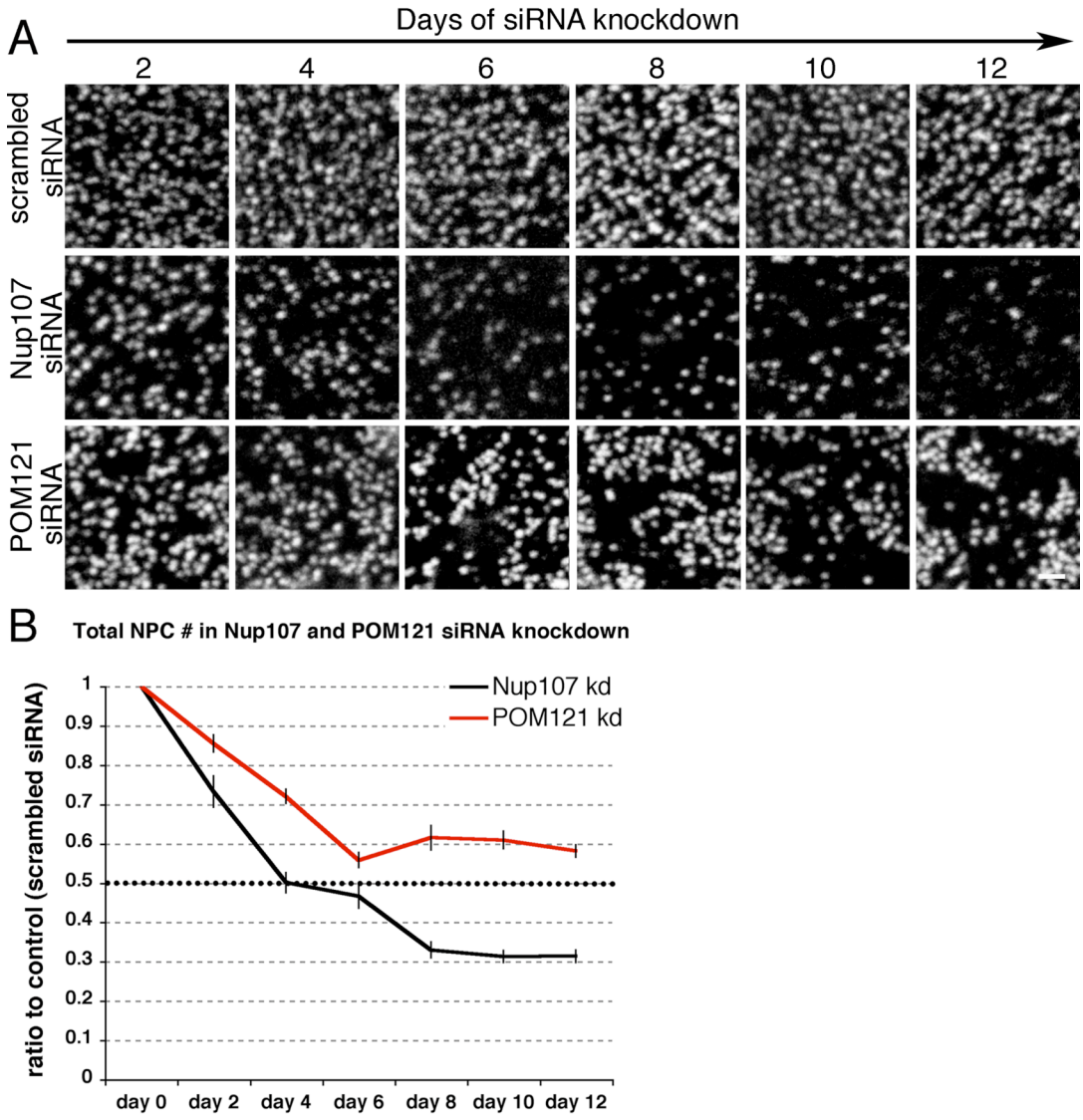

Figure 2: Extended Nup107 and POM121 siRNA treatments reduce total NPCs.

(A) U2OS cells were treated repeatedly every 48 hrs with scrambled, POM121 or Nup107 siRNA oligos for 12 days, fixed at indicated time points and immunostained with mAb414. (B) Quantification of mAb414 immunofluorescence (representing total NPCs per nucleus) over time, graphed as a ratio to control levels, N > 25 nuclei per time point.

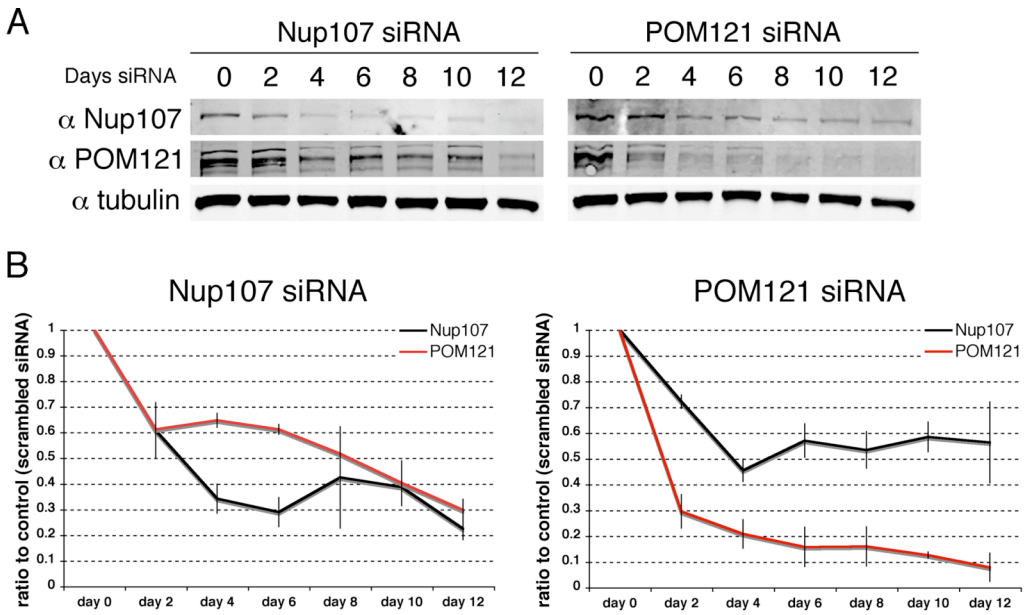

Figure 3: Extended Nup107 and POM121 treatments show distinct phenotypes.

(A) Western Blot analysis of U2OS cell lysates harvested every 48 hrs during a 12 day time course of POM121 or Nup107 siRNA treatment. (B) Quantification of protein levels in western blots against Nup107 and POM121 graphed as a ratio to control levels. N=3 independent Western blots. Error bars are standard error.

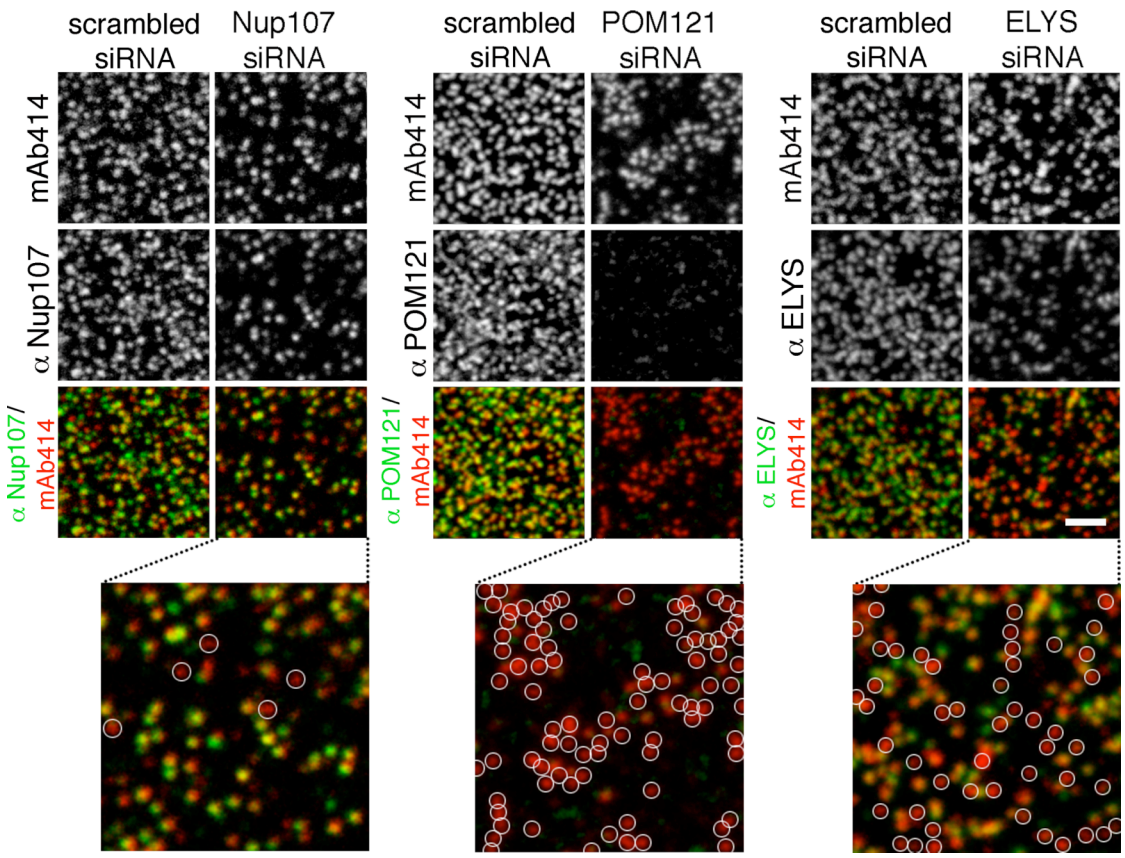

Figure 4: NPCs form in the absence of POM121 or ELYS.

Immunofluorescence staining of nuclear surfaces using mAb414 and antibodies against Nup107, POM121 or ELYS in U2OS cells treated with siRNA oligos for 4 days against the indicated Nup. White circles indicate NPCs lacking either Nup107, POM121 or ELYS. Scale bar 2μm.

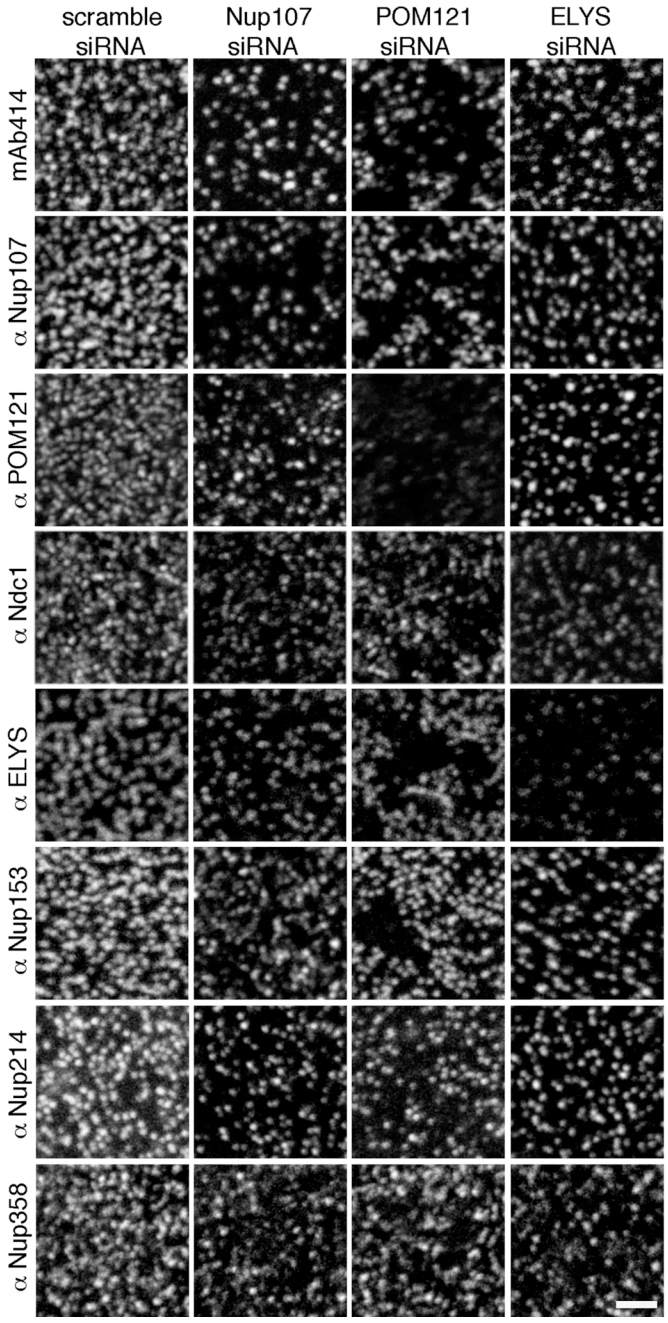

Figure 5: POM121 and ELYS siRNA depletions show unique phenotype.

Immunofluorescence staining of the nuclear surface in U2OS cells depleted of Nup107, POM121 or ELYS shows remaining NPCs containg Nups comprising multiple structural components of the NPC as well as Nups recruited late in NPC assembly, indicating NPCS under these conditions are complete NPCs. Scale bar 10μm.

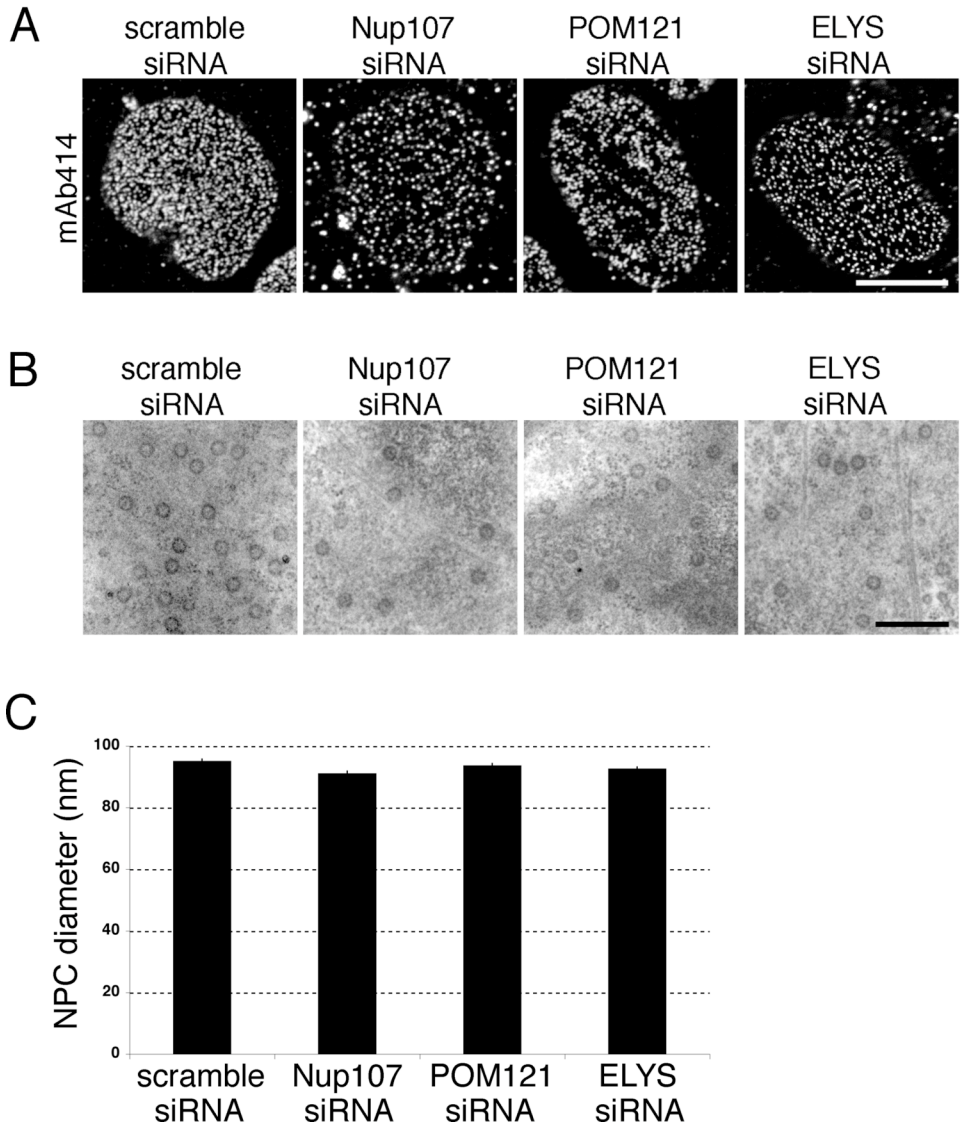

Figure 6: NPCs formed in Nup107, POM121 or ELYS depleted cells show no gross structural abnormalities

(A) mAb414 immunofluorescence staining of the nuclear surface shows a reduction in NPC density in U2OS cells depleted of Nup107, POM121 or ELYS for 72 hrs. Scale bar 10μm. (B) Transmission EM of the nuclear surface in U2OS cells depleted of Nup107, POM121 or ELYS shows a reduction in NPC density and that remaining NPCs under these conditions show no gross structural abnormalities. Scale bar 500nm. (C) Approximate NPC diameter as measured from TEM images shown in (B) show no significant difference between control cells and cells depleted of Nup107, POM121 or ELYS. N=50 NPCs.

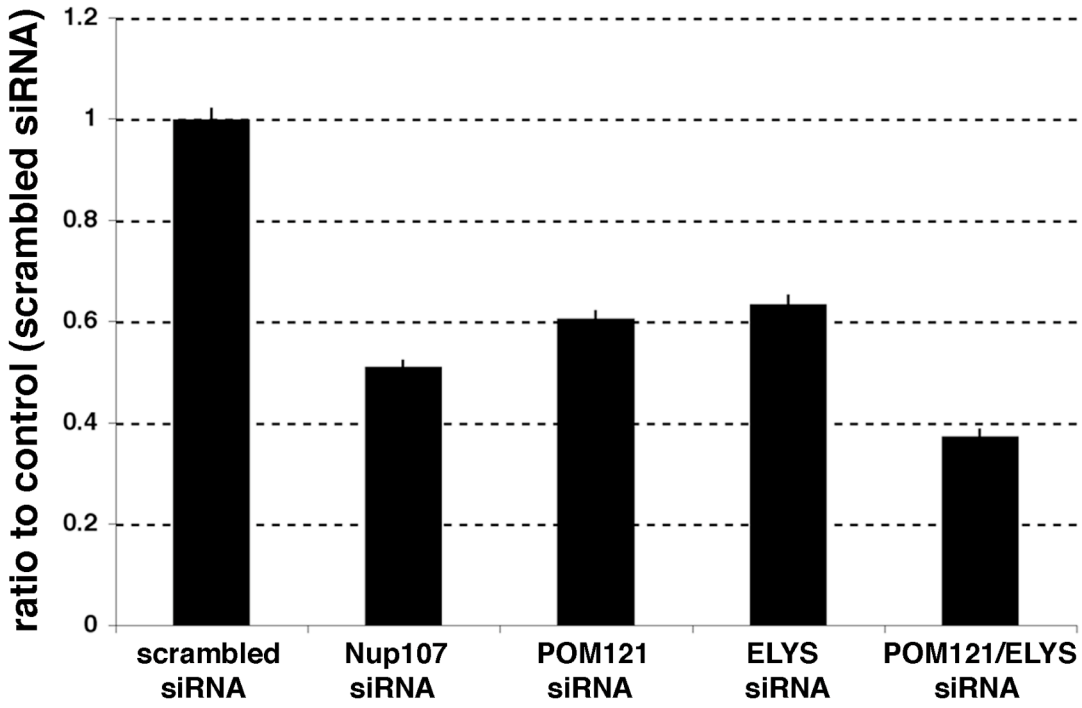

Figure 7: POM121 and ELYS play non-redundant roles in NPC assembly.

Quantification of mAb414 immunofluorescence in U2OS cells treated with siRNA oligos against indicated Nups, N>26 nuclei per condition. All error bars are standard error.

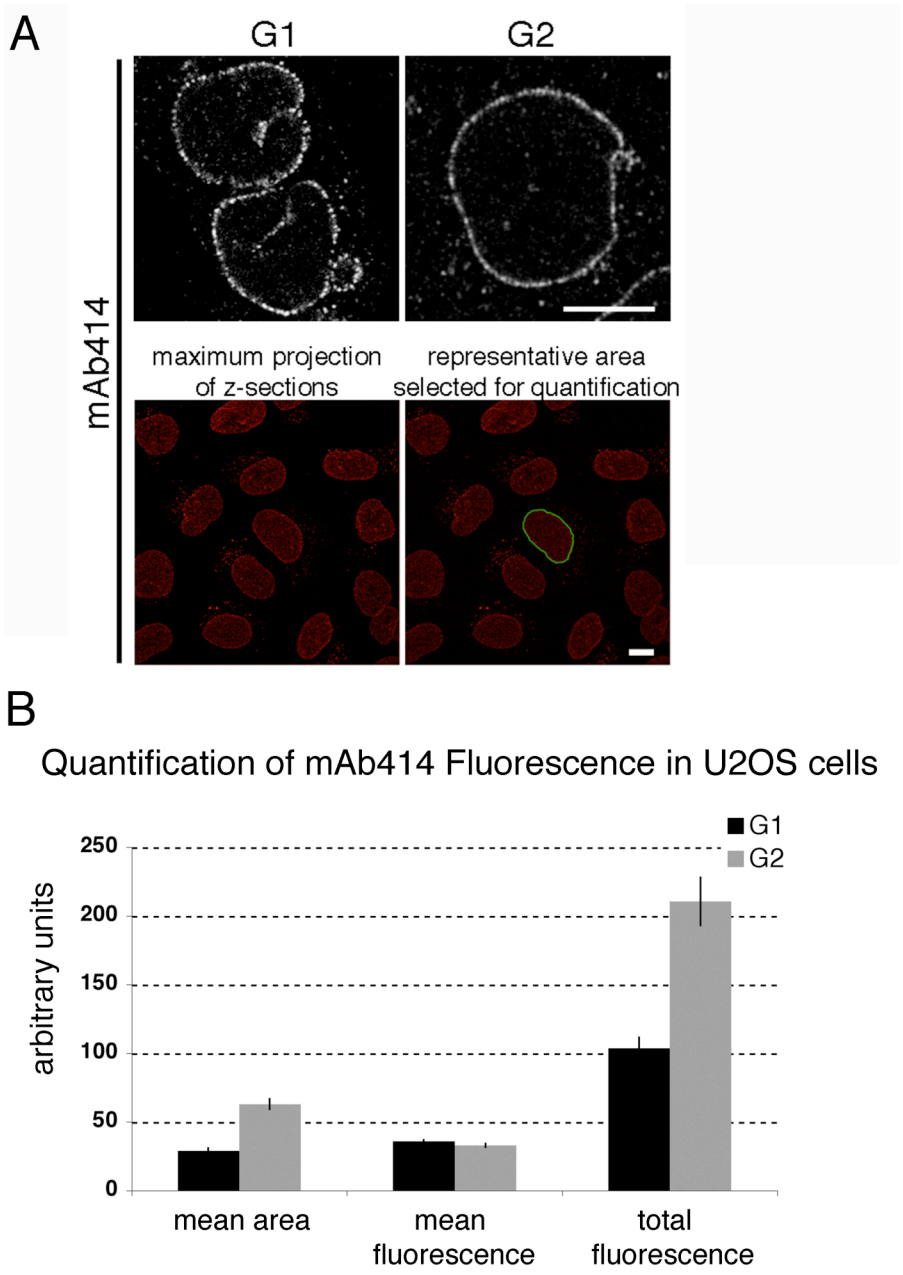

Figure S 1: Quantitative fluorescence reflects total NPC number

(A) Top panels: mAb414 immunofluorescence of nuclei in G1 and G2 illustrating change in nuclear size between growth phases. Bottom panels: example of maximum projections taken for quantification of total NPC number. Scale bars 10µm. (B) Validation of measuring total fluorescence signal by measuring pixel intensity and area of maximum projections of z-stacks taken through nuclei in G1 and G2, n > 28 nuclei per condition.

2.3.2 Elys is specifically required for post-mitotic assembly

The DNA binding Nup ELYS has been shown to be required for the targeting of the Nup107/160 complex to chromatin *in vitro*, which is critical for NPC formation during post-mitotic NE formation (Rasala et al., 2008). To test its role *in vivo*, we first knocked down ELYS (Figure 8) and monitored the recruitment of the Nup107/160 complex to chromatin during the anaphase/telophase transition using time lapse microscopy in living cells. We found that ELYS depletion strongly inhibited the recruitment of the Nup107/160 complex to chromatin, as visualized by 3GFP-Nup133, and therefore post-mitotic NPC assembly (Figure 8A, B). This implied that the observed ELYS independent formation of NPCs likely occurs in interphase. To directly test this we specifically monitored interphase nuclear pore formation by comparing NPC levels in G1 versus G2 nuclei. Cells treated with ELYS RNAi, which reduced the protein to < 20% of control levels (Figure 10A), exhibited a strong reduction in G1 pore numbers, yet NPC doubling in interphase remained efficient (Figure 11). To confirm that knockdown of ELYS had not simply prevented progression through the cell cycle and the associated insertion of new NPCs, the number of cyclin A-positive cells in each knockdown condition was quantified and shown to be comparable to control cells (Figure S2).

The overall reduction in NPC numbers at the NE but not in total protein levels of essential Nups (Figure 10A) in cells treated with ELYS siRNA can be

explained by a dramatic mis-localization of mAb414 signal from the NE to the cytoplasm (Figure 9A & B). Analysis of these cells by TEM showed the cytoplasmic signal to represent fully formed NPCs in the ER called annulate lamellae (Figure 9C). These experiments confirm previous results, and further, indicate that ELYS is required to target the Nup107/160 complex to chromatin specifically during post-mitotic pore assembly.

One reason why ELYS might be dispensable for interphase assembly could be that recruitment of the Nup107/160 complex to chromatin is either not rate limiting or not required when an intact NE is already formed. To test this directly, we investigated the requirement for ELYS in an *in vitro* system, in which NPC assembly into reforming versus intact NEs can be studied independently of cell cycle stage. We have established a *Xenopus* egg extract assay in which nuclei are pre-formed around sperm chromatin and new NPC insertion is monitored into the intact NE of expanding nuclei (D'Angelo et al., 2006). In the same system, NPC formation into the reforming NE can be analyzed when sperm chromatin is mixed with isolated membranes and cytosol (Anderson and Hetzer, 2007; Walther et al., 2003b). Using this approach, we first immuno-depleted either the Nup107/160 complex or ELYS from interphase extracts (Figure 10B) and then performed NPC assembly reactions. Consistent with previous reports, the depletion of either the Nup107/160 complex or ELYS inhibited NPC assembly in a reforming NE (Figure 12A, left panels) (Rasala et al., 2006; Walther et al., 2003a). In

contrast, when NEs were pre-formed in the presence of a limiting amount of cytosol (Anderson et al., 2008), washed and then incubated with the depleted extracts, the absence of the Nup107/160 complex prevented further NPC insertion whereas ELYS depletion did not (Figure 12A right panels, 12B). Additional evidence for ELYS' dispensability in interphase NPC insertion came from time course experiments performed in ELYS-depleted cytosol. While very few NPCs were detected in early time points (reforming NE), after extended incubation NPC levels practically matched control (mock-depleted) numbers (Figure 13). In contrast, even after four hours, nuclei formed with Nup107/160 depleted cytosol were unable to insert new NPCs.

Together, these results suggest that ELYS is required for NPC assembly into a reforming, but not pre-existing NE; and that it serves to spatially determine sites of new NPC assembly by targeting the Nup107/160 complex to chromatin.

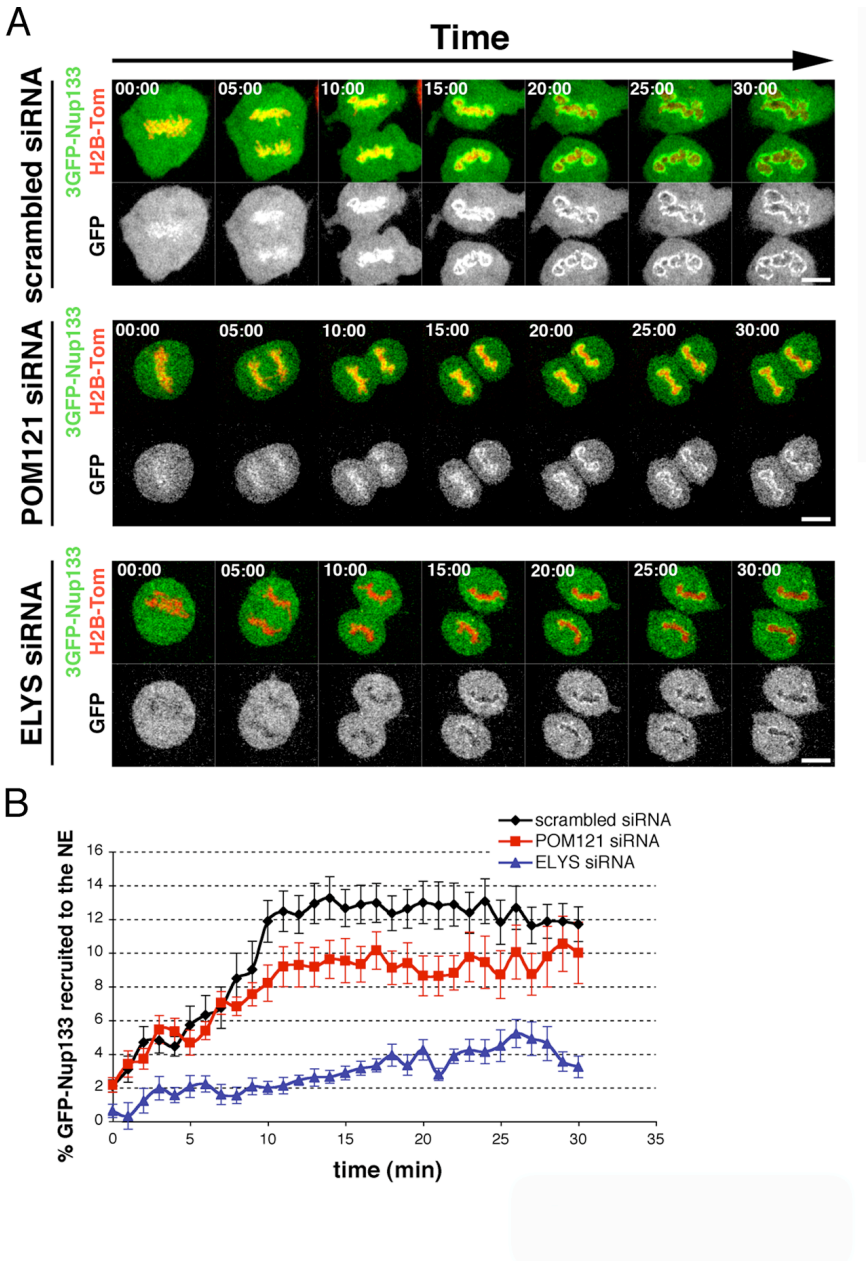

Figure 8: ELYS but not POM121 depletion inhibits Nup133 recruitment to post-mitotic chromatin.

(A) U2OS cells were transfected with 3GFP-Nup133 and H2B-tomato together with either scrambled, ELYS or POM121 specific siRNA oligos and imaged from metaphase for 30 minutes at the rate of 1 frame per minute. The onset of chromosome segregation marks t=0 (elapsed minutes are indicated for each frame). Scale bar 5 μm. (B) Quantification of GFP signal around chromatin over time, representing the Nup107/160 complex, to the reforming NE; N>12.

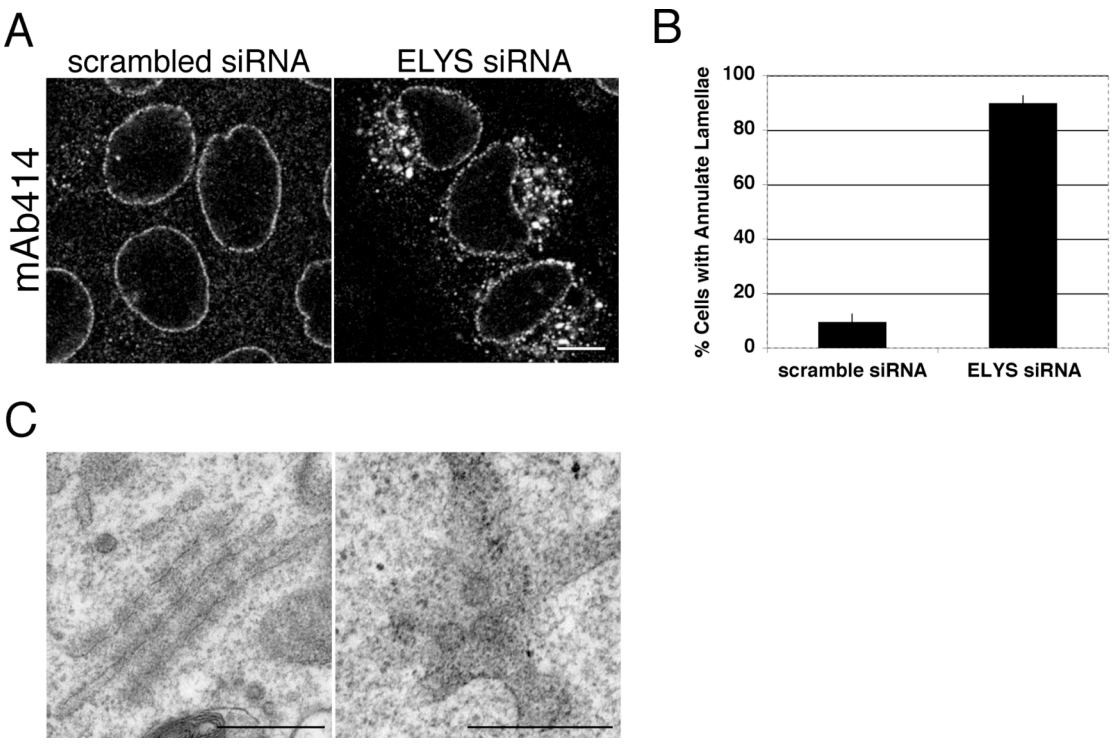

Figure 9: ELYS depletion induces mis-localization of Nups and annulate lamellae formation.

(A) Immunostaining with mAb414 shows a mis-localization of Nups in U2OS cells treated with ELYS specific siRNA oligos. Scale bar 5μm. (B) Quantification of cells containing cytoplasmic mAb414 signal in cells treated with control or ELYS specific siRNA oligos. N>160 cells. (C) Transmission electron microscopy shows annulate lamellae in U2OS cells treated with ELYS specific siRNA oligos. Scale bars 500 nm.

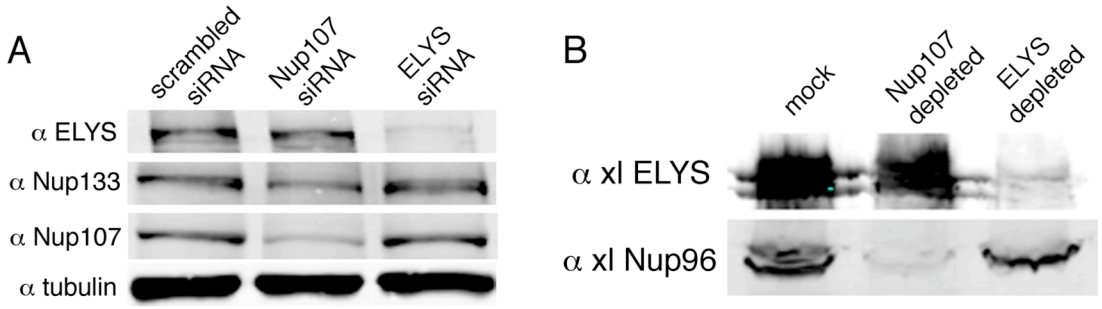

Figure 10: ELYS can be depleted independently of the Nup107/160 complex in cells and *Xenopus* egg extracts.

(A) Western blot analysis of lysates from U2OS cells treated with control, Nup107 or ELYS specific siRNA oligos. (B) Western blot analysis of immuno-depleted *Xenopus* cytosol shows specific depletion of either the Nup107 complex or ELYS.

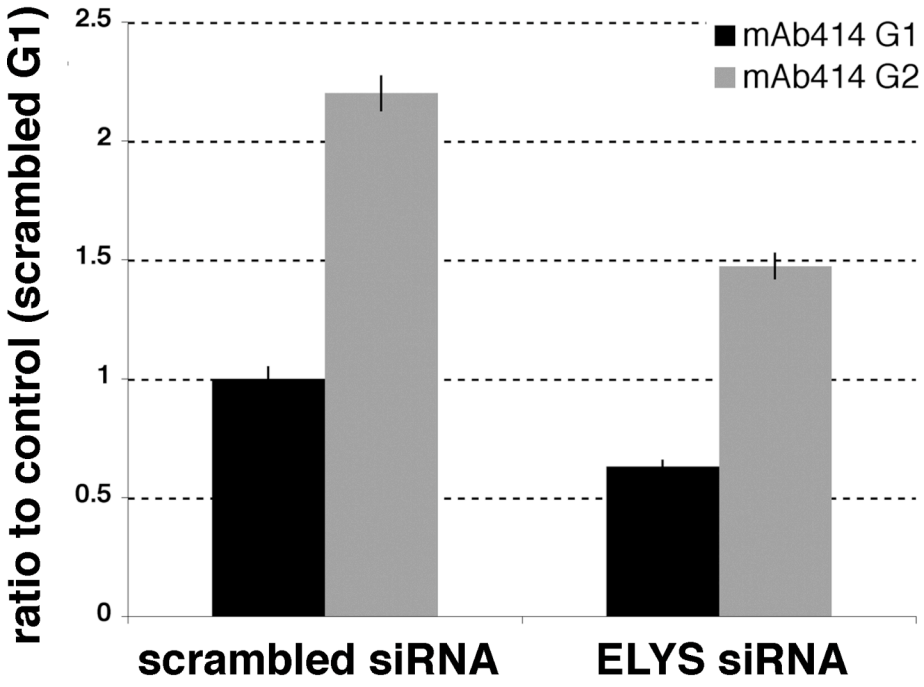

Figure 11: ELYS depletion in U2OS cells reveals a G1 specific defect in NPC assembly.

Quantification of total mAb414 fluorescent signal during G1 and G2 in U2OS cells transfected with control and ELYS siRNA oligos; N>98 nuclei per condition.

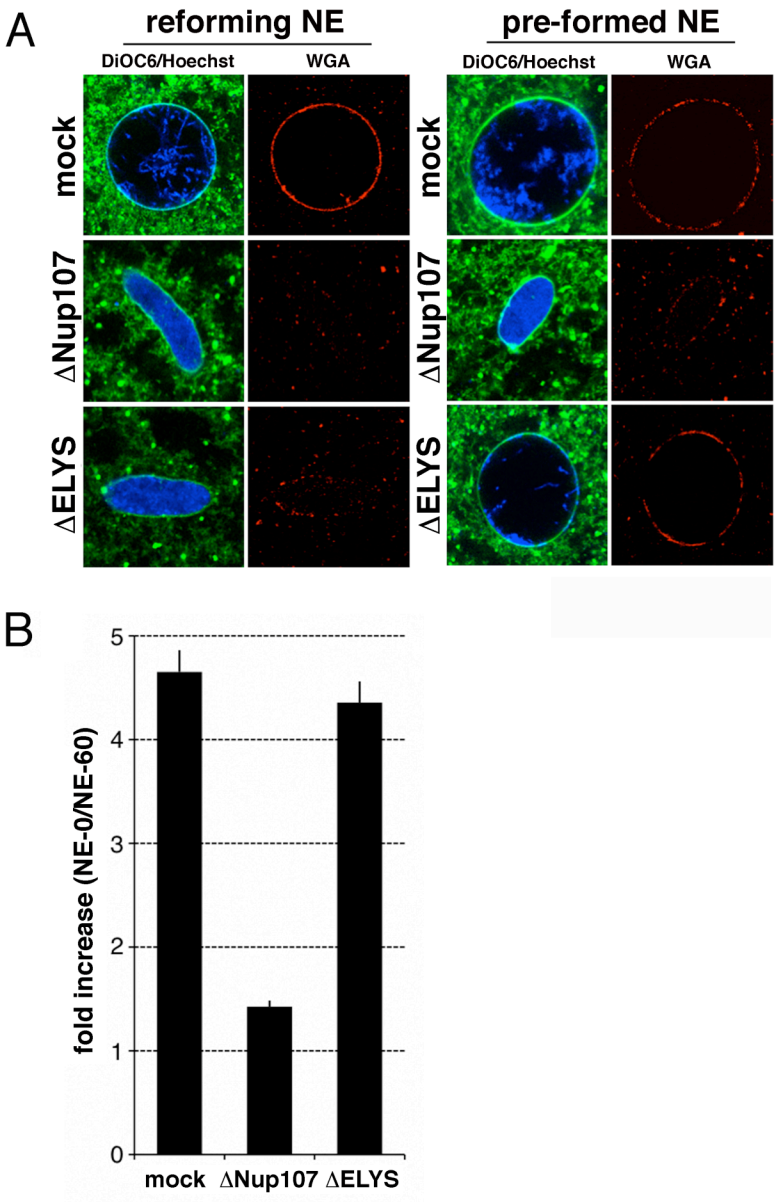

Figure 12: ELYS depletion specifically inhibits NPC assembly into reforming but not pre-formed nuclei, *in vitro*.

(A) *In vitro* nuclear assembly reactions using mock-, ELYS- or Nup107-depleted cytosol and either demembranated sperm heads (reforming NE) or washed, NE-enclosed sperm chromatin (pre-formed NE) were incubated for 60 min. Nuclei were incubated with DiOC6 (green) and fluorescent WGA (red) to visualize membranes and NPCs. (B) Quantification of total fluorescence, representing NPC number; N>150 nuclei. All error bars represent standard error.

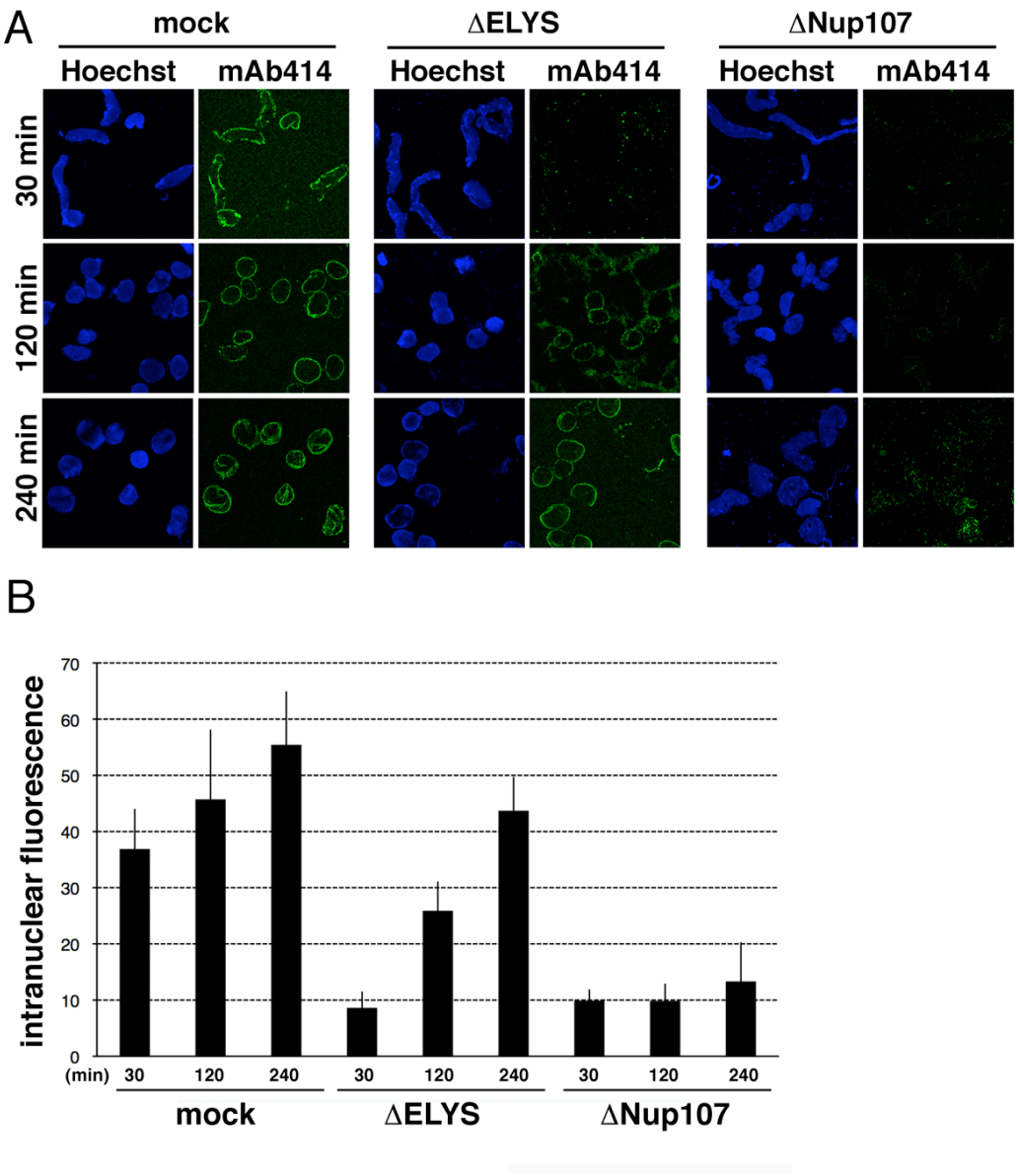

Figure 13: With extended incubations NPCs form in the absence of ELYS but not Nup107, *in vitro*.

(A) *In vitro* nuclear assembly reactions using mock-, ELYS- or Nup107-depleted cytosol were incubated for 240 minutes. Reactions were fixed and stained with mAb414 at indicated time points. (B) Quantification of total intranuclear fluorescence from time points illustrated in (A) shows efficient NPC insertion over time in ELYS- but not Nup107-depleted nuclei.

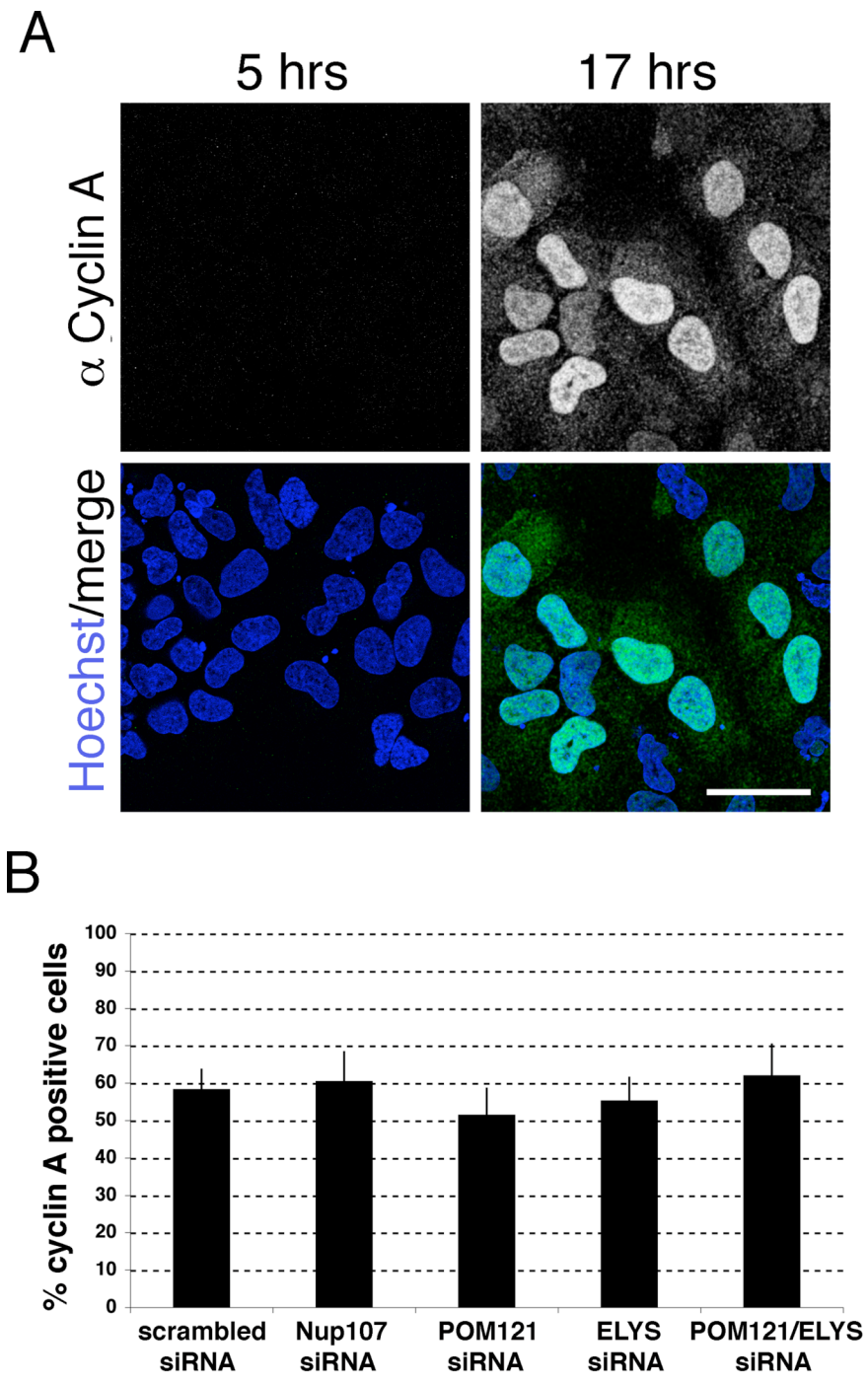

Figure S 2: Cell cycle progression in nucleoporin siRNA backgrounds

Cells at G1 and G2 time points were immunostained for Cyclin A. The number of G2 cells in each siRNA knockdown condition with positive Cyclin A staining were quantified and showed no difference from control cells.

2.3.3 POM121 is rate-limiting for interphase NPC assembly

Next we analyzed if other Nups play distinct roles in post-mitotic or interphase assembly of NPCs. We turned to POM121, since like ELYS it seemed to be dispensable for NPC formation but at the same time its depletion resulted in a general decrease in total NPCs (Figures 2, 4, 7). Consistent with our finding that ELYS and POM121 have non-redundant functions, we found that the knockdown of POM121 did not interfere with the recruitment of a 3GFP-Nup133 reporter to chromatin (Figure 8), and thus did not seem to be required for early events in post-mitotic NPC assembly. We noticed, however, that after formation of a closed NE (around 12 minutes post-anaphase onset (Anderson, Vargas et al. 2009)), the levels of Nup133 were reduced in the POM121 knockdown condition compared to control cells (Figure 8B). This indicated that POM121 might preferentially be required for NPC assembly once an intact NE has formed. To directly test this, we knocked down POM121 and examined the total pore number in cells synchronized in G1 and G2 (Figure 14 black & gray bars). Despite the efficient knockdown of POM121 protein to < 8% original levels, cells exhibited normal NPC numbers in G1, but an almost complete inhibition of the expected NPC doubling during interphase. Notably, both mAb414 reactive Nups and Nup107 were no longer recruited to the NE, suggesting that POM121 has a role in the initial steps of interphase pore assembly.

To further analyze the interphase specific role of POM121 in NPC formation, we performed an *in vitro* time course experiment using *Xenopus* egg extracts. We combined chromatin with membranes and cytosol and then monitored the rate of NPC assembly in the presence or absence of inhibitory α-POM121 antibodies (Figure 15). At early time points (post-mitotic NE formation) NPCs assembled at similar rates in both conditions. In contrast, NPC insertion during NE expansion (interphase) was specifically inhibited by the POM121 antibody.

These experiments indicate a specific role for POM121 in NPC insertion into an intact NE and suggest it is dispensible for post-mitotic assembly.

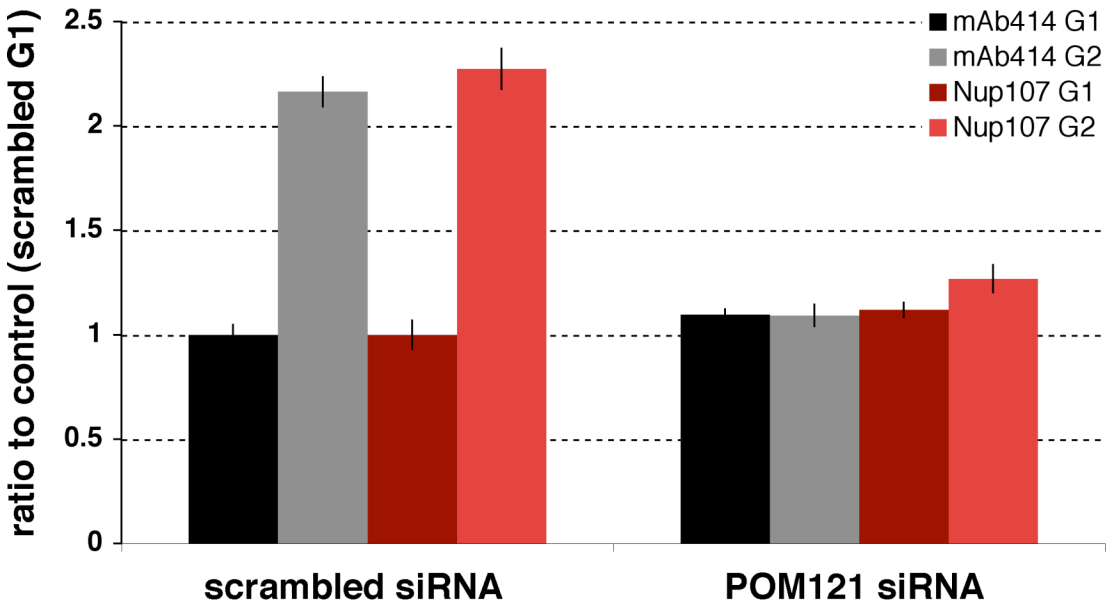

Figure 14: POM121 is required for interphase recruitment of Nup107 and NPC assembly.

Quantification of total mAb414 or a-Nup107 fluorescent signal during G1 and G2 in U2OS cells transfected with control or POM121 siRNA oligos. N>73 nuclei per condition.

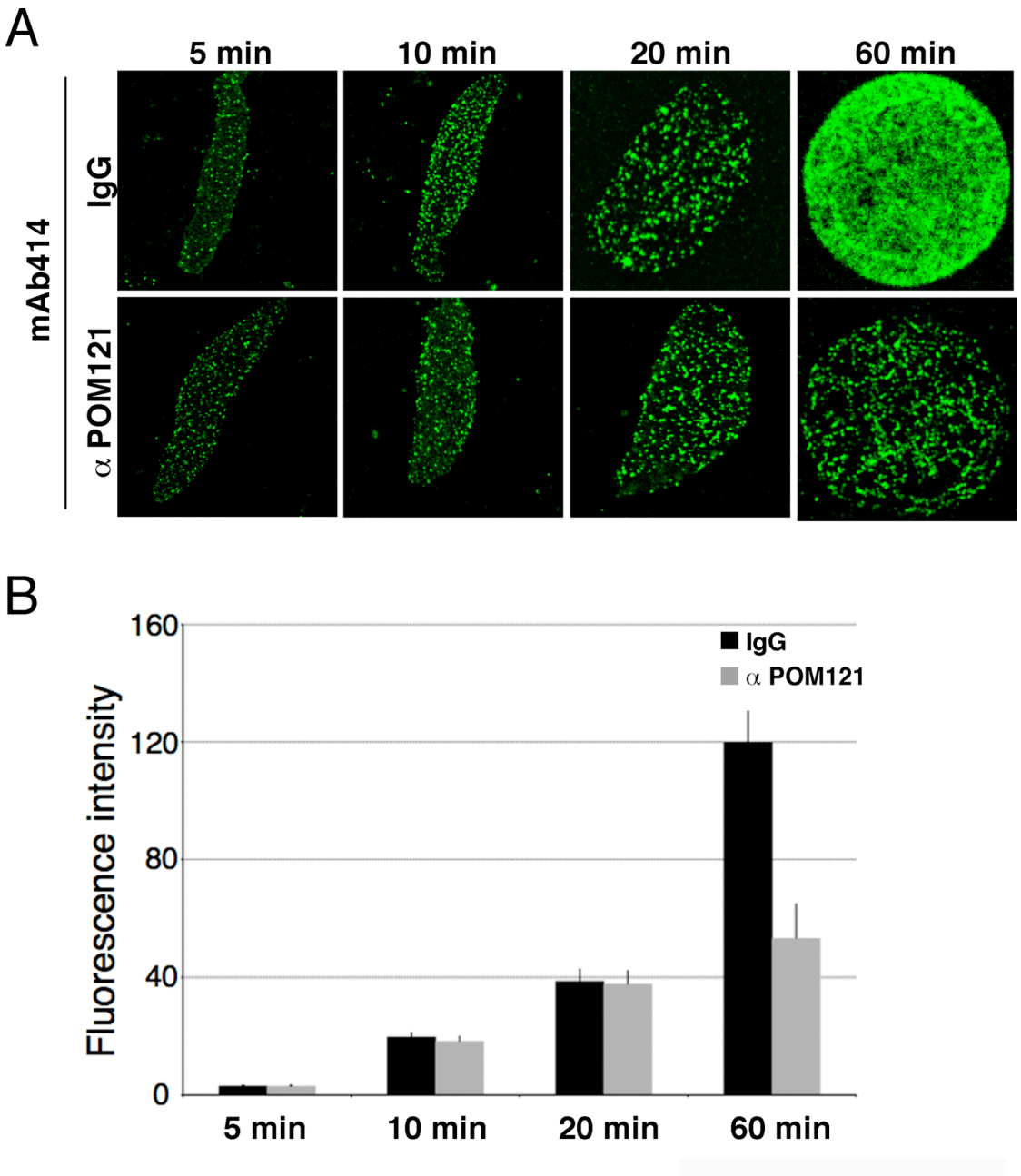

Figure 15: POM121 is required for NPC insertion in a closed NE *in vitro*.

(A) *In vitro* nuclear assembly reactions were performed in the presence of rabbit IgG or an inhibitory α-POM121 antibody. NPCs were visualized using mAb414 antibody. (B) Quantification of total fluorescence of conditions and time points illustrated in (A) shows a specific block in interphase NPC assembly in the presence of the α-POM121 antibody.

2.3.4 POM121 NLS is required for interphase NPC assembly

We have previously shown that NPC assembly in interphase involves the incorporation of the Nup107/160 complex from both sides of the NE (D'Angelo et al., 2006). While no classical import signals have been identified in these proteins, we identified a conserved bipartite nuclear localization signal (NLS) motif in the POM121 protein sequence (Figure 16A) and wondered if import of this transmembrane Nup through existing NPCs is critical for the assembly process. To determine if the NLS sequence in POM121 is functional and capable of mediating nuclear import we fused the POM121 sequence aa292-317 to 3GFP and expressed the construct in U2OS cells which were imaged in the presence or absence of the nuclear export inhibitor leptomycin B. While a mutant version of the 3GFP-NLS (methods 2.5.1) localized to the cytoplasm, the reporter protein with the original NLS sequence accumulated in the nucleus (Figure 16B). Similar results were obtained with the wildtype and mutant NLS sequences in the context of the cytoplasmic domain of POM121 (aa60-1199) (Figure 18). Further, a recombinant NLS containing fragment of POM121 (aa129-335) purified from bacteria directly interacts with importin α and this interaction is strongly diminished in the NLS mutant (Figure 17). We next asked if the NLS is critical for POM121 function and generated the same mutations in the NLS of the full-length protein (POM121mutNLS). When transfected into cells, the NLS mutant protein co-localized with NPCs as determined by mAb414 staining (Figure 18A) and exhibited similar residence

times at the NPC as determined by fluorescence recovery after photobleaching (FRAP) experiments (Figure 18B). This shows that the NLS mutant version of POM121 is able to stably incorporate into the NPC. As endogenous POM121 protein was still present under these conditions, we next depleted human POM121 in U2OS cells (Figure 19B), rescued with either the wild type or NLS mutant rat protein and examined mAb414 signal in G1 and G2 nuclei. Under these conditions, the specific block in interphase NPC assembly could be rescued with the wild type rat POM121 but not with the NLS mutant (Figure 19A). The results of these experiments suggest a functional NLS is critical for the role of POM121 in interphase NPC insertion. The observed incorporation of the POM121 NLS mutant protein is likely to occur during post-mitotic NPC assembly where the NE is reforming and nucleo-cytoplasmic transport is not required.

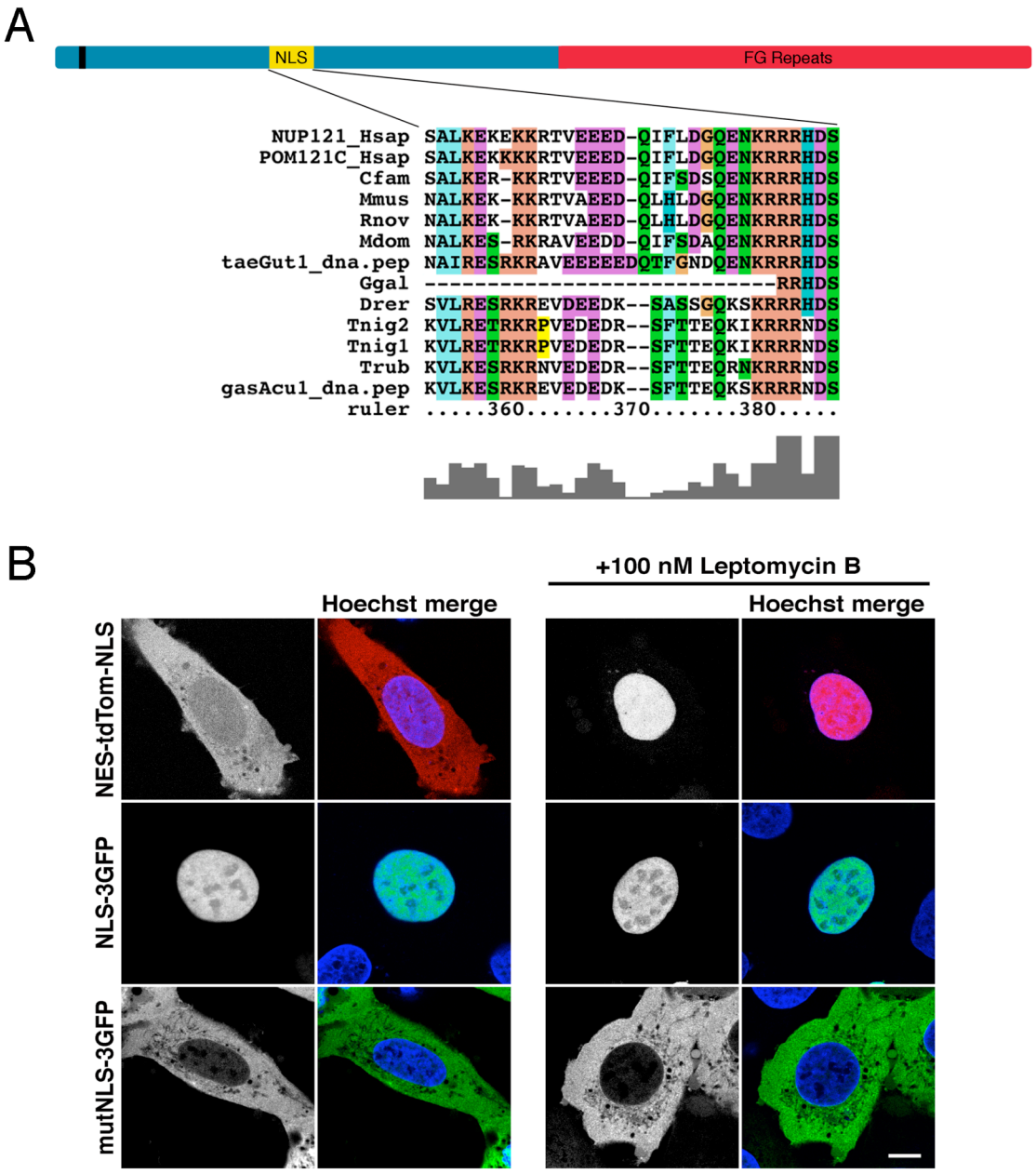

Figure 16: POM121 contains a functional NLS sequence.

(A) Schematic of POM121 topology and NLS motif sequence. (B) POM121 wtNLS and mutNLS sequence fused to 3GFP (green) or NES-tdTom-NLS (red) were transfected in U2OS cells and imaged with and without treatment with Leptomycin B for 2.5 hrs. Scale bar 5μm.

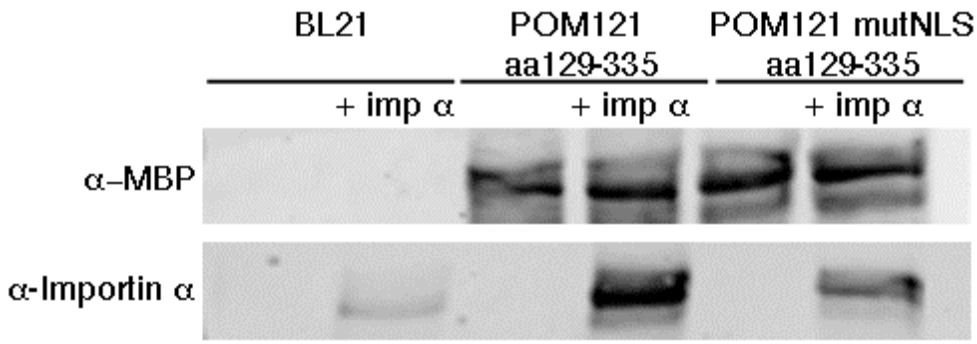

Figure 17: POM121 NLS interacts with importin α.

Wild type and mutNLS aa129-335 of POM121 were fused to an MBP tag and expressed in bacteria. Equal amounts of recombinant importin α was added to each of these (and control) lysates. Proteins were purified via the MBP tag by incubation with amylose resin and the washed beads analyzed by western blotting.

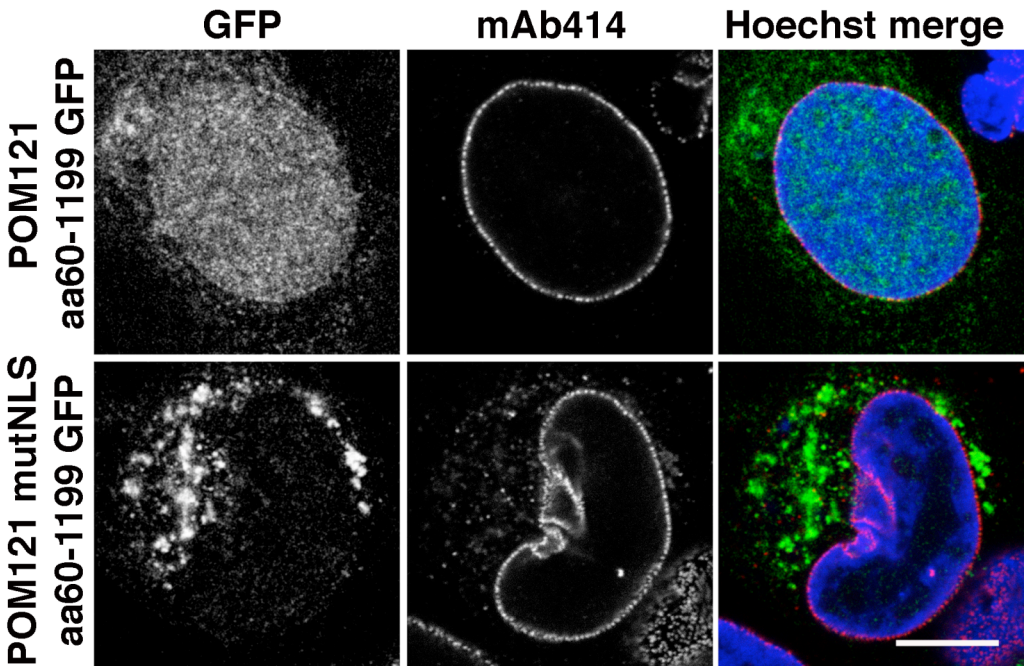

Figure 18: POM121 NLS is required for import of cytoplasmic domain of POM121

The cytoplasmic domain of POM121 (aa60-1199) wtNLS and POM121mutNLS was fused to GFP and expressed in U2OS cells. Cells were fixed and stained with mAb414. Scale bar 5μm.

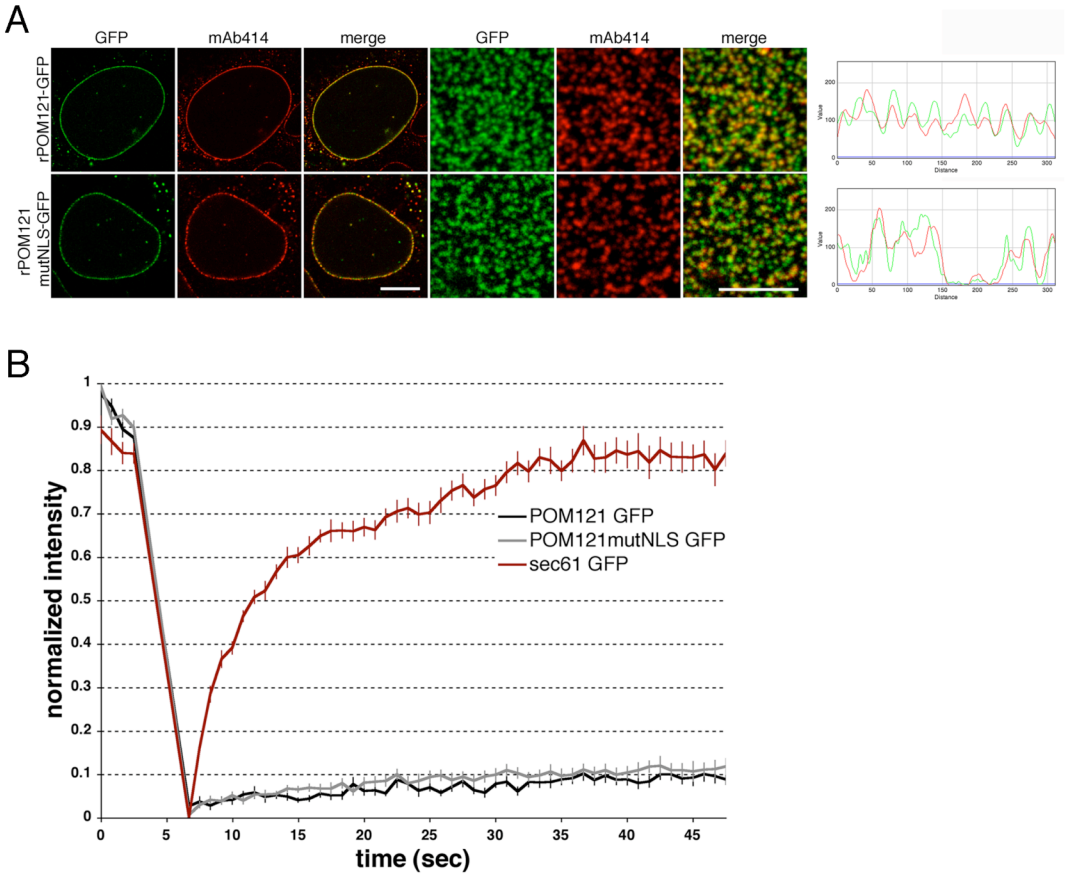

Figure 19: POM121mutNLS localizes to and behaves as wild type POM121 at NPCs

(A) U2OS cells transfected with either POM121-GFP or POM121mutNLS-GFP were fixed and stained with mAb414. Histograms show co-localization of GFP and mAb414 signal at the nuclear surface. Scale bars 5μm. (B) FRAP analysis of POM121-GFP and POM121mutNLS-GFP indicates the NLS mutation does not affect POM121 protein dynamics at the NPC. Results are compared to sec61-GFP, a highly mobile ER protein.

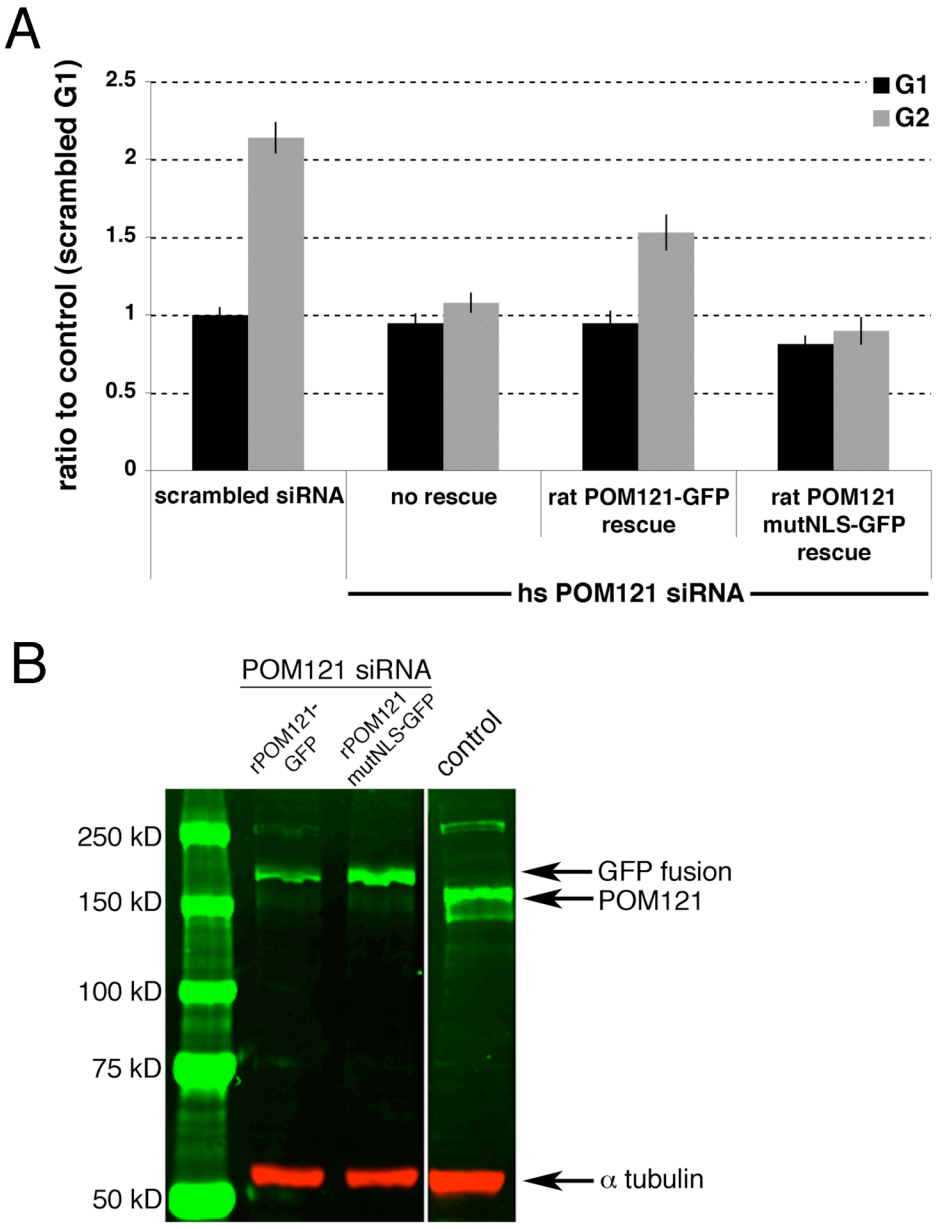

Figure 20: POM121 NLS is required for interphase NPC assembly

Quantification of total mAb414 fluorescent signal during G1 and G2 in control U2OS cells (scrambled) and in cells depleted of the endogenous human POM121 and rescued with either wt or mutNLS rat POM121; N>30 nuclei per condition. All error bars are standard error.

2.4 Discussion

We provide the first evidence that NPC assembly into an intact NE is distinct from nuclear pore formation at the end of mitosis into the reforming nuclear membrane. The key mechanistic distinctions are that interphase assembly can occur efficiently in cells depleted of ELYS, but specifically requires the transmembrane nucleoporin POM121. Interestingly, a functional NLS in POM121 is required during interphase but not post-mitotic NPC formation.

It is helpful to view the two NPC assembly pathways in the light of the differences in NE topology at the end of mitosis and in interphase. During NE reformation, membrane sheets re-emerge from the ER (Anderson and Hetzer, 2008a; Anderson et al., 2009; Lu et al., 2009) to surround chromatin, which is freely accessible to disassembled NPC components (Walther et al., 2003a; Walther et al., 2003b). Consistent with previous reports (Franz et al., 2007; Gillespie et al., 2007; Rasala et al., 2006), ELYS appears to mediate the rate-limiting step of Nup107/160 complex recruitment to chromatin. Since in the absence of ELYS, NPCs form in the ER as annulate lamellae, it appears that its major role is to spatially determine the site of NPC formation, the surface of chromatin. Another potentially critical aspect in evaluating the role of ELYS in NPC assembly is the cell cycle difference in the rate of NPC formation. While pores form in a matter of minutes between anaphase and telophase, interphase assembly is a much slower process that can last almost an hour

(D'Angelo et al., 2006). Thus the rapid targeting of the Nup107/160 complex and possibly other NE components by ELYS (Rasala et al., 2008) could be the rate-limiting step specifically during this cell cycle stage. Notably, ELYS is conserved among metazoa (Rasala et al., 2006) but no obvious homolog has been identified in yeast, which undergo closed mitosis, further supporting the idea that ELYS has a specific function in post-mitotic NPC assembly.

In contrast to post-mitotic NPC formation, targeting of the Nup107/160 complex to an intact NE does not depend on ELYS and thus chromatin association of Nups is unlikely to be rate limiting in interphase. Since NPC insertion in interphase occurs in a compartmentalized cell, other steps might be more critical. To accommodate the difference in NE topology, two evenly spaced flat membrane sheets, interphase assembly seems to involve a distinct set of events, presumably beginning with the fusion of the inner and outer nuclear membranes. We have shown that NPC insertion into an intact NE occurs by a *de novo* process from both sides of the NE (D'Angelo et al., 2006). It is conceptually consistent that a functional NLS in POM121 is specifically required for interphase NPC formation. Our results suggest that POM121 is delivered to the INM through existing NPCs, similar to the NLS-mediated transport of other INM proteins (King, Lusk et al. 2006). This requirement for active transport can explain why POM121 is rate-limiting in interphase, but not at the end of mitosis when proteins have free access to the forming INM. While the exact role of POM121 in the INM/ONM fusion process remains to be

determined, it seems logical that this transmembrane Nup is present both at the INM and ONM to participate in the coordinated bending of the nuclear membranes at sites of forming pores. As the N-terminal domain of POM121 facing the PNS is very short (30aa), it is likely this function requires interaction with additional proteins within the luminal space.

2.5 Methods

2.5.1 DNA constructs

Most DNA constructs were cloned using the Gateway system from Invitrogen (Table S1).

The pDONR207 was modified by the addition of 2GFP into restriction sites engineered in the pDONR207 vector (Invitrogen). rPOM121NLS was generated by PCR and cloned into this vector. The resulting entry clones were recombined with the vector pDEST53 to obtain the final 3GFP fusion construct.

The NES-tdTomato-NLS construct was obtained by adding NES (LQLPPLERLTL) with a linker (GGGG) to the amino-terminus and NLS (PPKKKRKVQ) with the same linker to the carboxy-terminus of tdTomato by PCR. The resulting fusion was then cloned into pCDNA6.2/C-Lumio using Gateway cloning (Invitrogen).

Two rounds of site directed mutagenesis were used to mutate the NLS of rPOM121 from KKKRTVAEEDQLHLDGQENKRRR to

*AAAA*TVAEEDQLHLDGQEN*AAAA*.

H2B-tdTomato was a gift from Tony Hunter's lab.

2.5.2 Antibody production, Western blotting and immunohistochemistry

Western blots were processed and analyzed using the Odyssey colorimetric infrared detection system. Blots were stained with fluorescent secondary antibodies emitting at 680nm and/or 800nm (Invitrogen/Rockland). A list of the primary antibodies is provided in Table S2.

2.5.3 Cell culture and transfection

U2OS cells were grown in DMEM with 10% fetal bovine serum with 1x antibiotic-antimycotic (Invitrogen). Cells were plated on coverslips or in 6 well plates 12 to 24h prior to transfection, and transfected with Lipofectamine 2000 (Invitrogen) as recommended by the manufacturer. For DNA and RNA oligo transfection, either 1 or 5μg of DNA plasmids or, 50 or 250 pmol of siRNA duplexes were used for cells plated in 24 or 6 well plates, respectively. For more efficient siRNA knockdown, cells were transfected a second time 48h later. Cells were fixed or lysed 24-48 hours after the second transfection. siRNA oligo duplexes were ordered from Invitrogen (Table S3).

For synchronization in mitosis, cells were treated with a final concentration of 0.6 μg/ml Nocodazole for 18 hrs. Mitotic cells were collected by shake off, washed in PBS and plated on glass coverslips in fresh medium. For G1 and G2 timepoints, cells were fixed and immunostained 5 and 19

hours after release.

For the Leptomycin B experiment U2OS were treated with 100 nM LMB for 2.5 hrs 24 hrs post transfection. Cells were washed in PBS, fixed in 4% paraformaldehyde and stained with Hoechst dye.

2.5.4 Cell imaging

Live cells were imaged at 37°C maintained by air stream incubator and enriched with CO2 (Solent Scientific). Time-lapse images were taken on a spinning-disk confocal microscope (Yokogawa) built around an inverted stage microscope (DMRIE2; Leica). Images were captured on an EM charge-coupled device digital camera (Hamamatsu Photonics) and acquired using SimplePCI v6.2 (Hamamatsu). Cells were imaged using a 63x oil emersion objective with a 1.4 numerical aperture (Leica).

Fixed cells were imaged using a scanning confocal microscope (DMRIE2; Leica) using a 63x oil emersion objective with a 1.4 numerical aperture (Leica). Images were aquired using the Leica Confocal Software (LCS) v1.5.

2.5.5 Fluorescence recovery after photobleaching

Fluorescence recovery after photobleaching (FRAP) was performed on sections of the nuclear rim in U2OS cells transiently expressing GFP fusion proteins using a scanning confocal microscope (DMRIE2; Leica) described above.

FRAP parameters used were: 4 pre-bleach scans, 4 bleaching scans followed by 50 post-bleach scans taken at 0.834 fps. Images and quantitative data were acquired using the Leica Confocal Software (LCS) v1.5. The data were analyzed and presented graphically in Microsoft Excel.

2.5.6 Electron Microscopy

Cells grown in 35mm plastic culture dishes were fixed using the protocol of Gilula et. al. (1978). The cells were fixed in 2.5% glutaraldehyde in 0.1M Na cacodylate buffer (pH7.3), washed and fixed in 1% osmium tetroxide in 0.1M Na cacodylate buffer. They were subsequently treated with 0.5% tannic acid followed by 1% sodium sulfate in cacodylate buffer and then dehydrated in graded ethanol series. The cells were cleared in HPMA (2-hydroxypropyl methacrylate: Ladd Research, Williston VT) and embedded in LX112 resin. Following overnight polymerization at 60˚C, small pieces of resin were attached to blank blocks using SuperGlue. Thin sections (70nm) were cut on a Reichert Ultracut E (Leica, Deerfield, IL) using a diamond knife (Diatome, Electron Microscopy Sciences, Hatfield PA), mounted on parlodion coated, copper, slot grids and stained in uranyl acetate and lead citrate. Sections were examined on a Philips CM100 TEM (FEI, Hillsbrough, OR) and data documented on Kodak SO-163 film for later analysis. Alternatively the samples were documented on a SIS Megaview III CCD camera (SIS, Lakewood CO). Negatives were scanned at 600 lpi using a Fuji FineScan 2750xl (Hemel Hempstead, Herts., UK) and converted to tif format for subsequent handling in

Adobe Photoshop.

2.5.7 Image analysis and statistics

Western blot bands were quantified and normalized to loading controls using Odyssey software.

To determine total NPC number, cells were immunostained with the listed antibodies. For each nucleus, the area and average fluorescence intensity were measured from maximum projections of 20 confocal z-series spanning the entire nucleus, using either Image J or Adobe Photoshop. Numerical data were analyzed and summarized graphically using Excel (Microsoft). Data for each experiment were collected from at least three independent experiments and combined for statistical analysis.

To quantify the recruitment of 3GFP-Nup133 during post-mitotic assembly, the contour of chromatin was drawn in the Adobe Photoshop software and transformed into a fringe. Due to the heterogeneity in expression levels, the acquisition parameters and level adjustments differed from cell to cell and thus we quantified the recruitment of 3GFP-Nup133 as the ratio of protein present at the nuclear periphery to total protein, using the equation below:

% 3GFP-Nup133 recruited to the nuclear periphery = fringe area x (avg fringe fluorescence – avg cytoplasm fluorescence) / [section area x (avg section fluorescence – background fluorescence)]

2.5.8 *Xenopus* egg extract preparation and immunodepletion

Extract and sperm chromatin preparation, fluorescent labeling of membranes, and immunofluorescence were performed as previously described (Hetzer et al., 2000; Walther et al., 2003b). Immunodepletion of the cytosol was performed by 3 cycles of either α-xELYS or α-xNup107 antibodies (Table S2) coupled to protein A Pansorbin cells (Calbiochem). Each cycle was carried out for 10 minutes and the resulting cleared cytosol was used to perform *in vitro* nuclear assembly reactions as previously described (Lohka and Masui 1983). Depletion was confirmed by Western blotting.

2.5.9 Bacterial expression of recombinant protein and biochemistry

Expression of MBP-POM121 (aa129-335), MBP-POM121mutNLS (aa129-335) and HIS-importin α in BL21 bacteria was induced with 250 mM IPTG for 5 hrs at 37°C. Cell pellets were homogenized by two rounds of emulsification and soluble fractions isolated by centrifugation. Equal amounts of HIS-importin α lysate were added to each of the MBP lysates. These and MBP lysates alone were incubated with amylose resin at 4°C overnight. Beads were washed three times, boiled and bound protein analyzed by Western blotting.

Table 1: DNA constructs

Name	Source	Entry vector	Destination vector
rPOM121-GFP	rPOM121,cDNA library	pDONR207	pcDNA6.2 DEST47
rPOM121NLS-3GFP (aa 292-317)	rPOM121	pDONR207 2GFP (modified)	pcDNA6.2 DEST53
rPOM121 (aa 60-1199) - GFP	rPOM121	pDONR207	pcDNA6.2 DEST47
Sec61-GFP	hSec61β (aa 1-65)	pDONR207	pcDNA6.2 DEST47
3GFP-mNup133	mNup133	pDONR207 2GFP (modified)	pcDNA6.2 DEST53

Table 2: Antibodies

Antibodies generated by Hetzer lab						
Protein	**Antigen used**	**Host**	**Vector**	**Protein purification method**	**Dilution for WB**	**Dilution for IHC**
mPOM121	aa 448-647	Rabbit	pDEST15	Glutathione agarose (Sigma-Aldrich)	1:1000	1:500
hNDC1	aa 296-485	Guinea Pig	pDEST15	Glutathione agarose (Sigma-Aldrich)	N/A	1:200
mNup153	aa 1-198	Rabbit	pDEST15	Glutathione agarose (Sigma-Aldrich)	N/A	1:200
xNup107	aa 76-171	Rabbit	pDEST15	Glutathione agarose (Sigma-Aldrich)	immunodepletion	
xNup96	aa 1-150	Rabbit	pDEST15	Glutathione agarose (Sigma-Aldrich)	1:1000	N/A
mNup107	aa 600-926	Rabbit	pHis9-GW	Ni-NTA (Qiagen)	Affinity purified 1:1000	Affinity purified 1:1000
hNup133	aa 67-478	Rabbit	pHis9-GW	Ni-NTA (Qiagen)	1:200	N/A

Gifts and commercially available antibodies				
Protein	**Host**	**Lab / Vendor**	**Dilution for WB**	**Dilution for IHC**
Cyclin A	Rabbit	Tony Hunter Lab	N/A	1:200
hELYS	Rabbit	Iain Mattaj Lab	1:2000	1:1000
xELYS	Rabbit	Iain Mattaj Lab	1:2000	1:1000
xPOM121	Rabbit	Iain Mattaj Lab (Antonin et al, 2005)	Inhibitory antibody	
Control IgG	Rabbit	Santa Cruz	Immunodepletion / inhibition	
Lamin A	Rabbit	Sigma-Aldrich	1:750	
α-tubulin	Mouse	Sigma-Aldrich	1:5000	1:1000
6His epitope	Mouse	Sigma-Aldrich	1:5000	N/A
Centromere protein	Human	Antibodies Inc	N/A	1:2000
NPC (mAb414)	Mouse	Covance	1:5000	1:1000

Table 3: RNAi oligo sequences

Target	Sequence
unspecific	UAGAUACCAUGCACAAUCCdTdT
hELYS	UCGUGGAAAGUUUGCUGCAGGGAAA
hPOM121	CAGUGGCAGUGGACAUUCAdTdT
hNup107	CUGCGAAUACACUUUCUUCdTdT
hNup96	GCACAAAUUGUGAAGCACUdTdT

2.6 Acknowledgements

I would like to acknowledge Christine Doucet for her experiment in Figure 8, Martin Hetzer for his experiments in Figures 12, 13 and 15. I would like to thank Roberta Schulte for performing the immunodepletions of *Xenopus* extracts used in Figure 13 and Malcolm Wood for his help with the transmission electron microscopy in Figures 6 and 9. Also Gerard Manning for his help in generating the POM121 alignment shown in Figure 16.

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Chapter 3: Early steps of NPC assembly in interphase

3.1 Summary

Most published experiments regarding NPC assembly, both in cells and *in vitro*, are relevant to nuclear pore assembly at the end of mitosis. As the experiments described in Chapter 2 illustrate definitive mechanistic differences between interphase and post-mitotic NPC assembly, there is now a deficit of information regarding the interphase assembly pathway. Experiments described in this chapter provide evidence that POM121 and the Nup107 complex are the earliest characterized Nups required for this process. We show that POM121 is required for membrane fusion and that the mechanism of recruitment of the Nup107 complex in interphase is distinct from post-mitosis, in that it is assembled at sites of forming NPCs.

3.2 Introduction

While little is known specifically regarding interphase NPC assembly we do have some important insights into this process. We know that it occurs *de novo* and requires essential components from both sides of the nuclear envelope (D'Angelo, Anderson et al. 2006). We also know that, relative to post-mitotic assembly, it takes a significantly longer amount of time to create a nuclear pore, likely reflecting the added requirement of a double membrane fusion event. The transmembrane Nups have long been suggested as likely

candidates for possible mediators of this INM/ONM fusion event. However, as gp210 is not required in some tissues (Eriksson, Rustum et al. 2004) and the essential nature of both NDC1 and POM121 has been controversial, it is unclear how or even whether they are indeed involved in this context of membrane fusion. To date, no published experiments have directly addressed this question.

3.3 Results

3.3.1 POM121 precedes the Nup107/160 complex at new assembly sites during interphase

Our POM121 knockdown experiment in U2OS cells showed a clear inhibition of interphase assembly as measured by mAb414 recruitment to the NE (Figure 14). Interestingly, in this experiment further recruitment of Nup107 between G1 and G2 was also prevented (red and pink bars), as was recruitment of our Nup133-3GFP reporter once the NE had fully formed (Figure 8). While it has been shown that post-mitotic ELYS targeting of the Nup107/160 complex to chromatin subsequently recruits POM121 and ER membranes (Rasala, Ramos et al. 2008), our results indicate that POM121 precedes the Nup107 complex at sites of new pore assembly in interphase.

As interphase NPC assembly requires 30-60 min (D'Angelo et., 2006), we wondered if NPC intermediates could be detected that contain POM121 but not the Nup107/160 complex. To test this we triple immuno-stained

dividing cells, which double their NPCs between G1 and G2 (Maul, Price et al. 1971), with antibodies against POM121, Nup107 and Nup96. As expected, most fluorescent loci contained all three of these Nups, indicating successful incorporation of the Nup107/160 complex. However, we found many of the fluorescent sites analyzed contained POM121 signal alone (Figure 21 & 22). Importantly, treatment with siRNA oligos against either Nup107 or Nup96 resulted in a significant increase in POM121 only signal, indicating true intermediates (Figure 22). Of particular interest we also found fluorescent loci containing either POM121 and Nup96 without Nup107 or POM121 with Nup107 but not Nup96 (Figure 21). Notably, Nup96 and Nup107 were not detected without associated POM121 signal.

To confirm these results in another system we made use of an α-Nup133 antibody previously shown in our lab to inhibit NPC assembly in *Xenopus* extracts. Nuclei assembled in the presence of control IgG assembled normally and immuno-stained as expected for both POM121 and Nup107. In contrast, nuclei assembled in the presence of the inhibitory antibody contained normal levels of POM121 but not Nup107, indicating that prevention of Nup107 complex incorporation in these nuclei had no effect on the localization of POM121 to the NE (Figure 23). Together these results support the conclusion that POM121 precedes the Nup107/160 complex at forming NPCs during interphase and raise questions regarding the mechanism of recruitment of the Nup107/160 complex to these sites.

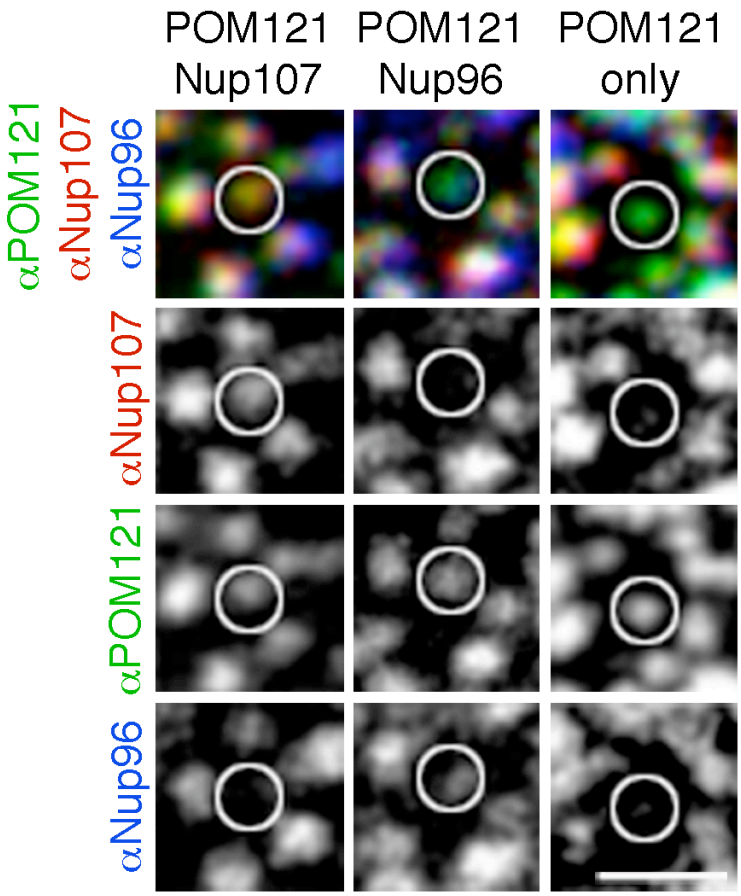

Figure 21: NPC intermediates in U2OS cells.

U2OS cells were triple-immunostained with antibodies against POM121, Nup96 and Nup107 and the nuclear surfaces imaged in three channels by confocal microscopy. White circles indicate NPC intermediates. Scale bar 2μm.

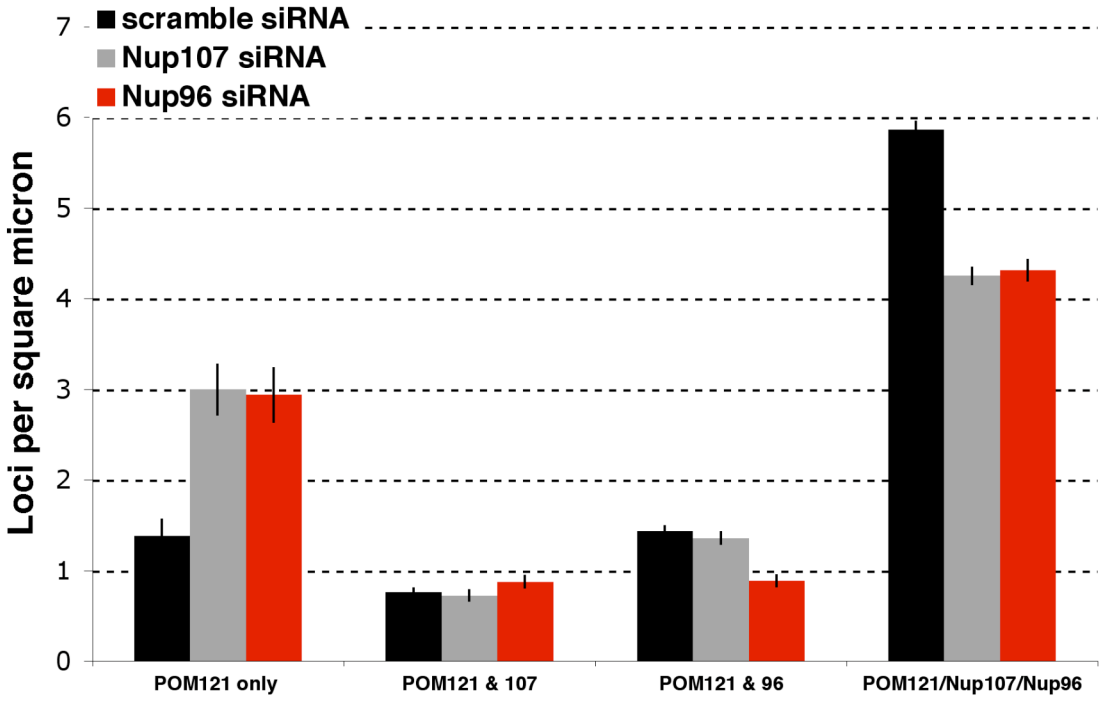

Figure 22: Quantification of NPC intermediates in Nup107 and Nup96 knockdown.

U2OS cells treated with scrambled, Nup107 or Nup96 specific siRNA oligos twice over 4 days were triple immunostained for POM121, Nup107 and Nup96. NPC intermediates were quantified by comparing three color overlays shown in Figure 21. N = 30 fields per knockdown condition.

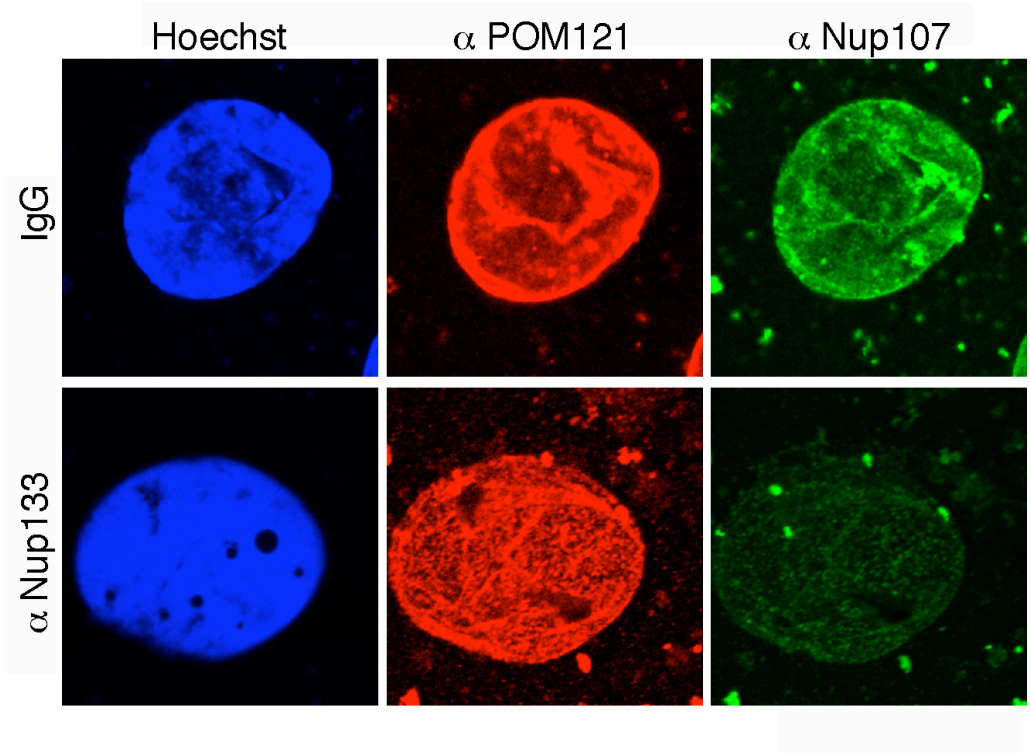

Figure 23: Inhibitory αNup133 antibody prevents Nup107 but not POM121 recruitment to the NE, *in vitro*.

In vitro nuclei were assembled from *Xenopus* extracts for 60 min in the presence of either rabbit IgG or an inhibitory antibody against Nup133. Nuclei were fixed and immunostained for POM121 and Nup107.

3.3.2 Nup107 protein level and recruitment to sites of interphase NPC assembly are Nup96 dependant.

Immunofluorescence detection of NPC intermediates containing POM121 with either Nup107 or Nup96, but not both, raised the unexpected possibility that the Nup107/160 complex might be assembled at sights of new NPC assembly rather than arriving at these sites as an intact complex as previously assumed.

Consistent with previous results, the knockdown of Nup107 in cells by siRNA treatment leads to a similar reduction in Nup133 protein levels (Boehmer, Enninga et al. 2003). Somewhat surprisingly, when we analyzed total protein levels in U2OS cells treated with siRNA oligos against Nup107 we saw very little reduction in Nup96 levels. In contrast, when cells were treated with siRNA oligos specific for Nup96, we observed a reduction in not only Nup96 levels but also Nup107 and Nup133 (Figure 24). This result indicated a non-reciprocal relationship in the regulation of total protein levels within the Nup107/160 complex. To examine this possibility directly at the NE we performed quantitative immunofluorescence with antibodies against Nup107, Nup96 and mAb414 in U2OS cells treated with scrambled, Nup107 or Nup96 specific siRNA oligos (Figure 25). The results mimicked those obtained by western blotting in that Nup107 knockdown had little effect on Nup96 recruitment to the NE while Nup96 depletion prevented proper localization of both Nup96 and Nup107. Notably, both siRNA treatments resulted in a similar

reduction in complete NPCs as indicated by mAb414 staining. To obtain data at the resolution of individual NPCs, we turned to the quantification of NPC intermediates performed in Figures 21 and 22. While we did find some loci containing only POM121 and Nup107, this number did not change in either of our siRNA treatments (Figure 22). However, the number of POM121/Nup96 alone intermediates, while responding to Nup96 knockdown, did not change with Nup107 depletion. The number of POM121/Nup96 intermediates in the Nup96 knockdown cells is similar to that of POM121/Nup107 intermediates under all conditions; perhaps indicating that these intermediates actually reflect a low level of background staining.

Together these three experiments clearly show that Nup107 total protein levels and its localization to the NE are linked to Nup96, but not vice versa. Further they indicate that, during interphase, components of the Nup107/160 complex exist and are recruited to forming pores either individually or in smaller protein complexes.

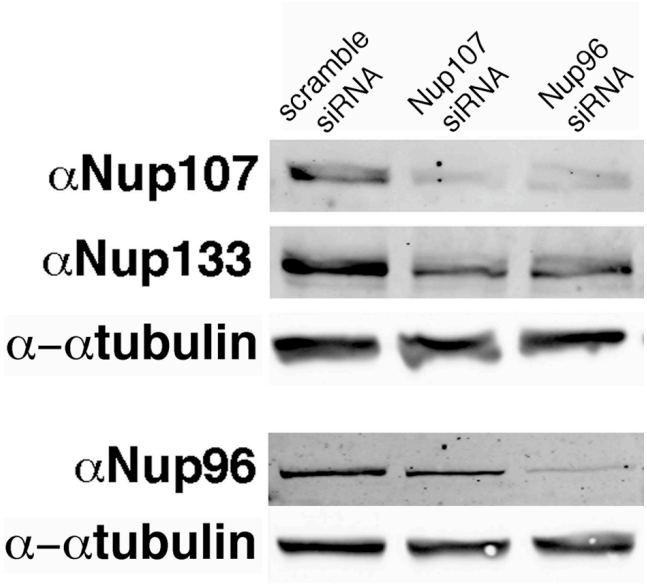

Figure 24: Nup107 and Nup133 protein levels are linked to Nup96.

Western blot analysis of whole cell lysates in U2OS cells treated with scrambled, Nup107 or Nup96 siRNA oligos twice over 4 days.

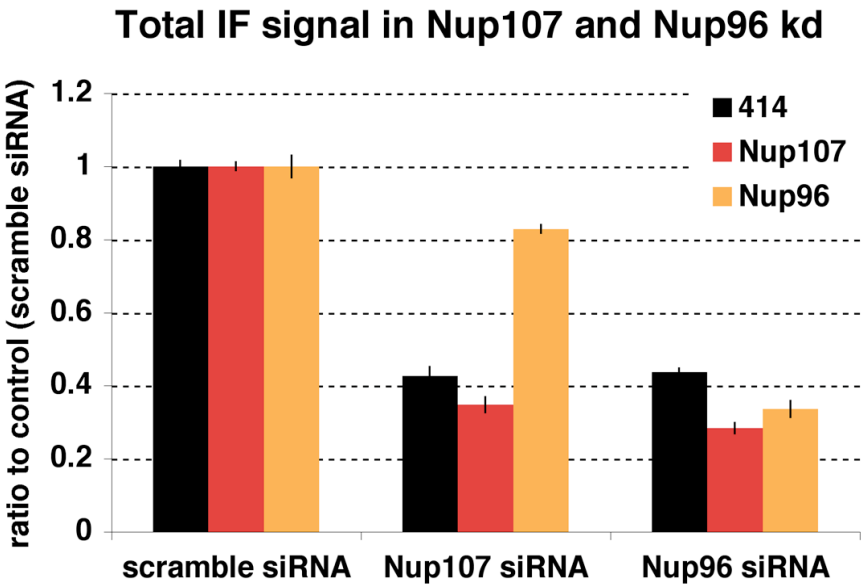

Figure 25: Nup107 recruitment to the NE depends on Nup96

Quantitative immunofluorescence analysis of Nup107, Nup96 and mAb414 reactive Nups at the NE in U2OS cells treated with scrambled, Nup107 or Nup96 specific siRNA oligos twice over 4 days. N>50 nuclei per condition.

3.3.3 POM121 implicated in bringing ONM/INM together to facilitate fusion

Although it is still unresolved how the fusion of the INM and ONM occurs and which proteins mediate this event, our results show that POM121 precedes the Nup107/160 complex and functions as an early player in interphase NPC assembly. To further analyze this we turned to a recently established method to functionally monitor INM/ONM fusion (Dawson et al., 2009). In brief, *Xenopus* egg extracts were incubated with WGA-Sepharose beads to deplete glycosylated Nups, many of which are critical for establishing the permeability barrier of the NPC. When sperm chromatin and membranes are mixed with these extracts, lacking FG repeat Nups, the forming nuclei contain a closed NE but the assembled NPCs remain permeable for diffusion of molecules up to ~ 150 kDa. Under these conditions the formation of new pores (holes in the NE) can be detected by the influx of a fluorescently labeled 70 kDa dextran, which in mock-depleted cytosol is excluded from nuclei (Figure 26A). When we preassembled nuclei for 60 min and added either mock depleted cytosol or WGA depleted cytosol in the presence of the calcium chelator BAPTA, a known inhibitor of NPC formation, the dextran influx was strongly inhibited (Figure 26A & B), suggesting the INM and ONM had not fused. Next, we added WGA depleted cytosol in the absence or presence of control or inhibitory Fab fragments of an α-POM121 antibody. In reactions with control antibodies including Nup107, Nup96, ELYS and NDC1 (data not

shown), nuclear influx of the dextran was detected 5 min after the addition of WGA depleted cytosol. In contrast, no influx was observed in the presence of the α-POM121 antibody, providing evidence that POM121 may be involved in the formation of a pre-pore/hole in an intact NE.

Next, nuclei were assembled for 30 minutes under normal conditions and then either control IgG or inhibitory α-Nup133 or α-POM121 antibodies were added to the reactions for an additional 60 min. In these conditions there was an influx of the 70kD dextran only in the case of the α-Nup133 antibody, providing further support for the involvement of POM121, but not the Nup107 complex, in membrane fusion (Figure 27).

To investigate the potential role of POM121 in membrane fusion in more detail we analyzed the *in vitro* assembled nuclei shown in Figure 23 by TEM. These nuclei containing POM121 but not Nup107 or Nup133 at the NE seemed likely to contain potential NPC intermediates or "pre-pores". We observed that compared to control nuclei the nuclear envelopes of these nuclei contained few fully formed NPCs and showed a unique phenotype at the nuclear envelope. The nuclei assembled in the presence of the inhibitory α-Nup133 antibody contained many sites where the INM and ONM were in close proximity but apparently not fused and with no electron dense material indicative of a normal NPC (Figure 28).

In an attempt to investigate this phenomenon in cells we performed several TEM experiments. We first performed immuno-gold staining of

POM121 in NEs isolated from wild-type U2OS cells. In support of our hypothesis we found examples of POM121 staining not only at NPCs but also at areas of the NE where the two membranes displayed a dramatic decrease in luminal spacing similar to the *in vitro* results, perhaps representing "pre-pores" (Figure 29). TEM analysis of the NE in intact cells and comparison of controls with cells depleted of Nup96 revealed several examples of potential pre-pores, however, due to the nature of TEM it was difficult to conclude whether or not this phenotype was more prevalent in the cells treated with Nup96 siRNA oligos.

As expected, in all conditions examined, diameters of NPCs measured in transverse section averaged ~ 80nm (Figure 30 black arrows). Reflecting this dimension, the lengths of the closely opposed membranes in our *in vitro* experiments and wild type and Nup96 depleted cells averaged ~ 107nm (Figure 30, top panel, white arrow). Perhaps indicative of its function in membrane fusion, TEM analysis of cells over-expressing a recombinant POM121 construct revealed long stretches of the nuclear envelope coming together (Figure 30, bottom panel, white arrow). These dramatic stretches of altered NE ranged from 162nm to 684nm. Together, these results suggest that POM121 is required for the process of membrane fusion prior to recruitment of Nup96 and/or other members of the Nup107/160 complex and indicate a role for POM121 not necessarily in the actual fusion of INM/ONM but in bringing the two leaflets of the NE together in order to promote fusion.

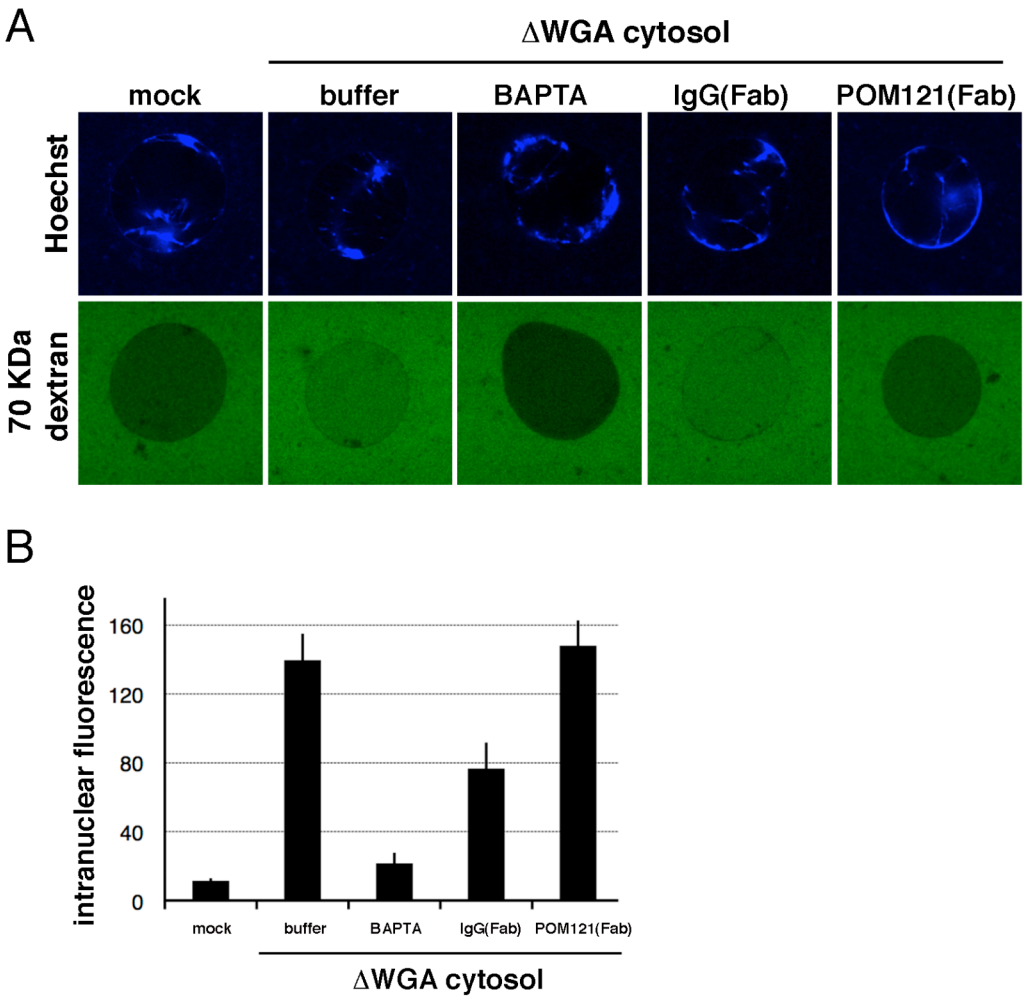

Figure 26: Inhibitory POM121 Fab fragments inhibit INM/ONM fusion, *in vitro*.

(A) Assembled nuclei were incubated with mock- or WGA-depleted cytosol for 30 min in the presence of buffer, BAPTA, IgG or POM121 Fab fragments. Fluorescently labeled 70 kDa dextran (green) was added to probe for INM-ONM fusion. (B) Quantification of intranuclear 70 kDa dextran signal in conditions described in (B), N>150 nuclei per condition

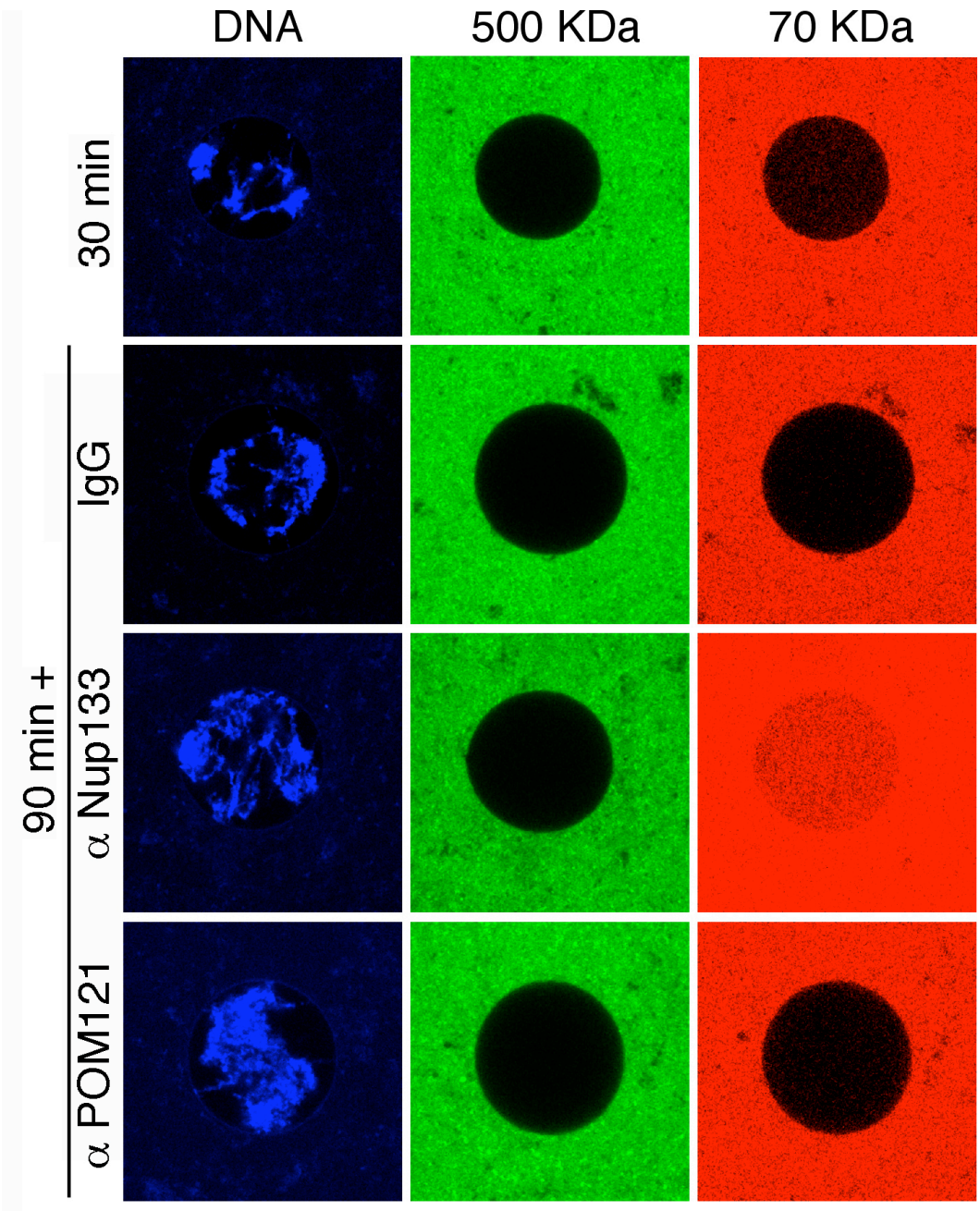

Figure 27: Inhibition of Nup133 but not POM121 allows nuclear influx *in vitro*

Nuclei were assembled in vitro from Xenopus extracts under standard conditions for 30 min. Either control IgG or inhibitory antibodies against Nup133 or POM121 were added to the assembly reaction. Nuclear integrity and NE fusion events were imaged using 70kD and 500kD fluorescent dextrans 60 minutes after antibody addition.

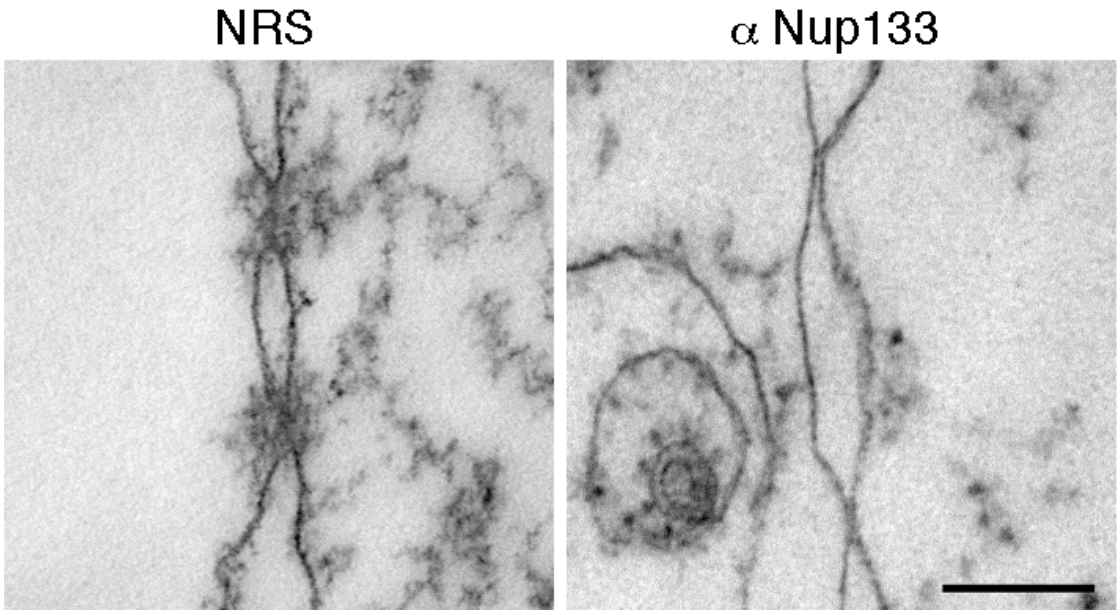

Figure 28: Inhibition of Nup133 recruitment in *in vitro* nuclear assembly reveals NPC intermediates.

Nuclei were assembled *in vitro* from *Xenopus* egg extracts under normal conditions (left) or in the presence of and inhibitory α-Nup133 antibody (right). Nuclei were fixed, processed and imaged by transmission electron microscopy.

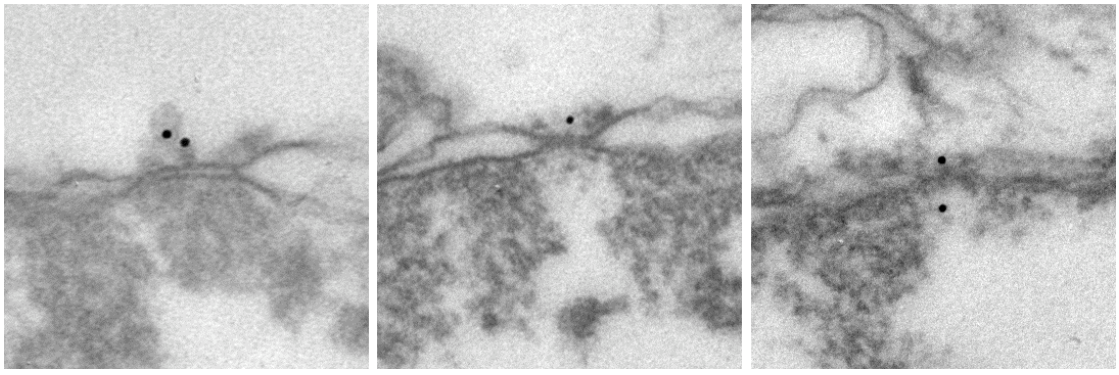

Figure 29: POM121 localizes to NPCs and potential pre-pores.

Nuclear envelopes were isolated from wildtype U2OS cells and stained with immunogold against endogenous POM121. Samples were fixed, processed and imaged by transmission electron microscopy.

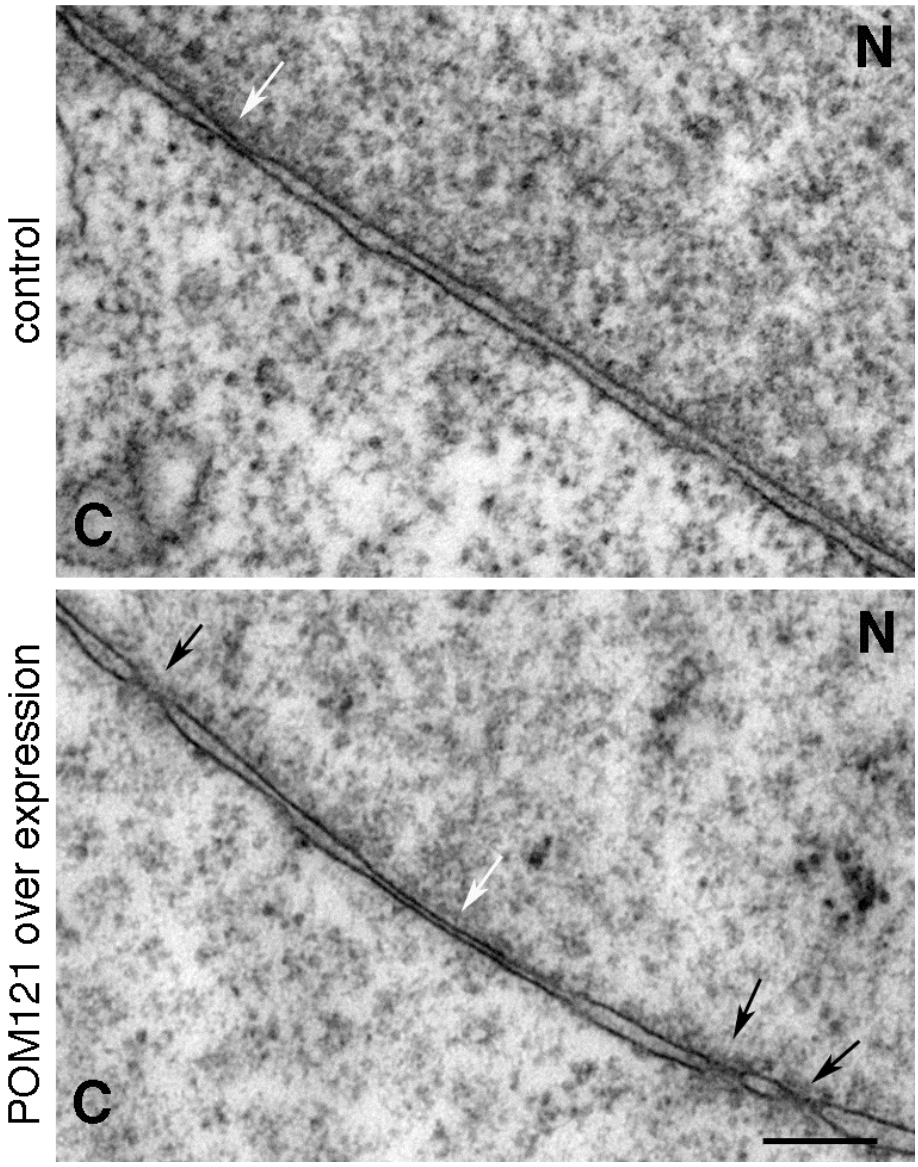

Figure 30: POM121 over expression increases length of closely opposed membranes.

Potential NPC pre-pores were identified in wild-type U2OS cells (top panel) by TEM of the nuclear envelope. In cells over expressing POM121 we observed long stretches of NE where the INM and ONM show decreased spacing. White arrows indicate pre-pores/close membranes, black arrows indicate NPCs. Scale bar 200 nm.

3.4 Discussion

Experiments presented in this chapter shed light on early steps in interphase NPC assembly; implicating POM121 in the initial fusion event and subsequent recruitment of the Nup107/160 complex. Several lines of evidence indicate that during interphase, POM121 is present at newly forming pores before the Nup107/160 complex becomes incorporated. First, we readily detected NPC assembly intermediates that contain POM121 but not members of the Nup107/160 complex. Secondly, depletion of POM121 inhibits recruitment of both endogenous Nup107 and 3GFP-Nup133 to the NE, specifically in the context of a closed NE. Thirdly, nuclei assembled *in vitro* from *Xenopus* extracts in the presence of an inhibitory α-Nup133 antibody allow nuclear influx of 70 kD fluorescent dextran and while Nup107 staining is reduced, levels of POM121 at the NE are unchanged.

As ELYS is required for Nup107/160 complex recruitment to sites of new pore assembly specifically following mitosis, there must be a unique mechanism of recruitment during interphase. Results in this chapter indicate that following INM/ONM membrane fusion, the Nup107/160 complex is assembled piece wise with Nup96 preceding Nup107. We noticed a non-reciprocal relationship in which Nup107 seems to require Nup96 both for total protein levels in the cell and recruitment to the NE and NPCs. These experiments compliment those from the Wozniak lab (personal communication) showing a direct interaction of POM121 with Nup160, located

adjacent to Nup96 in the complex (Lutzmann, Kunze et al. 2002). Further, Nup107 and Nup133 (adjacent to each other at the other end of the complex) seem to be co-regulated, at least at the level of total protein as determined by Western blotting (Boehmer, Enninga et al. 2003). Data not included in this dissertation but published with experiments in Chapter 2 show that a membrane curvature sensing domain (ALPS) (Drin, Casella et al. 2007) in Nup133 is required for its recruitment to the NE and new NPC insertion specifically during interphase. Together these experiments begin to shape a model in which a direct interaction with POM121 could target a subset of Nup107/160 complex Nups to sites of INM/ONM fusion, presumably on both sides of the NPC. Targeting of curved membranes by Nup133 could target itself with Nup107 to these sites. Analysis of Nup160, Seh1, Sec13 and Nup85 and their roles in this model remain.

As INM and ONM fusion does not occur in the presence of an inhibitory α-POM121 antibody, POM121 appears to play a critical role, either directly or indirectly, in this process subsequently recruiting the Nup107/160 complex. TEM analysis of nuclei assembled with an inhibitory α-Nup133 antibody reveal potential pre-pores as they are enriched in sites where the INM and ONM are brought close together at lengths comparable to the size of an NPC. These intermediates are readily detected in U2OS cells and further, in cells over expressing POM121 these become long stretches of close membranes. Since in these experiments we did not find sites of actual membrane fusion or holes

in the NE, it is possible that recruitment of Nup107/160 complex components (Nup96/Nup160) to these sites is required for stabilization of the initial fusion event and without it the membranes return to their normal position/spacing.

These experiments show that while POM121 appears to play a role in INM/ONM fusion, that role involves bringing the two membranes of the NE together. How this leads to fusion of the membranes is unclear. The localized decreased in the PNS is reminiscent of the mechanism of SNARE/SM mediated membrane fusion of membranes in endo and exocytosis. In the zippering model of membrane fusion two membranes are physically brought together via bundling of protein domains extending towards each other from either membrane until it becomes energetically favorable to fuse. Alternatively, SM clasping proteins are designed to bind to specific SNARE bundles, inducing conformational changes forcing two membranes together to induce fusion (Sudhof and Rothman 2009). As there are only 30 aa of POM121 facing the PNS it seems likely that interaction with one or more additional proteins within the luminal space would be required to facilitate fusion via a similar type of mechanism. Additionally, interaction of POM121 molecules located in the two leaflets of the NE with a luminal protein could provide a mechanism for coordinating membrane fusion from both sides of the NE.

3.5 Methods

3.5.1 Fluorescent labeling of primary antibodies and generation of Fab fragments.

To perform the triple staining experiment, the α-Nup107 antibody was biotinylated and labeled with fluorescent streptavidin 568nm. The α-POM121 antibody was affinity purified and directly coupled to a 488 nm fluorophore using the APEX antibody labeling kit from Invitrogen.

The Fab fragment of the inhibitory α-xPOM121 was generated using the Pierce Micro Preparation kit.

3.5.2 WGA depletion of Xenopus cytosol

Biotinylated wheat germ agglutinin (WGA) (Biomeda) was immobilized under saturating conditions on streptavidin-coated magnetic beads (Invitrogen). After excessive washing, beads were incubated with freshly prepared cytosol (membrane free) (Walther, Alves et al. 2003) rotating on ice for 30 min. Depleted cytosol was immediately snap frozen and stored in liquid nitrogen. Depletion of Nups was verified by Western blotting using mAb414 antibody (Millipore).

3.5.3 Nuclear envelope isolation and immunogold staining

Nuclear envelopes were isolated from U2OS cells by re-suspending U2OS cell pellets through a 27.5 gauge syringe several times. Broken nuclei were centrifuged onto poly-L-lysine coated cover slips and stained with an

affinity purified α-POM121 primary antibody and a gold labeled α-rabbit secondary antibody. Samples were then processed for TEM imaging as in section 2.5.6.

3.6 Acknowledgements

I would like to thank Martin Hetzer for his experiments in Figures 23, 26 and 27 and Malcolm Wood for his help with the TEM experiments in Figures 28, 29 and 30.

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Chapter 4: Conclusions and Future Directions

4.1 Conclusions

Experiments described in this dissertation provide the first direct evidence for mechanistic differences in nuclear pore complex assembly during the two phases of the cell cycle in which it occurs. We first show that the transmembrane Nup POM121 and the DNA binding ELYS/Mel28 are unique from other Nups in that they are not required for the formation of individual NPCs yet their depletion reduces total NPC numbers. We show that ELYS is specifically required for targeting of the Nup107/160 complex to post mitotic chromatin and its depletion results in dramatic mislocalization of NPCs to the cytoplasm. Cell culture and *in vitro* experiments show that ELYS is not required for nuclear growth or NPC insertion into a closed NE.

To accommodate the topology of a closed nuclear envelope, interphase assembly seems to involve a distinct set of events, beginning with fusion of the inner and outer nuclear membranes. POM121 is required specifically for interphase NPC formation and appears to be involved in a narrowing of the PNS leading to INM/ONM fusion and subsequent recruitment of the Nup107/160 complex. Its role in interphase NPC assembly depends on a functional NLS sequence likely indicating a mechanism for coordination of membrane fusion from both sides of an intact NE.

We also provide evidence that in contrast to post-mitotic NPC assembly, when the Nup107/160 is targeted as a complete entity to chromatin via ELYS, this essential complex appears to assemble at sites of new pores during interphase. Data from our lab shows that recruitment of Nup133 to sites of new NPCs in interphase requires a membrane curvature sensing ALPS domain (Drin, Casella et al. 2007). As several experiments show that localization of Nup107 to the NE and NPCs is dependent on Nup96 but not vice versa it is possible that a Nup96 containing subunit of the Nup107/160 complex participates in stabilization of a POM121 dependent "pre-pore" hole in the NE. A Nup107/Nup133 subunit could then be targeted to these holes via the ALPS domain of Nup133.

Together these results indicate there are two distinct pathways leading to NPC assembly, correlating to phases of the cell cycle, and illustrate key protein components and mechanistic differences specific to those pathways.

4.2 Future Directions

There are several interesting questions and aspects of this project requiring additional study. First and perhaps most obvious is further analysis of the role of POM121 in INM/ONM fusion and identification of other involved proteins. As the luminal domain of POM121 is only ~30 amino acids long, it seems likely that the localized observed decrease in the PNS and a fusion event would require interactions with proteins occupying the PNS. The other

transmembrane Nup, NDC1, has been shown to be required for NPC assembly and has three loops between its transmembrane domains facing the PNS. However, these luminal domains are even shorter than that of POM121 and both its longer N and C termini face the nucleo/cytoplasm.

A member of the LINC complex family of proteins, Sun1 is an INM transmembrane protein, shown to homodimerize, with its C terminus extending into the PNS. Interestingly, images of Sun1 siRNA knockdown in HeLa cells seem to phenocopy those of POM121 in that NPCs are no longer uniformly distributed in the NE (Liu, Pante et al. 2007). Authors of this study conclude this depletion induces NPC clustering, however, in light of our data it is possible that Sun1 is involved in interphase NPC insertion. In support of this hypothesis a deletion mutant of Sun1 removing its luminal domain displays the same "clustering" phenotype. A separate study of Sun1 and Sun2 shows that siRNA mediated knockdown of these proteins results in a widening of the PNS (Crisp, Liu et al. 2006). As our TEM images of cells over expressing POM121 clearly show increased narrowing of the PNS, it will be interesting to see if the either of these proteins interact with POM121.

NPCs display a static and relatively uniform spacing in the NE, with NPC density remaining constant during the cell cycle (Daigle, Beaudouin et al. 2001). How total pore number and NPC spacing is regulated is currently unknown. Since most Nups utilized in post mitotic assembly are recycled components from the mother cell, these questions are most relevant to the

context of interphase NPC insertion. As the Sun proteins have been shown to interact with the lamin meshwork underlying the NE, an interaction with POM121 would also have interesting implications regarding determination and maintenance of the regular spacing of NPCs within the NE.

Experiments in Chapter 3 indicate that POM121 precedes the Nup107/160 complex at sites of new pore formation in interphase and that recruitment of Nup107 depends on Nup96. In light of this data and the direct interaction of POM121 with Nup160 (Wozniak, personal communication), it is notable that we have noticed the siRNA knockdown of Nup96 has a rapid and dramatic effect on NPC number, more so than Nup107 or Nup133. A possible explanation for this result is a difference in protein stability between members of the Nup107/160 complex. While Nup107 is remarkably stable (D'Angelo, Raices et al. 2009) and recycled through cell divisions, the majority of Nup96 appears to be degraded during mitosis (Chakraborty, Wang et al. 2008).

Further, Nup96 is generated with Nup98 via a proteolytic cleavage event from a precursor protein. While both Nup96 and Nup98 are components of the NPC proper, recent data from our lab shows that Nup98 has an additional role in regulating gene transcription. Of particular interest are chromatin immuno-precipiation experiments indicating binding to its own promoter as well as those of other Nups including four members of the Nup107/160 complex and NDC1. Regulation of Nup transcription and NPC number is poorly understood and this Nup98/Nup96 precursor protein provides

a potentially elegant mechanism in which one product (Nup98) mediates transcription of essential Nups and the other (Nup96) is a physically limiting factor in NPC assembly. With Nup96 degraded during mitosis, this molecular regulator would be reset with each cell division.

Additionally, while the Nup107/160 complex has been implicated in celly cycle progression via its function at mitotic kinetochores and centrosomes (Loiodice, Alves et al. 2004; Orjalo, Arnaoutov et al. 2006), recent experiments show that artificially maintaining high levels of Nup96, specifically, during mitosis slows the G1/S transition (Chakraborty, Wang et al. 2008). As, following proteolytic cleavage of the precursor protein, Nup96 has been shown to interact with Nup98 (Fontoura, Dales et al. 2001), this delay could be occurring via indirectly reducing levels of functional Nup98. High levels of Nup96 sequestering Nup98 could negatively affect cell cycle progression via inhibition of its transcriptional functions and/or mRNA export of cell cycle specific transcripts at the NPC. Underscoring the physiological relevance of Nup96-Nup98 in cell cycle progression are chromosomal translocations of this gene resulting in abnormal gene expression and a variety of leukemias (Nakamura 2005).

Membrane fusion at the nuclear envelope and regulation of pore number and density are some of the longest standing questions remaining in the field. Further uncovering mechanistic details of NPC assembly, regulation and how they relate to cell function, cell cycle and human disease will provide

exciting advancements in better understanding the function of the nucleus; the organelle protecting our most precious possession - the genome.

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