

Capture of AT-rich Chromatin by ELYS Recruits POM121 and NDC1 to Initiate Nuclear Pore Assembly

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Assembly of the nuclear pore, gateway to the genome, from its component subunits is a complex process. In higher eukaryotes, nuclear pore assembly begins with the binding of ELYS/MEL-28 to chromatin and recruitment of the large critical Nup107-160 pore subunit. The choreography of steps that follow is largely speculative. Here, we set out to molecularly define early steps in nuclear pore assembly, beginning with chromatin binding. Point mutation analysis indicates that pore assembly is exquisitely sensitive to the change of only two amino acids in the AT-hook motif of ELYS. The dependence on AT-rich chromatin for ELYS binding is borne out by the use of two DNA-binding antibiotics. AT-binding Distamycin A largely blocks nuclear pore assembly, whereas GC-binding Chromomycin A₃ does not. Next, we find that recruitment of vesicles containing the key integral membrane pore proteins POM121 and NDC1 to the forming nucleus is dependent on chromatin-bound ELYS/Nup107-160 complex, whereas recruitment of gp210 vesicles is not. Indeed, we reveal an interaction between the cytoplasmic domain of POM121 and the Nup107-160 complex. Our data thus suggest an order for nuclear pore assembly of 1) AT-rich chromatin sites, 2) ELYS, 3) the Nup107-160 complex, and 4) POM121- and NDC1-containing membrane vesicles and/or sheets, followed by (5) assembly of the bulk of the remaining soluble pore subunits.

INTRODUCTION

The possession of a nuclear envelope (NE) that encompasses the genome is the defining characteristic of all eukaryotes. The envelope consists of double nuclear membranes, hundreds to thousands of nuclear pore complexes (NPCs), and in higher eukaryotes, a nuclear lamina. Bidirectional transport of protein and RNA molecules through the nuclear envelope is mediated exclusively by NPCs, large structures ~60–125 MDa in size (Reichelt *et al.*, 1990; Macara, 2001; Quimby and Corbett, 2001; Goldfarb *et al.*, 2004; Pemberton and Paschal, 2005; Patel *et al.*, 2007).

In higher eukaryotes the nuclear envelope, including pore complexes, disassembles at mitosis as a prelude to spindle assembly and chromosome segregation (Burke and Ellenberg, 2002; Margalit *et al.*, 2005; Prunuske *et al.*, 2006). This disassembly then necessitates nuclear envelope reformation around each set of segregated chromosomes toward the end of mitosis, a process that involves both nuclear membrane recruitment and nuclear pore formation.

Analysis of the pore subunits produced by mitotic disassembly has provided the most useful clues to nearest neighbor interactions within the vertebrate pore. Vertebrate nuclear pores are comprised of ~30 different proteins or nucleoporins (Nups) in 8–32 copies each, to give a 500–1000 protein structure (Cronshaw *et al.*, 2002). At mitosis the massive vertebrate pore disassembles into ~14 soluble subunits, each with a distinct protein composition, whereas the

integral membrane pore proteins, POM121, NDC1, and gp210, segregate into endoplasmic reticulum (ER) sheets and vesicles (Gerace *et al.*, 1982; Wozniak *et al.*, 1989; Greber *et al.*, 1990; Hallberg *et al.*, 1993; Ellenberg *et al.*, 1997; Yang *et al.*, 1997; Cotter *et al.*, 1998; Daigle *et al.*, 2001; Vasu and Forbes, 2001; Liu *et al.*, 2003; Suntharalingam and Wentz, 2003; De Souza *et al.*, 2004; Hetzer *et al.*, 2005; Schwartz, 2005; Lau *et al.*, 2006; Madrid *et al.*, 2006; Mansfeld *et al.*, 2006; Stavru *et al.*, 2006). Although the majority of soluble mitotic pore subunits consist of 1–3 nucleoporins, one key subunit is quite large: the Nup107-160 complex contains 9–10 different proteins and is critical not only for pore structure and function but, most relevant to this study, to the early steps of nuclear pore assembly (Belgareh *et al.*, 2001; Vasu *et al.*, 2001; Harel *et al.*, 2003b; Walther *et al.*, 2003a).

Late in anaphase, nuclear pore assembly commences with the soluble and integral membrane pore proteins coming together coincident with the newly forming nuclear membranes. Postmitotic NPC assembly is a stepwise process, but one only beginning to be understood. The end point is known and consists of 1) the massive central scaffold of the pore with eight spoke-like elements, 2) eight cytoplasmic filaments, and 3) eight shorter nuclear filaments that meld to form the nuclear pore basket. Certain nucleoporin subunits have been classified by immunofluorescence on intact cells into early, mid-, or late-assembling, but the order of assembly of the majority of subunits has been unknown (Chaudhary and Courvalin, 1993; Bodoor *et al.*, 1999; Haraguchi *et al.*, 2000; Belgareh *et al.*, 2001; Daigle *et al.*, 2001; Rabut *et al.*, 2004; Rasala *et al.*, 2006; Franz *et al.*, 2007). A recent study has made some headway on this order (Dultz *et al.*, 2008) and confirmed that the Nup107-160 complex is a very early subunit.

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An equally perplexing problem has been the timing and role of membrane assembly in the postmitotic assembly of nuclear pores. Two major mechanistic models have been proposed that differ substantially in regard to the role of the nuclear membranes in this process. One model, and considerable data, proposes that NPCs assemble *within* patches of double nuclear membranes as soon as those membranes begin to form on the surface of chromatin in late anaphase (Macaulay and Forbes, 1996; Goldberg *et al.*, 1997; Harel *et al.*, 2003a; Anderson and Hetzer, 2007; Baur *et al.*, 2007; M. Hetzer, personal communication). In this model, because assembly occurs at a site on the double membranes, a distinct and unique fusion event must occur between the inner and outer nuclear membranes for pore assembly to proceed. Indeed, precedent exists for an inner/outer nuclear membrane fusion event: this occurs during S-phase nuclear pore assembly in vertebrates (Maul *et al.*, 1972) and in yeast, that possess an intact nucleus throughout the cell cycle (Mutvei *et al.*, 1992; Winey *et al.*, 1997). A second model for postmitotic pore assembly proposes that most or all the soluble subunits are assembled on the chromatin in late mitosis and the nuclear membranes then encircle and seal around this structure toward the end of the process (Sheehan *et al.*, 1988; Burke and Ellenberg, 2002; Walther *et al.*, 2003a; Burke, 2007; Antonin *et al.*, 2008). Consistent with and important to both models are the findings that a subset of targeting and initiating nucleoporins, i.e., ELYS and the Nup107-160 complex, can bind to chromatin even in the absence of membranes (Walther *et al.*, 2003a,b; Baur *et al.*, 2007; Franz *et al.*, 2007; Gillespie *et al.*, 2007). Clearly, important questions remain unanswered as to how the process of nuclear pore assembly is initiated, ordered, and regulated.

The vertebrate protein ELYS has been shown to play the earliest known role in initiating and targeting nuclear pore assembly to the chromatin (Rasala *et al.*, 2006; Franz *et al.*, 2007). Mutations in MEL-28, the *Caenorhabditis elegans* homologue of ELYS, show clear defects in nuclear envelope morphology and function, consistent with this role (Fernandez and Piano, 2006; Galy *et al.*, 2006). Vertebrate ELYS is a large 270-kDa protein with putative nuclear localization signal (NLSs), nuclear export signals (NESs), WD repeats and an AT-hook DNA-binding motif (Kimura *et al.*, 2002). ELYS was originally proposed to be a transcription factor involved in murine embryonic hematopoiesis (Kimura *et al.*, 2002). The subsequent finding that knockout mice lacking ELYS die well before hematopoiesis, however, suggested an earlier and broader role for ELYS in the cell (Okita *et al.*, 2004).

A link between ELYS and the vertebrate nuclear pore was first identified in a mass spectrometry analysis of proteins that coimmunoprecipitate with the largest nuclear pore subunit, the Nup107-160 complex; ELYS was the most prominent protein discovered in this search (Rasala *et al.*, 2006). When RNAi-mediated knockdown of ELYS was performed in human cultured cells, a large reduction of pore number in the nuclear envelope was detected, together with an unexpected increase in pore-containing membranes in the cytoplasm known as annulate lamellae (AL; Rasala *et al.*, 2006; Franz *et al.*, 2007). These studies revealed that the most vital role of ELYS is to target pore assembly specifically to the chromatin periphery. In the absence of ELYS, nucleoporin assembly occurs within ER membranes to produce cytoplasmic pores. Although both ELYS and the Nup107-160 complex have been shown to bind to chromatin in *Xenopus* nuclear reconstitution extracts early in pore assembly, ELYS is now known to target the Nup107-160 complex there (Franz *et al.*, 2007). The immunodepletion of either ELYS or the Nup107-160 complex in *Xenopus* nuclear reconstitution

studies results in nuclei that have intact nuclear membranes, but are devoid of nuclear pores (Harel *et al.*, 2003b; Walther *et al.*, 2003a; Franz *et al.*, 2007; Gillespie *et al.*, 2007). The most recent study found that a 208-amino acid fragment of the ELYS C-terminus that contains among other sequences putative NLSs and an AT-hook motif (rATH) acts to prevent endogenous ELYS from chromatin binding and, because of that prevents nuclear pore assembly, nuclear import, and ultimately DNA replication (Gillespie *et al.*, 2007).

In this study, we have dissected the molecular role of ELYS in the early steps of nuclear pore assembly through the use of targeted deletion and point mutation analysis, sequence-specific DNA-binding antibiotics, and analysis of recruitment of the soluble and integral membrane pore proteins. We find the chain of assembly involves AT-rich DNA, ELYS, the Nup107-160 complex, and POM121, which together effectively mark the sites where pore assembly initiates. The recruitment of the remaining soluble pore subunits depends on the presence of the integral membrane pore proteins and membrane vesicle fusion.

MATERIALS AND METHODS

Antibodies, Constructs, and Protein Expression

To generate the xELYS antiserum, *Xenopus* ELYS cDNA (LOC397707) was purchased from ATCC (Manassas, VA). The extreme C-terminus of this clone was PCR amplified using oligos 5'-CGGGATCCGAAATAAAGTTGATTCTCTCTC-3' and 5'-ACGCGTCGACTCATCTCTTTCGCCGCGT-3' and subcloned into pET28a. Recombinant, his-tagged protein was expressed in *Escherichia coli* BL21 expression cells, purified on Ni-NTA agarose (Qiagen, Valencia, CA), and used to immunize a rabbit. Anti-*Xenopus* ELYS antibody was affinity purified as in Orjalo *et al.* (2006) and used for immunofluorescence and immunoblotting. Because *Xenopus* ELYS is sensitive to degradation, *Xenopus* egg or cell lysates were prepared for immunoblotting by heating at 60°C for 10 min in 1× sample buffer (62.5 mM Tris, pH 6.8, 10% glycerol, 2% SDS, 50 mM DTT, supplemented with bromophenol blue). Other antibodies used in this study included anti-xNup160, anti-hNup133, anti-rat Nup98 GLFG (Harel *et al.*, 2003b); anti-hNup85, anti-*Xenopus* POM121, anti-gp210 (Harel *et al.*, 2003a); anti-xNup43, anti-xNup37 (Orjalo *et al.*, 2006); anti-Nup93, anti-hNup205 (Miller and Forbes, 2000); anti-Tpr (Shah *et al.*, 1998); anti-*Xenopus* importin β (Rasala *et al.*, 2006); anti-xNup155 (S. Vasu and D. J. Forbes, unpublished data); anti-mNup53, anti-xNDC1 (V. Delmar and D. J. Forbes, unpublished data); anti-xNup50 (R. Sekhorn, unpublished data); anti-Orc2, anti-RCC1, anti-Mcm3 (generous gifts from Z. You, Washington University, St. Louis, MO); anti-FG nucleoporin antibody mAb414 (used to probe for Nup358, Nup214, Nup153, and Nup62 by immunoblot) and anti-GST (Covance, Berkeley, CA); anti-human importin α (BD Transduction Laboratories, Lexington, KY), anti-GAPDH (Calbiochem, San Diego, CA), and anti-ribophorin (Serotec, Oxford, United Kingdom).

To generate recombinant GST- Δ AT-hook, the above oligos and cDNA clone were used and the PCR product was subcloned into pGEX-6P-3 (GE Healthcare, Uppsala, Sweden). To generate recombinant GST-AT-hook+, oligos 5'-CGGGATCCACCAATAATGTTCTCT-3' and 5'-ACGCGTCGACTCATCTCTTTCGCCGCGT-3' were used and the PCR product was subcloned into pGEX-6P-3. Stratagene's QuickChange Site-directed Mutagenesis Kit (La Jolla, CA) was utilized to generate the GST-AT-hook 2R→A double point mutant using mutagenesis oligos 5'-GTTCGGCCTCAAAAACGGCAGCGCACCTCAAAAACAAAAGC-3' and 5'-GCTTTGTGTTTTGGAGGTGCGCCTGCCGTTTTGAGGCCGGAAC-3' and following the manufacturer's protocol. All recombinant, glutathione S-transferase (GST)-tagged proteins were expressed in *E. coli* BL21 expression cells and purified on glutathione Sepharose 4B beads (GE Healthcare).

RanQ69L was expressed, purified, and loaded with GTP as in Orjalo *et al.* (2006).

Nuclear and Annulate Lamellae Reconstitution Reactions

Cytosolic and membrane vesicle fractions of *Xenopus* egg extracts were prepared as in Powers *et al.* (1995). Nuclei were reconstituted at room temperature, by mixing *Xenopus* egg membrane vesicle and cytosolic fractions at a 1:20 ratio with an ATP-regeneration system and sperm chromatin (Macaulay *et al.*, 1995). Recombinant proteins (Figure 3) or buffer (0.35% ethanol), Distamycin A, or Chromomycin A₃ (Sigma, St. Louis, MO; Figure 4) were added to the cytosol and membranes on ice at the specified concentrations before chromatin addition. Note that Chromomycin A₃ is highly toxic and should be handled with care.

AL were assembled for 2 h at room temperature, by mixing *Xenopus* egg membrane vesicle and cytosolic fractions at a ratio of 1:8, supplemented with glycogen as in Meier *et al.* (1995). Recombinant proteins, buffer (0.35% ethanol) or Distamycin A, or 2 mM GTP γ S were added to the reactions, as indicated. AL were diluted in 1 \times ELB (10 mM HEPES, pH 7.6, 50 mM KCl, 25 mM MgCl₂), and pelleted through a 30% sucrose cushion. The membrane pellet was solubilized with SDS-containing sample buffer and subjected to immunoblot analysis.

Nuclear Import

To assay for nuclear import in the presence or absence of Distamycin A and Chromomycin A₃, green fluorescent protein (GFP)-M9 transport substrate (a generous gift from A. Lachish-Zalait and M. Elbaum, Weizmann Institute, Rehovot, Israel) was added to reconstituted nuclei 60 min after the start of assembly and fixed 5 min later in 3% paraformaldehyde. The DNA was stained with Hoechst.

Immunofluorescence

For direct immunofluorescence, mAb414, affinity purified anti-POM121, or anti-xELYS were coupled to Alexa fluor dyes (Molecular Probes, Eugene, OR). To assay for the presence of nuclear pores or nucleoporins, nuclear reactions were stopped on ice 1 h after the start of assembly. Directly labeled antibodies were added to the reactions for at least 10 min. The nuclei were mounted on mounting media containing 3,3'-dihexyloxycarbocyanine (DHCC) membrane dye (green images) and Hoechst, or fixed with 3.2% formaldehyde, incubated with octadecyl rhodamine B chloride (R18, Molecular Probes) membrane dye (red images), and mounted on Vectashield with DAPI (Vector Laboratories, Burlingame, CA). Images were acquired using an Axiovert 200M (Carl Zeiss, Thornwood, NY) at a magnification of 63 \times using an oil objective (Carl Zeiss) with a 1.3 NA at 23°C and with Immersol 518F (Carl Zeiss) as the imaging medium. Images were recorded using a Coolsnap HQ (Photometrics, Tucson, AZ) camera and Metavue software (Molecular Devices, Downingtown, PA).

POM121 Pulldown

His-tagged *Xenopus* POM121 protein fragment aa 164-435 was expressed from a pET28a vector in *E. coli* BL21 expression cells and purified on Ni-NTA agarose (Qiagen). xPOM121 aa 164-435 was coupled to CnBr-Sepharose CL4B beads (GE Healthcare) prepared according to the manufacturer's instructions. Beads (5 mg) containing POM121 fragment or the control His-GFP (25 μ g) were incubated with membrane-free *Xenopus* egg cytosol that had been diluted 1:20 in PBS with 1 mM PMSF and a protease inhibitor mixture (P8340; Sigma). This was incubated at room temperature with tumbling for 1 h. The beads were washed three times with PBS. Proteins were eluted with 100 mM glycine, pH 2.5, and neutralized with 100 mM Tris-HCl, pH 7.9. SDS-PAGE and immunoblotting were performed as in Shah *et al.* (1998).

Anchored Chromatin and Anchored Nuclei Reactions

Protocols were adapted from Macaulay and Forbes (1996). Crude nucleoplasm was prepared by heating egg cytosol to 100°C for 5 min. The denatured proteins were removed from nucleoplasm by microcentrifugation at 14,840 \times g for 20 min. Demembrated sperm chromatin was decondensed by addition of 2 volumes of crude nucleoplasm for ~10 min at room temperature. Decondensation state was monitored by fluorescence microscopy. Decondensed chromatin was diluted to 2500 sperm/ μ l in 1 \times ELB (10 mM HEPES, pH 7.6, 50 mM KCl, 25 mM MgCl₂). Diluted decondensed sperm chromatin, 50 μ l, was allowed to settle by gravity onto poly-L-lysine-treated coverslips (12 mm; Fisher Scientific, Pittsburg, PA) for 2 h in a humidified chamber. The chromatin-coated coverslips were then washed with 1 \times ELB and blocked with 5% BSA/ELB for 20 min. For the anchored chromatin experiments, membrane-free *Xenopus* egg cytosol (which was subjected to an additional centrifugation at 14,840 \times g for 20 min and designated as membrane-free by the absence of the integral membrane proteins ribophorin and gp210), an ATP-regenerating system, 25 μ g/ml nocodazole, and recombinant proteins or antibiotics (where indicated) were combined on ice for a final volume of 30–40 μ l and then added to the chromatin-coated coverslips. Chromatin-binding reactions were allowed to continue for 20–60 min. Coverslips were washed three times with 1 \times ELB-K (10 mM HEPES, pH 7.6, 125 mM KCl, 25 mM MgCl₂) to remove all unbound proteins. Chromatin-bound proteins were solubilized with SDS-containing sample buffer and subjected to immunoblotting analysis.

Anchored nuclei reactions were conducted as above, by mixing *Xenopus* egg membrane vesicle and cytosolic fractions at a ratio of 1:10, an ATP-regenerating system, 25 μ g/ml nocodazole, and recombinant proteins or antibiotics (where indicated) on ice for a final volume of 30–40 μ l. Reactions were incubated with chromatin-coated coverslips for 1 h at room temperature. GTP γ S, 2 mM, was included in the reaction, where indicated, to prevent membrane vesicle fusion.

RESULTS

Anti-ELYS Antibody Demonstrates That ELYS Is Abundant in Nuclear Pores, But Not Annulate Lamellae Pores

To investigate the molecular mechanism for ELYS function in nuclear pore assembly, we utilized a nuclear reconstitution system derived from *Xenopus* egg extract. The egg contains large stores of disassembled pore subunits for future cell division; extracts of *Xenopus* eggs have been well characterized for studies of nuclear assembly and nuclear pore assembly (Forbes *et al.*, 1983; Lohka and Masui, 1983; Newport, 1987). To study the role of ELYS, we generated an antibody to aa 2358-2408 of the *Xenopus* ELYS protein (LOC397707). This antibody recognized a protein of the expected size (~270 kDa) in both *Xenopus* egg cytosol and XL177 cultured cell lysates (Figure 1B). Immunofluorescence revealed that the antibody stains *Xenopus* in vitro-reconstituted nuclei in a punctate nuclear rim pattern that colocalizes, as expected, with known nucleoporins containing phenylalanine-glycine repeat domains (FG-Nups; Figure 1A).

The anti-xELYS antibody was next used to biochemically probe for the presence of ELYS in nuclear pores and AL pores. AL are cytoplasmic stacks of membranes containing structures identical to pores, typically found in rapidly dividing cells such as gametes and tumor cells (Kessel, 1992; Meier *et al.*, 1995). AL can be readily assembled in vitro in *Xenopus* egg extracts in the absence of added chromatin (Dabauvalle *et al.*, 1991; Meier *et al.*, 1995; Miller and Forbes, 2000). When immunoblots were probed with the anti-ELYS antibody, they showed that ELYS biochemically purifies with reconstituted nuclei, but not with AL pore complexes assembled in vitro (Figure 1C, compare lanes 1 and 2). This data authenticates the antibody and further supports the conclusion that ELYS acts to target pore assembly to the chromatin periphery (Rasala *et al.*, 2006; Franz *et al.*, 2007), rather than functioning as a structural component of the pore.

The ELYS C-Terminus Contains Both AT-Hook and non-AT-Hook Chromatin-binding Domains

ELYS has a putative AT-hook DNA-binding motif (Kimura *et al.*, 2002) and is indeed chromatin associated (Galy *et al.*, 2006; Franz *et al.*, 2007; Gillespie *et al.*, 2007). To better understand the interaction between chromatin and ELYS, we set out to specifically mutate the ELYS AT-hook motif to test whether it actually plays a role in the chromatin binding of ELYS. This, though possibly assumed from recent work (Gillespie *et al.*, 2007), has never been tested. We expressed a GST-tagged fragment corresponding to the C-terminal 128 aa of *Xenopus* ELYS that contains the eight amino acid AT-hook motif, KPRGRPPK (AT-hook+, Figure 2A). We also expressed an identical fragment, but one into which we had introduced two arginine (R) \rightarrow alanine (A) point mutations in the AT-hook motif to give KPAGAPPK (underscoring indicates mutated to alanine) (AT-hook-2R \rightarrow A, Figure 2A). These arginine residues have been shown to be crucial for the interaction between AT-hook motifs and DNA in proteins such as HMGA1/HMG-I(Y) and Taf1 (Huth *et al.*, 1997; Metcalf and Wassarman, 2006).

To test the ability of the ELYS GST-AT-hook+ and GST-AT-hook-2R \rightarrow A fragments to bind to chromatin, we added decreasing concentrations of the fragments to egg cytosol and incubated this with "anchored chromatin" on coverslips (i.e., coverslips coated with decondensed *Xenopus* sperm chromatin packets; Macaulay and Forbes, 1996). After 20 min of incubation and subsequent washing, any chromatin-

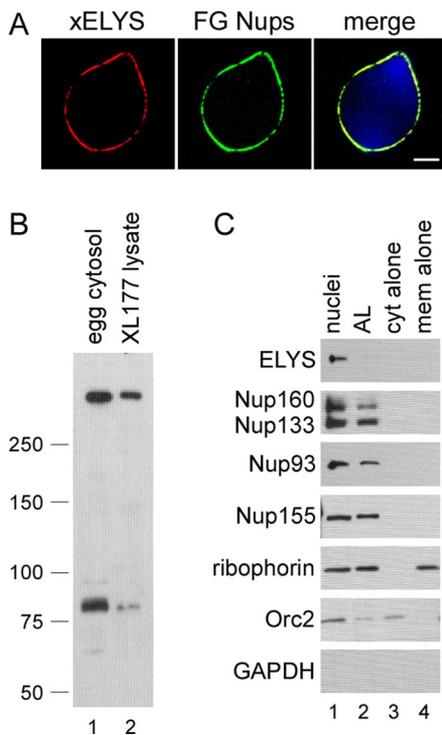


Figure 1. ELYS is abundant in nuclear pores, but not in annulate lamellae (AL) pores. (A) *Xenopus* reconstituted nuclei were stained with directly labeled anti-xELYS-AF568 (red) and mAb414-AF488 (FG-Nups, green). A merge shows the two localized in an overlapping pattern at the nuclear rim. The DNA is stained with DAPI. Scale bar, 5 μ m. (B) Anti-*Xenopus* ELYS antibody recognizes a full-sized protein band of the expected size of ~270 kDa and a smaller band (~80 kDa) in *Xenopus* egg cytosol (lane 1) and XL177 *Xenopus* cell lysates (lane 2). (C) Nuclear pores were assembled in the presence of *Xenopus* egg membranes, cytosol, and sperm chromatin (nuclei, lane 1). AL pores were assembled in the presence of *Xenopus* egg membranes and cytosol (AL, lane 2). Reactions containing cytosol only (lane 3) or membranes only (lane 4) were used as controls. All reactions were spun through a 30% sucrose cushion, with the heavier components, including the nuclei (lane 1), AL (lane 2), and membrane vesicles (lane 4) pelleting, whereas the soluble proteins (lane 3) remained in the supernatant. The pellets were solubilized with SDS-containing sample buffer, and the presence of ELYS was determined by immunoblot. ELYS was enriched in the nuclear pore assembly reaction, whereas the rest of the soluble Nups tested (Nup160, Nup133, Nup93, and Nup155) purified with both nuclear and AL pores. Orc2, a nuclear protein not associated with pores, is enriched in the purified nuclei (lane 1). Ribophorin, an integral membrane ER protein, copurifies with the membranes. GAPDH, a cytosolic protein not associated with pores, is absent.

bound proteins were solubilized and analyzed. Immunoblot analysis revealed that the ELYS GST-AT-hook+ fragment bound to chromatin (Figure 2B). However, we found that ELYS GST-AT-hook-2R→A also bound to chromatin with near identical affinity (Figure 2B). This data suggested that either the putative AT-hook motif does not contribute to ELYS chromatin-binding ability or there might exist an additional chromatin-binding domain elsewhere in the C-terminus of ELYS.

To test for an additional chromatin binding domain, we expressed a smaller fragment of the ELYS C-terminus, corresponding to the last 51 aa (aa 2359-2408) and lacking the AT-hook motif (Δ AT-hook, Figure 2A). ELYS GST- Δ AT-hook was indeed able to bind to chromatin, albeit with approximately a two- to threefold lower affinity compared

with the longer GST-AT-hook+ (Figure 2C). GST, used as a control, did not bind to anchored chromatin (Figure 2C). To confirm that ELYS AT-hook motif indeed binds to chromatin, we tested ELYS aa 2281-2359, which contains the AT-hook motif but lacks the second chromatin-binding domain, for chromatin binding. This smaller AT-hook+ fragment bound to chromatin, but the 2R→A mutant of that fragment did not (data not shown). Together the data indicate that the C-terminal 128 amino acids of *Xenopus* ELYS contain two chromatin-binding domains, an AT-hook motif and a second domain.

The AT-Hook Motif Itself Is Required for the Dominant Negative Effect of the ELYS C-Terminus on Nuclear Pore Assembly

The ELYS GST-tagged recombinant protein fragments AT-hook+, AT-hook-2R→A, and Δ AT-hook are all capable of chromatin binding, albeit with slightly varying affinities (Figure 2). We next tested whether these distinct ELYS fragments competed with endogenous ELYS for binding to chromatin. The addition of 5 or 10 μ M ELYS AT-hook+ to egg cytosol readily blocked endogenous full-length ELYS binding to anchored chromatin (Figure 3A, lane 3 and 6, top strip). Higher concentrations (10 μ M) of ELYS AT-hook-2R→A also blocked endogenous ELYS chromatin binding. However, in the presence of 5 μ M AT-hook-2R→A, we observed considerable ELYS chromatin binding (Figure 3A, lanes 4 and 7, top strip). The ELYS Δ AT-hook fragment did not cause a major loss of endogenous ELYS chromatin binding at either concentration (Figure 3A, lanes 5 and 8, top strip). Thus, the ELYS fragment containing a functional AT-hook, AT-hook+, most efficiently outcompeted endogenous ELYS for chromatin binding. (Lane 1 shows an aliquot of total cytosol run on the gel as a control for immunoblotting.) Addition of the ELYS AT-hook+ fragment also efficiently blocked the chromatin binding of the Nup107-160 complex (Figure 3A, lanes 3 and 6, second strip), reinforcing the conclusion that the binding of the Nup107-160 complex to chromatin is dependent on ELYS chromatin binding.

To compare the effects of ELYS AT-hook+, AT-hook-2R→A, and Δ AT-hook on nuclear pore assembly, we reconstituted nuclei *in vitro* in the presence of the recombinant fragments and probed for nuclear pores using directly labeled anti-FG-nucleoporin antibody (Alexa-568-mAb414). This antibody has been used extensively to indicate the presence of mature nuclear pores *in vivo* and in reconstituted nuclei (i.e., Harel *et al.*, 2003a,b; D'Angelo *et al.*, 2006; Franz *et al.*, 2007). The addition of high concentrations (15 μ M) of ELYS fragments mutant in or deleted for the AT-hook, i.e., ELYS GST-AT-hook-2R→A or ELYS GST- Δ AT-hook, had no detrimental effects on nuclear pore assembly (Figure 3B, FG-Nups). Specifically, the rims of the assembled nuclei stained brightly with directly labeled anti-FG Nup antibody, mAb414, all along their length, typical of normal nuclear pore assembly. Moreover, nuclear membrane recruitment and fusion were normal, as determined by a continuous and smooth nuclear rim stain with the membrane dye DHCC (Figure 3B, DHCC). In contrast, the addition of an equivalent concentration of ELYS GST-AT-hook+ had no effect on nuclear membrane fusion (Figure 3B, DHCC), but severely inhibited nuclear pore assembly (Figure 3B, FG-Nups, second column). This ELYS GST-AT-hook+ phenotype of membrane-enclosed, but pore assembly-inhibited nuclei mimics the effects of ELYS immunodepletion (Franz *et al.*, 2007). Although both the AT-hook+ and AT-hook 2R→A mutant give rise to smaller nuclei, the most distinguishing difference between them is the absence of nuclear pores in the AT-hook+ nuclei, demonstrating that nuclear

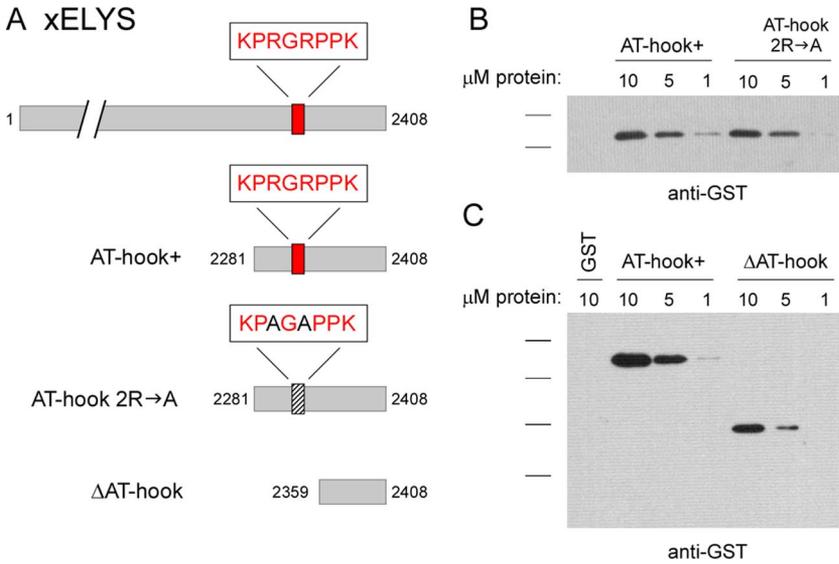


Figure 2. The ELYS C-terminus contains at least two chromatin-binding domains. (A) Cartoons representing the xELYS C-terminal fragments used in this study. The red box represents the AT-hook motif, the black and white striped box represents the arginine (R) to alanine (A) AT-hook motif double point mutant. (B and C) Anchored chromatin-binding assays in which chromatin-coated coverslips were incubated with *Xenopus* egg cytosol plus the indicated amounts of (B) GST-AT-hook+, GST-AT-hook 2R→A, or (C) GST, GST-AT-hook+, GST-ΔAT-hook. Immunoblots were probed with anti-GST antibody. GST-AT-hook+, GST-AT-hook 2R→A, and GST-ΔAT-hook all bound to the anchored chromatin with varying affinities, whereas GST did not. Dashes represent molecular-weight markers 55 and 40 kDa (B) and 55, 40, 33, and 24 kDa (C).

pore assembly is exquisitely sensitive to a change in the eight-amino acid AT-hook of ELYS. Near the completion of this work, a study was published that showed that a longer C-terminal recombinant

of *Xenopus* ELYS, which the authors termed rATH, bound to chromatin, inhibited endogenous ELYS and members of the Nup107-160 complex from binding to chromatin, and blocked NPC assembly (Gillespie *et al.*, 2007). rATH (208 aa;

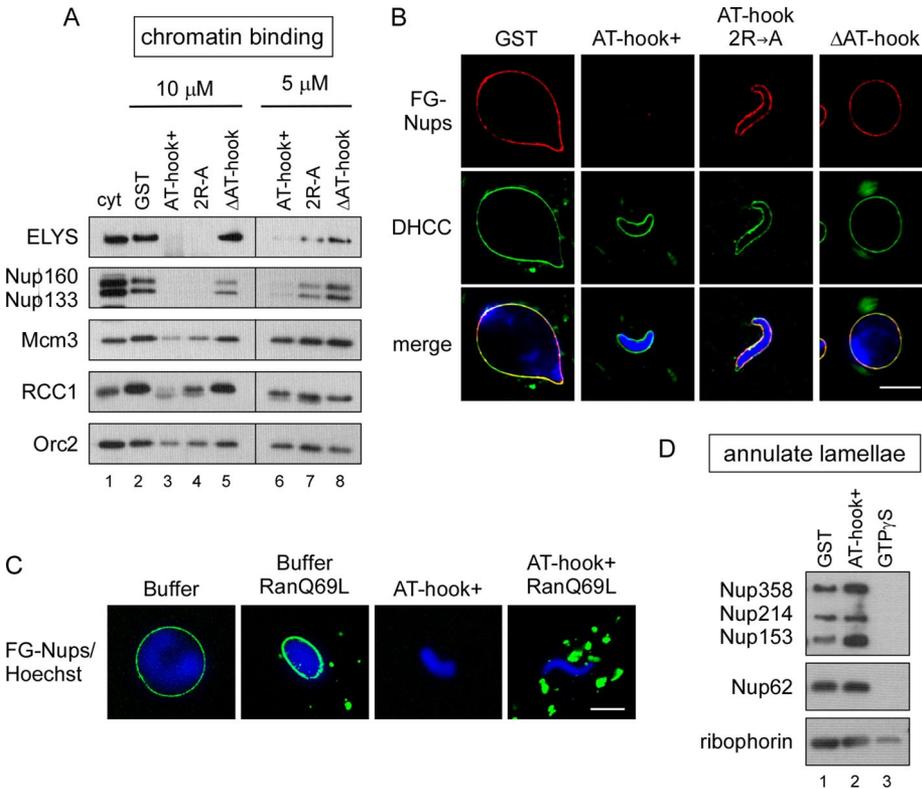


Figure 3. The AT-hook motif itself is required for the dominant negative effect of ELYS' C-terminus on nuclear pore assembly. (A) Anchored chromatin binding assay in which chromatin-coated coverslips were incubated with *Xenopus* egg cytosol plus 10 μ M GST, or 5 or 10 μ M GST-AT-hook+, GST-AT-hook 2R→A, or GST-ΔAT-hook. Immunoblot analysis using revealed the relative amounts of chromatin binding for endogenous ELYS, Nup107-160 complex members Nup160 and Nup133, Mcm2-7 complex member Mcm3, the RanGEF RCC1, and Orc2. (The apparent mobility shift of RCC1 was not reproducibly observed.) Each lane contains bound protein derived from an equal number of sperm chromatin immobilized on poly-L-lysine-treated coverslips, except for the Cyt lane (3 μ l of *Xenopus* egg cytosol diluted 1:10), which is shown as a control for immunoblotting (lane 1). (B) Reconstituted nuclei were assembled in the presence of 15 μ M GST, GST-AT-hook+, GST-AT-hook 2R→A, or GST-ΔAT-hook for 1 h. The presence of mature nuclear pores was visualized by staining for the FG-Nups using directly labeled mAb414-AF568 (red). Membrane fusion was determined by continuous DHCC stain of the nuclear membranes (green).

DNA was visualized with DAPI (blue, merge). Scale bar, 10 μ m. (C) Reconstituted nuclei were assembled in the presence of buffer or GST-AT-hook+, either in the presence or absence of 30 μ M RanQ69L-GTP. The FG-Nups, representing mature nuclear pores, were stained with directly labeled mAb414-AF488 (green) and then fixed with 2% formaldehyde before being visualized by fluorescent microscopy. DNA was stained with Hoechst (blue). Scale bar, 10 μ m. (D) Annulate lamellae (AL) were assembled by combining purified *Xenopus* cytosol with membranes in the presence of either 20 μ M GST (lane 1) or GST-AT-hook+ (lane 2) for 2 h. The addition of 2 mM GTP γ S to reactions containing membranes and cytosol inhibits AL assembly and is shown for comparison (lane 3). All membranes, including membrane-associated proteins, were purified by high-speed centrifugation through a 30% sucrose cushion and were solubilized by SDS-containing sample buffer. Immunoblot analysis using mAb414 revealed that equal amounts of the soluble FG-nucleoporins, Nup358, Nup214, Nup153, and Nup62, assembled into AL supplemented with excess GST or GST-AT-hook+. Integral membrane protein ribophorin served as a loading control.

aa 2200-2408) contains within it the smaller AT-hook+ fragment of ELYS used here (128 aa; aa 2281-2408; Figure 2A), and the rATH data are consistent with our own AT-hook+ data. However, that study did not in any way demonstrate that the AT-hook motif itself was the operationally important component of the 208 aa rATH fragment or whether other distinct chromatin-binding domains were present. Our data demonstrate, for the first time, the importance of the specific amino acids of the AT-hook motif to nuclear pore assembly.

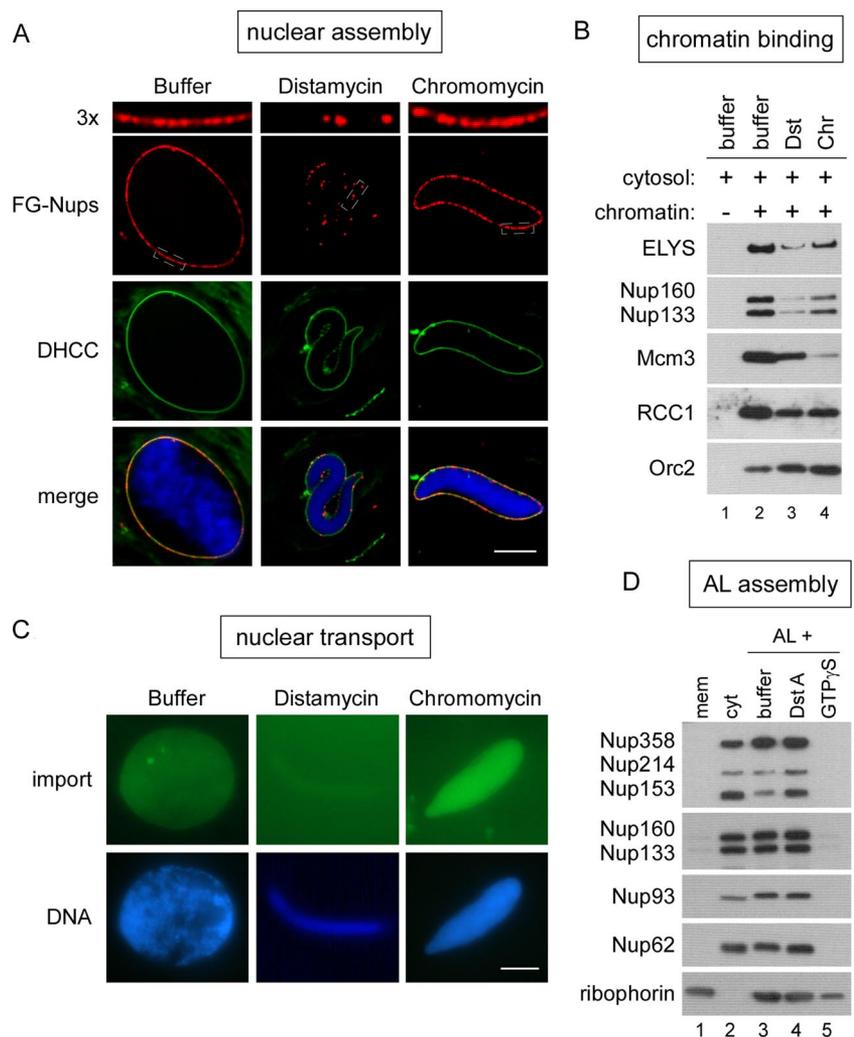
During the above experiment, we also observed that addition of the inhibitory AT-hook+ fragment in the anchored chromatin assay reduced the amount of chromatin-bound RCC1, the RanGEF, to some extent (Figure 3A, lane 3). The fact that the nuclear membranes assemble well in the AT-hook+ condition (Figure 3B) implies that there is adequate RCC1 and RanGTP present to promote nuclear assembly and thus not the cause of the pore assembly defect. However, to better demonstrate that the pore defect has nothing to do with Ran levels, either in our studies or those of Gillespie *et al.* (2007), we assembled nuclei in the presence of

ELYS GST-AT-hook+ with or without excess Ran-GTP. Indeed, the nuclear pore defect induced by AT-hook+ was not reversed by excess Ran-GTP, i.e., no nuclear pores were seen on the surface of chromatin (Figure 3C, right panel). Interestingly, abundant FG Nup-containing structures, typical of AL, appeared in the cytoplasm under the Ran⁺ conditions, further suggesting that the pore assembly defect is specific to pore assembly on the chromatin. Thus, the AT-hook motif itself is required for the dominant negative effect of the ELYS C-terminus on nuclear pore assembly.

The Dominant Negative Fragment of ELYS Does Not Block the Assembly of Annulate Lamellae

To more definitively test whether GST-AT-hook+ only acts at the chromatin level, rather than on another step in pore assembly, we tested this fragment on AL assembly *in vitro*. When interphase egg extract is incubated in the absence of a source of chromatin or DNA, AL containing cytoplasmic pores have been shown to readily form *in vitro* (Dabauvalle *et al.*, 1991; Meier *et al.*, 1995; Miller and Forbes, 2000). Annulate lamellae were assembled in the presence or absence

Figure 4. The antibiotic Distamycin A inhibits nuclear pore assembly. (A) Reconstituted nuclei were assembled in the presence of buffer, 10 μ M Distamycin A (AT-binder), or 10 μ M Chromomycin A₃ (GC-binder). Nuclear pores were stained with directly labeled mAb414-AF568 (FG-Nups, red). High-magnification views of FG-staining nuclear pores (red) are shown above the red FG-Nup panels. Membrane fusion was determined by continuous DHCC stain of the nuclear membranes (green). DNA was stained with DAPI (blue, merge). Approximately 80% of the nuclei assembled in the presence of 10 μ M Distamycin contained little to no visible nuclear pore staining, whereas the majority of the nuclei assembled in the presence of buffer or 10 μ M Chromomycin displayed normal nuclear pore staining. Scale bar, 5 μ m. (B) Anchored chromatin-binding assay in which chromatin-coated coverslips were incubated with *Xenopus* egg cytosol plus buffer (lane 2), 10 μ M Distamycin A (lane 3), or 10 μ M Chromomycin A₃ (lane 4). Immunoblot analysis revealed the effect of each antibiotic on the relative amounts of chromatin binding for endogenous ELYS, Nup107-160 complex members Nup160 and Nup133, Mcm2-7 complex member Mcm3, the RanGEF RCC1, and Orc2. Chromatin was not added to the reaction in lane 1 to control for nonspecific binding to the BSA-treated coverslips. In this experiment, each lane, with the exception of lane 1, contains bound protein derived from an equal number of sperm chromatin which had been immobilized on poly-L-lysine-treated coverslips. (C) To assess NPC function, nuclei were assembled in control, Distamycin (10 μ M), and Chromomycin A₃ (10 μ M) conditions for 1 h, before incubation with GFP-M9 transport substrate for 5 min. Reactions were stopped with 4% formaldehyde and assessed by fluorescence microscopy for nuclear import. Chromatin was visualized with Hoechst DNA dye. Representative nuclei are shown. (D) Annulate lamellae (AL) were assembled *in vitro* by incubating *Xenopus* egg membranes with cytosol, either in the presence of buffer or 10 μ M Distamycin A (Dst A; lanes 3 and 4). The purified membrane fractions were probed by immunoblotting (lanes 3–5). A reaction assembled in the presence of 2 mM GTP γ S, which blocks membrane fusion and inhibits AL assembly (Meier *et al.*, 1995), was used as a negative control (lane 5). Distamycin A did not affect the assembly of soluble pore proteins Nup160, Nup133, Nup93, or Nup62 into AL. Mem, 3 μ l of *Xenopus* egg membrane fraction diluted 1:20, and cyt, 3 μ l of *Xenopus* egg cytosol diluted 1:10, are shown for comparison (lanes 1 and 2). Ribophorin was used as a loading control.



of equimolar amounts of GST or ELYS GST-AT-hook+ and then isolated. Immunoblot analysis revealed that there was no difference in the amounts of tested nucleoporins assembled into AL pore complexes in the presence of GST-AT-hook+ compared with that with GST alone (Figure 3D, lanes 1 and 2). An assembly reaction containing 2 mM GTP γ S, known to inhibit AL formation (Meier *et al.*, 1995), is shown for comparison (Figure 3D, lane 3). Clearly, the ELYS AT-hook+ fragment that blocked nuclear pore assembly did not block AL pore assembly and thus acts at the surface of the chromatin to deny endogenous ELYS access.

The Antibiotic Distamycin A, Which Binds AT-rich DNA, Blocks Nuclear Pore Assembly

AT-hook motifs, found in a subset of DNA/chromatin-binding proteins, such as the nonhistone chromosomal high mobility group HMG protein family, are known to bind specifically to the minor groove of DNA at AT-rich sequences (Reeves and Nissen, 1990; Aravind and Landsman, 1998; Reeves, 2001). The importance of the ELYS AT-hook motif that we demonstrated above using the 2R→A point mutation implies that AT-rich DNA may play a role in NPC assembly. To analyze this further, we assembled reconstituted nuclei *in vitro* in the presence of two sequence-specific antibiotics: 1) Distamycin A, which binds to the minor groove of AT-rich regions of DNA, and 2) Chromomycin A₃, which binds to the minor groove of GC-rich regions of DNA. These two antibiotics have been used previously to define the binding specificities for certain DNA/chromatin-binding proteins, including histone H1 (Kas *et al.*, 1989), topoisomerase II (Bell *et al.*, 1997), and the nuclear envelope protein Lamin B (Rzepecki *et al.*, 1998). Histone H1 and topoisomerase II were prevented from DNA binding by Distamycin A *in vitro*, whereas *in vivo* Lamin B was prevented from chromatin binding by Chromomycin A₃ and, to a lesser extent, Distamycin A. On addition to nuclear assembly reactions, neither Distamycin nor Chromomycin affected nuclear membrane recruitment or membrane vesicle fusion at any concentration tested; intact nuclear membranes assembled in both conditions (Figure 4A, DHCC), although in both cases the nuclear were smaller.

In contrast, however, we found that the AT-rich DNA binding antibiotic Distamycin A (10 μ M) severely disrupted nuclear pore assembly (Figure 4A, FG Nups). Many fewer (0–3) nuclear pores were observed per 5- μ m linear run of nuclear envelope than were seen in the same length of nuclear envelope in control nuclei (Figure 4A, Buffer, FG-Nups). Importantly, addition of the GC-rich DNA binding antibiotic Chromomycin A₃ showed little effect on pore number per linear run of nuclear envelope (Figure 4A, Chromomycin, FG Nups). It should be noted, however, that at very high concentrations of Chromomycin A₃ (>50 μ M; data not shown), we did observe nuclear pore assembly defects, possibly resulting from some global alteration of chromatin structure or composition such that ELYS was lost through a more nonspecific process.

To ascertain that Distamycin A blocks NPC assembly specifically through effect on DNA, rather than a nonspecific effect of the antibiotic on pore proteins, we tested whether Distamycin had an effect on the assembly of cytoplasmic AL pore complexes. We found that Distamycin A clearly had no effect on AL pore assembly (Figure 4D).

Both Distamycin A and AT-hook motif proteins bind to the minor groove of AT-rich DNA. The data above suggested that Distamycin A inhibits NPC assembly by preventing endogenous ELYS from binding to such AT-rich sites. To test this conclusion, we asked whether Distamycin

blocked the binding of ELYS to chromatin. For this, an anchored chromatin assay was performed in the presence of either Distamycin A or Chromomycin A₃. Immunoblot analysis of the chromatin-bound proteins revealed that Distamycin A did indeed dramatically reduce the amount of ELYS, as well as the Nup107-160 complex, bound to chromatin (Figure 4B, lane 3). Chromomycin A₃ affected ELYS and the Nup107-160 complex to a significantly lesser extent (Figure 4B, lane 4).

Interestingly, Mcm3, which was previously implicated to interact with ELYS (Gillespie *et al.*, 2007), was affected by the antibiotics in an opposite manner to that of ELYS and the Nup107-160 complex (Figure 4B, lanes 3 and 4).

Finally, we assessed the antibiotic treated nuclei for NPC function by performing nuclear import assays with the transportin substrate GFP-M9. Clearly, Chromomycin A₃ did not block the formation of functional nuclear pores, because GFP-M9 accumulated to high levels in the Chromomycin A₃-treated nuclei (Figure 4C). In contrast, nuclei assembled in the presence of Distamycin imported very little (Figure 4C) or not at all (data not shown). Thus, the data points toward a mechanism in which ELYS initiates pore assembly specifically on AT-rich chromatin.

ELYS, the Nup107-160 Complex, and Nup153 Are the Only Soluble Pore Subunits Found to Bind Chromatin in the Absence of Membranes

In *Xenopus*, NPCs are assembled from 14 soluble subunits (Figure 5A) and three integral membrane proteins. Our data, together with others, demonstrates that NPC assembly begins with the chromatin binding of ELYS and is followed by recruitment of the Nup107-160 complex (Franz *et al.*, 2007; Gillespie *et al.*, 2007). The next step in NPC assembly has remained unknown.

We hypothesized that the step in NPC assembly that immediately follows the chromatin-binding of ELYS and the Nup107-160 complex is either 1) the recruitment of additional soluble pore subunits to the chromatin/ELYS/Nup107-160 precursor in a membrane-independent manner or 2) the recruitment of one or more integral membrane pore protein(s) with its associated membrane vesicle or sheet. To address the first possibility, we asked whether additional soluble pore subunits can bind to chromatin in the complete absence of membranes. Only a fraction of the nucleoporins have been previously tested for their ability to bind chromatin (Walther *et al.*, 2003ab; Baur *et al.*, 2007; Franz *et al.*, 2007; Gillespie *et al.*, 2007). Here we set out to perform a near comprehensive analysis. Previous chromatin-binding experiments were done by incubating chromatin and cytosol together at room temperature, followed by fixation and purification to remove any unbound soluble proteins, and identification by immunofluorescence (Walther *et al.*, 2003a,b; Franz *et al.*, 2007). This type of analysis had three potential problems: 1) few nucleoporins have been looked at, 2) fixation before the separation of unbound soluble proteins from chromatin-bound proteins could well lead to false positives, and 3) any membrane contamination that occurred could induce full pore assembly in the membrane-containing regions and give the appearance in immunofluorescence of a false-positive association of nucleoporins with the chromatin, especially in the presence of Ran, as shown by Baur *et al.* (2007).

In this study chromatin binding was assayed biochemically by incubating anchored chromatin with cytosol, and bound proteins were detected by immunoblotting. A large number of nucleoporins was tested in a single experiment, while simultaneously monitoring for any membrane con-

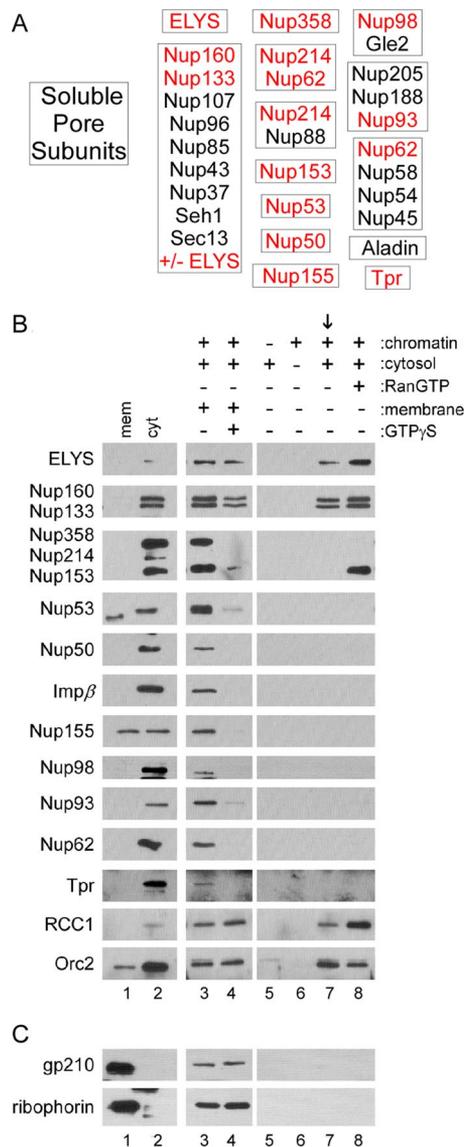


Figure 5. Only ELYS and the Nup107-160 pore subunits bind chromatin in the absence of membranes and RanQ69L-GTP. (A) A diagram representing the known soluble nucleoporin subunits (boxed). It should be noted that we were unable to probe for Aladin because of the lack of an anti-*Xenopus* Aladin antibody. Nucleoporins highlighted in red were assayed for their ability to bind to anchored chromatin by immunoblotting. (B and C) Anchored chromatin binding assay in which chromatin-coated coverslips were incubated with: *Xenopus* egg cytosol plus membranes to assemble anchored nuclei (lane 3); egg cytosol plus membranes plus 2 mM GTP γ S to block membrane vesicle fusion (lane 4); buffer alone (lane 6), membrane-free *Xenopus* egg cytosol (lane 7, arrowhead), or the identical cytosol plus 30 μ M RanQ69L-GTP (lane 8). A coverslip not coated with chromatin was incubated with cytosol (lane 5) as a control for nonspecific protein binding. In this experiment, Orc2 serves as a loading control. Mem, *Xenopus* egg membrane fraction diluted 1:20, and cyt, *Xenopus* egg cytosol diluted 1:10, are shown as a control for immunoblotting with the various antibodies (lanes 1 and 2). It should be noted that we did not detect chromatin binding of Nup358 in the presence of excess RanGTP, as was previously published (Walther *et al.*, 2003b). (C) Immunoblots using antibodies to the integral membrane proteins gp210 and ribophorin show that membrane vesicles are not present in the *Xenopus* egg cytosol (lanes 2 and 5–8).

tamination. The cytosol used was shown in this manner to be devoid of membrane proteins (Figure 5C, lanes 2 and 5–8; ribophorin and gp210).

When chromatin-coated coverslips were incubated with cytosol completely devoid of membranes in this assay, the only pore subunits that bound to chromatin were found to be ELYS and the Nup107-160 complex (Figure 5B, lane 7). In the positive control, where we assembled complete nuclei by addition of membranes and cytosol to the anchored chromatin templates, all of the tested soluble pore subunits (Figure 5A, red) were found associated, except for Nup214, possibly because of the relatively low affinity of mAb414 for Nup214 (Figure 5B, lane 3).

Next, when excess RanGTP was added to membrane-free cytosol, only one additional nucleoporin subunit was observed to bind to chromatin: Nup153 (Figure 5B, lane 8). Nup153 has previously been shown to bind to chromatin in the presence of RanGTP and to biochemically interact with the Nup107-160 complex (Vasu *et al.*, 2001; Walther *et al.*, 2003b). This data indicates that the majority of soluble nuclear pore subunits do not bind to chromatin in the absence of membranes, even when excess RanGTP is present, in these experimental conditions.

Finally, when membranes and cytosol were added to anchored chromatin in the presence of GTP γ S, a compound that blocks membrane vesicle fusion (Macaulay and Forbes, 1996), again only ELYS and the Nup107-160 complex bound to the chromatin templates without substantial binding of other nucleoporin subunits (Figure 5B, lane 4). Thus, the *in vitro* assembly of the bulk of the soluble nuclear pore subunits requires the presence of membranes vesicles and importantly, requires membrane vesicle fusion.

POM121 Interacts with the Nup107-160 and Nup93-205 Pore Subunits

The data above suggested that the recruitment of membranes must follow the chromatin binding of ELYS and the Nup107-160 complex. Three pore integral membrane proteins, POM121, NDC1, and gp210, exist in vertebrates. POM121 appears early in nuclear assembly, making its binding partners in the nuclear pore of particular interest (Gerace *et al.*, 1982; Chaudhary and Courvalin, 1993; Hallberg *et al.*, 1993; Bodoor *et al.*, 1999; Drummond and Wilson, 2002; Antonin *et al.*, 2005; Dultz *et al.*, 2008). Furthermore, RNAi knockdown of POM121 has, in many but not all cases, shown POM121 to be required for nuclear pore formation (Antonin *et al.*, 2005; Mansfeld *et al.*, 2006; Funakoshi *et al.*, 2007).

The ~120-kDa POM121 protein in *Xenopus* and mammals is a single transmembrane protein with the bulk of the protein accessible for interaction with other nucleoporin subunits (Figure 6A; Hallberg *et al.*, 1993; Soderqvist and Hallberg, 1994). However, the C-terminal third of POM121 contains the FG repeat motifs present in a number of Nups that are thought to bind transport receptors such as importin β and transportin (Hallberg *et al.*, 1993). A fragment of POM121, presumed to be available for interaction within the scaffold of the nuclear pore, but one that lacked FG repeats (in order to avoid transport receptor binding), was used to search for soluble nucleoporins that link to the critical POM121 protein. Using this fragment (aa 144-435, Figure 6A), pull-downs were performed with *Xenopus* egg extracts. Nucleoporins that bound to the POM121 fragment in pull-downs were identified by immunoblotting (Figure 6B). Notably, pore subunits containing Nup358, Nup214, Nup155, Nup62, and Nup53, showed no affinity for the POM121 fragment (Figure 6B, lanes 3 and 5). Importin α and β bound,

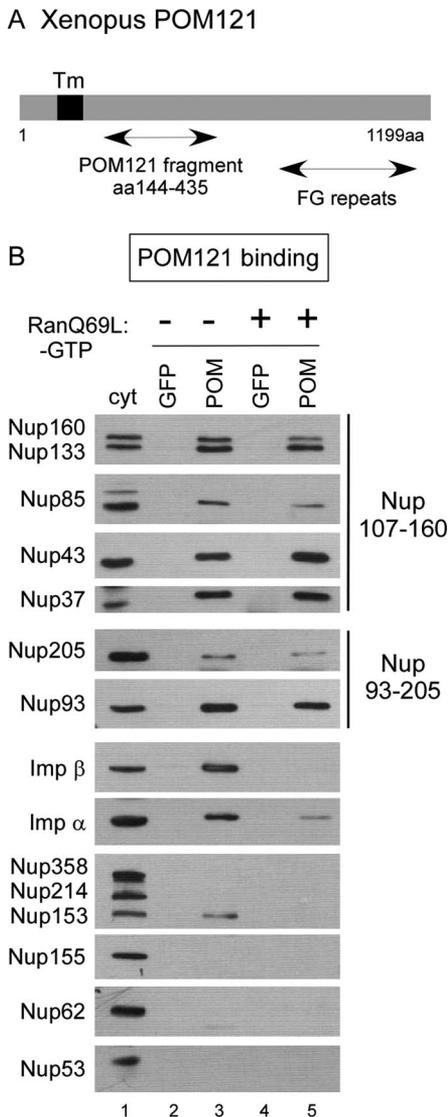


Figure 6. POM121 binds the Nup107-160 and the Nup93-205 complexes in the absence of FG Nups, Nup53, and Nup155. (A) A map of POM121 and the fragment used. (B) The *Xenopus* POM121 fragment aa 144-435 (POM) was coupled to beads and mixed with *Xenopus* egg cytosol in pulldown reactions, in the presence or the absence of 10 μ M RanQ69L-GTP. his-GFP beads were used as a control. The bound proteins were probed by immunoblotting with different anti-nucleoporin and import factor antibodies, as shown in the figure. Nup160, Nup133, Nup85, Nup43, and Nup37 of the Nup107-160 complex, and Nup93 and Nup205 of the Nup93-205 complex specifically bound to the POM121 fragment. Cyt, *Xenopus* egg cytosol diluted 1:10, is shown as a control for immunoblotting with the various antibodies (lane 1).

but were largely removed by RanQ69L-GTP (Figure 6B, compare lanes 3 and 5). The FG nucleoporin Nup153 also bound to the POM121 beads, but it too was removed by RanQ69L-GTP, suggesting an indirect interaction, perhaps through its known binding to importin β (Shah and Forbes, 1998; Shah *et al.*, 1998; Ben-Efraim and Gerace, 2001; Walther *et al.*, 2003b).

Strikingly, the Nup107-160 complex bound strongly to the POM121 fragment (Figure 6B, lane 3; see Nup160, Nup133, Nup85, Nup43, and Nup37). Its binding was unaffected by RanQ69L-GTP (Figure 6B, lane 5), indicating that the inter-

action is not mediated through importin α or β . A lesser amount of ELYS was also observed to bind to the POM121 beads (data not shown) and may associate with POM121 through its interaction with the Nup107-160 complex (Rasala *et al.*, 2006). In addition, Nup93 and to a smaller extent Nup205, members of the Nup93-188-205 complex, also bound the POM121 beads (Figure 6B, lane 3 and 5). None of the above proteins bound to GFP negative control beads (Figure 6B, lane 2 and 4).

We conclude that the Nup107-160 complex and the Nup93-188-205 complex, two key subunits of the nuclear pore's central scaffold (Krull *et al.*, 2004), bind to POM121 in vitro. Because these two soluble pore subunits do not strongly interact with one another in *Xenopus* egg extracts, even in the presence of RanGTP (Vasu *et al.*, 2001 and Figure 5B), they may bind to POM121 aa 144-435 independently. Notably, the very strong and specific interaction of the Nup107-160 complex with POM121 implies that the recruitment of POM121 could be the next step in pore assembly after recruitment of ELYS and the Nup107-160 complex to chromatin.

ELYS and the Nup107-160 Complex Recruit POM121- and NDC1-containing Membrane Vesicles

We wanted to determine whether the recruitment of POM121-containing membrane vesicles is dependent on chromatin-bound ELYS/Nup107-160 or is found in nuclear membranes independent of ELYS. To test this, we used the tools developed to produce ELYS-minus nuclei. Specifically, we assembled nuclei in the presence or absence of ELYS GST-AT-hook+ or in the presence or absence of Distamycin A, and assayed for POM121. When nuclei were assembled in the presence of GST or GST-AT-hook-2R \rightarrow A, anti-POM121 antibodies stained the nuclear rims in a normal punctate manner (Figure 7A). In contrast, when nuclei were assembled in the presence of GST-AT-hook+, no POM121 stain could be visualized (Figure 7A, middle panel). This indicates that either POM121 was not recruited to ELYS-minus nuclei or that it was recruited but could not oligomerize into a detectable entity in the absence of ELYS. When nuclei were assembled in the presence of 10 μ M Distamycin, again POM121 antibodies failed to stain the nuclear membranes (Figure 7B). Thus, like the ELYS GST-AT-hook+ fragment, Distamycin A prevents either the recruitment of POM121-containing membrane vesicles to nuclei or the assembly of POM121 into visible protein oligomers.

A recent study showed that POM121 and NDC1 are co-enriched in the same set of membrane vesicles in *Xenopus* egg extracts, but are separate from the membrane vesicles that contain gp210 (Antonin *et al.*, 2005; Mansfeld *et al.*, 2006). NDC1 is the only integral membrane pore protein conserved in both yeast and higher eukaryotes and has been shown to be essential for nuclear pore assembly (Chial *et al.*, 1998; West *et al.*, 1998; Lau *et al.*, 2004; Lau *et al.*, 2006; Madrid *et al.*, 2006; Mansfeld *et al.*, 2006; Stavru *et al.*, 2006).

To determine whether the recruitment of NDC1 was dependent on chromatin-bound ELYS, ELYS-minus anchored nuclei were tested for the presence of NDC1 by immunoblotting. This approach has the advantage over immunofluorescence of allowing one to determine the presence of a pore membrane protein in nuclei, even if the protein is not oligomerized. Anchored nuclei were assembled in the presence of GST, GST-AT-hook+ or GST- Δ AT-hook and assayed for the presence of NDC1 and gp210 by immunoblot analysis (Figure 7C). We found that nuclei assembled in the presence of GST and ELYS Δ AT-hook contained all the nucleoporins tested, including ELYS, Nup160, Nup133,

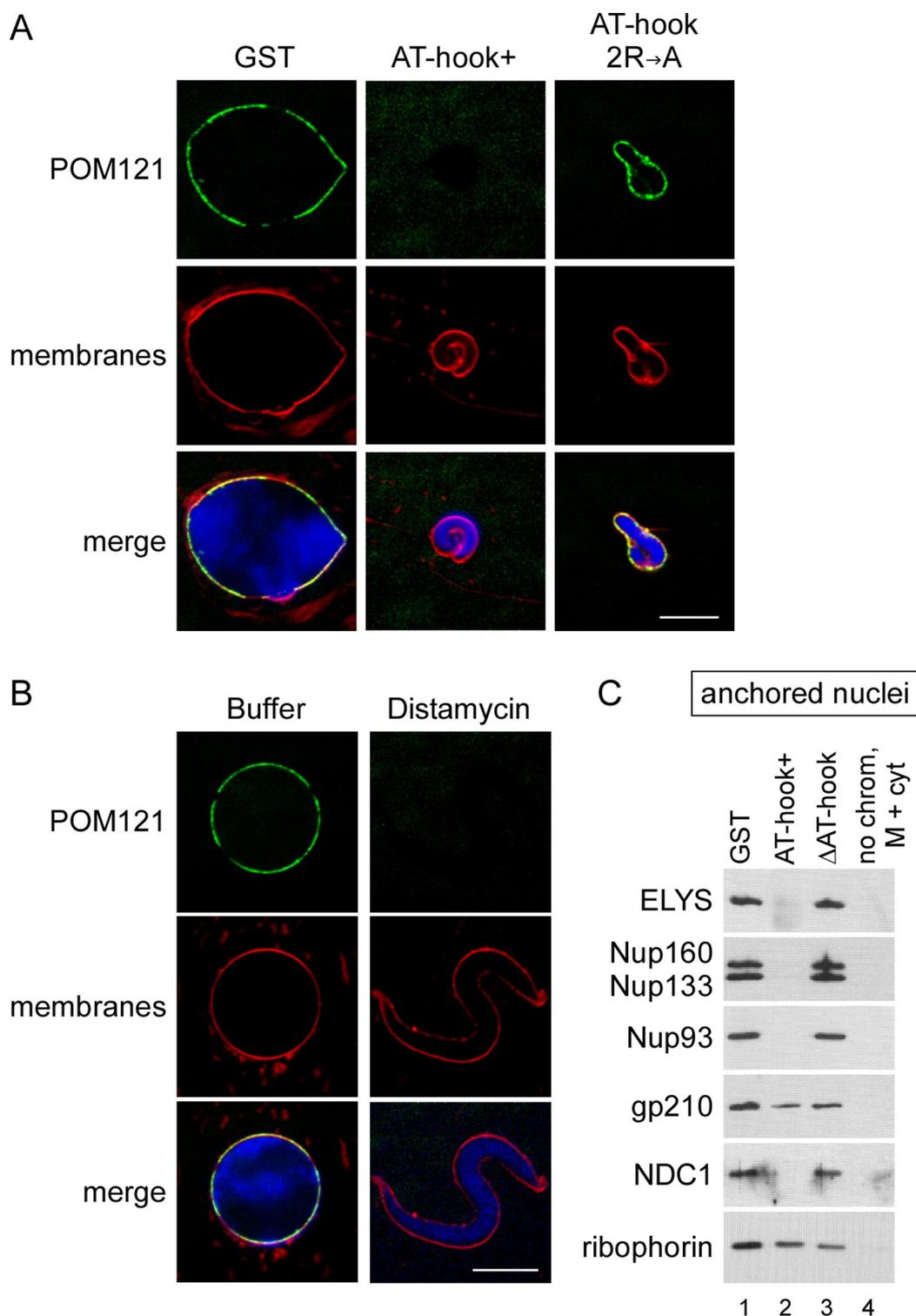


Figure 7. Chromatin-bound ELYS/Nup107-160 complex recruit POM121- and NDC1-containing membrane vesicles. (A) Reconstituted nuclei were assembled in the presence of 15 μ M GST, GST-AT-hook+, or GST-AT-hook-2R→A. The presence of POM121 in the nuclear membranes was probed for with directly labeled anti-POM121-AF488 (green). Membranes were stained with R18 membrane dye (red). DNA was stained with Hoechst (blue). Nuclei were fixed before mounting onto slides. Scale bar, 10 μ m. (B) Reconstituted nuclei were assembled in the presence of buffer or 10 μ M Distamycin A and processed as in A. Scale bar, 10 μ m. (C) Anchored nuclei were assembled by incubating chromatin-coated coverslips with membranes and cytosol for 1 h, in the presence of either 15 μ M GST, GST-AT-hook+, or GST- Δ AT-hook. A coverslip not treated with chromatin, but incubated with membranes, M, and cytosol, cyt, was used as a control for nonspecific binding (lane 4). The recruitment of integral membrane pore proteins NDC1 and gp210, in the presence (GST and GST- Δ AT-hook, lane 1 and 3) or absence (GST-AT-hook+, lane 2) of chromatin-bound ELYS/Nup107-160 complex, was determined by immunoblot analysis.

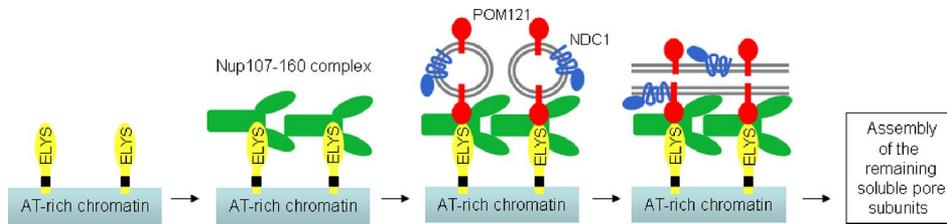


Figure 8. A model for the early steps in NPC assembly. On the basis of our findings, we propose a model for the early steps in NPC assembly. The first step in pore assembly is the binding of ELYS (yellow) to AT-rich chromatin sites, selected via the high-affinity AT-hook motif (black box) and strengthened by its second chromatin binding domain. ELYS then acts as a bridge between the chroma-

tin and the Nup107-160 complex (green; Rasala *et al.*, 2006; Franz *et al.*, 2007; Gillespie *et al.*, 2007). Chromatin-bound ELYS/Nup107-160 complex actively recruits the integral membrane pore proteins POM121 (red) and NDC1 (blue), likely through interaction with the cytosolic domain of POM121. After membrane vesicle fusion, the rest of the soluble pore subunits assemble and a complete nuclear pore is formed. (Note, other vesicles/sheets bind to the chromatin via different linkages such as lamins to form the bulk of the nuclear membranes, but are not shown here.)

Nup93, and the pore integral membrane proteins gp210 and NDC1 (Figure 7C, lanes 1 and 3), as expected from the normal nuclear phenotype observed after addition of these proteins in Figure 3B. However, anchored nuclei assembled in the presence of the AT-hook+ fragment lacked not only ELYS, Nup160, Nup133, and Nup93, but also lacked NDC1 (Figure 7C, lane 2). In contrast, these nuclei did contain gp210 and the ER/nuclear membrane protein ribophorin (Figure 7C, lane 2). We thus conclude that it is the recruitment of POM121/NDC1-containing membrane vesicles to nuclei that requires ELYS and the Nup107-160 complex to be present on chromatin, whereas the recruitment of gp210-containing vesicles does not.

DISCUSSION

The chromatin-binding protein ELYS targets nuclear pore assembly to the surface of chromosomes as nuclei form at the end of mitosis. In this study we sought the molecular underpinnings of the action of ELYS in the early steps of nuclear pore assembly using an *in vitro* system. Emphasizing that ELYS is the chromatin-targeting marker for nuclear pore assembly, we show that ELYS is not needed for AL pore formation (Figure 1). We found that the C-terminus of ELYS contains two chromatin-binding domains, an AT-hook motif, and a downstream chromatin-binding domain (Figures 2 and 3, and data not shown). Excess wild-type ELYS AT-hook+ fragment blocks nuclear pore assembly, by blocking the recruitment of ELYS and the Nup107-160 complex, whereas a mutant in the AT-hook motif of this fragment does not (Figure 3). Distamycin A, an AT-rich DNA-binding antibiotic, phenocopies this inhibition of pore assembly, whereas Chromomycin A₃, a GC-rich binding antibiotic, does not (Figures 4 and 7). Although endogenous ELYS and the Nup107-160 complex strongly bind to chromatin in the absence of membranes, the remainder of the 14 soluble subunits of the pore, with the possible exception of Nup153, requires membrane recruitment and fusion to associate with chromatin-initiated nuclear intermediates (Figure 5). By biochemical means, POM121 was found to bind the Nup107-160 complex, arguing that POM121 membrane recruitment is the step that follows ELYS/Nup107-160 chromatin binding (Figure 6). Importantly, the inhibition of ELYS chromatin binding also inhibits the recruitment of POM121 and NDC1 integral membrane proteins to forming nuclei (Figure 7).

A Model for the Early Steps in Nuclear Pore Assembly

The data above support a model for the early steps in nuclear pore assembly where the binding of ELYS to AT-rich

chromatin via the conserved AT-hook motif and an adjacent non-AT-hook domain marks the sites of nuclear pore assembly (Figure 8). The binding of ELYS to AT-rich DNA tracts “seeds” the chromatin with pore initiation sites. Chromatin-bound ELYS recruits the Nup107-160 complex to nuclear pore initiation sites (Franz *et al.*, 2007; Gillespie *et al.*, 2007). The recruitment of pore membrane components NDC1 and POM121 then occurs via an interaction between the Nup107-160 complex and the cytoplasmic domain of POM121. Assembly of the remaining soluble pore subunits would then follow.

ELYS/Nup107-160 Recruit Key Pore Membrane Proteins POM121 and NDC1 to the Nuclear Envelope

Targeting of pore membrane proteins POM121 and NDC1 to ELYS-marked sites *in vitro* involves recruitment of a specific pool of vesicles (Figure 7). POM121 and NDC1 are known to be coenriched in the same membrane vesicles in *Xenopus* egg extracts, while gp210 is contained in separate vesicles (Antonin *et al.*, 2005; Mansfeld *et al.*, 2006). The *in vitro* system used here allowed us to separate the binding of POM121/NDC1-containing membranes from the independent binding of membranes involved in more general nuclear membrane assembly, which apparently includes gp210. Extrapolating from our data to *in vivo* conditions, one must consider that nuclear membrane assembly involves the binding of ER membrane tubules that contain a mixture of ER and nuclear membrane proteins, including the integral membrane pore proteins. Potentially, chromatin-bound ELYS/Nup107-160 complex could, *in vivo*, attract POM121 and NDC1 proteins, as they diffuse laterally in the forming inner nuclear membrane to place at least one copy directly above the pore-seeding points on chromatin.

Previous studies have indeed hypothesized a connection between the Nup107-160 complex, or its yeast homologue the Nup84 complex, and membranes (Heath *et al.*, 1995; Li *et al.*, 1995). Sec13 is a member of both the Nup107-160 complex and COPII vesicle-transport complexes (Siniosoglou *et al.*, 1996; Fontoura *et al.*, 1999; Harel *et al.*, 2003b; Bickford *et al.*, 2004; Loiodice *et al.*, 2004). Other members of the Nup84 complex also show similarity to proteins of the vesicular-transport complexes COPI, COPII, and clathrin (Devos *et al.*, 2004). Indeed, the Nup107-160 complex member, Nup133, contains a membrane-curvature-sensing motif with the ability to bind to liposomes (Drin *et al.*, 2007). Lastly, the latest structural model of the yeast NPC places the Nup84 complex adjacent to the nuclear membranes (Alber *et al.*, 2007; Hsia *et al.*, 2007). All these are consistent with our finding of key biochemical and functional interactions between the Nup107-160 complex and POM121 and NDC1.

Yeast Lack a Direct ELYS Homologue

As essential as ELYS appears to be in metazoans, the yeasts *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* lack a large canonical ELYS homologue. However, a small gene in *S. pombe* encoding 295 aa bears considerable homology to aa 694–972 in the large human ELYS protein (2266 aa). We find no readily apparent *S. cerevisiae* homologue to either ELYS or the small *S. pombe* gene. Lacking the majority of ELYS structure, nonmetazoans such as yeast may require a different initiation device for pore assembly in their intact nuclei. Considering that yeast do not undergo open mitosis and thus do not disassemble their NPCs, it is possible that a targeting protein such as ELYS is not required. However, if yeast nuclear pores are indeed initiated from chromatin sites, then a prediction from the ELYS precedent would be that any mechanism that acts to anchor the yeast Nup84 complex to chromatin would be sufficient to initiate new pore assembly.

Nuclear Pores Are Initiated on AT-rich Chromatin

Two *Xenopus* ELYS isoforms have been published, one of 2201 aa (Galy *et al.*, 2006) and one of 2408 aa (Gillespie *et al.*, 2007), the latter being that used here. Upstream of the AT-hook, however, we note that the larger isoform differs from other vertebrate homologues in that it contains seven copies of ~31-aa repeat, which we observe to contain sequences closely related to AT-hook motifs, as defined by Huth *et al.* (1997). *Xenopus*, with its rapid cell division in early development where cell division takes place every 30 min for the first 12 divisions (Newport and Kirschner, 1982), might potentially use these excess AT-hook-like sequences of ELYS to accomplish rapid nuclear pore assembly.

Our point mutation and antibiotic studies demonstrate that ELYS preferentially binds to and NPC assembly is preferentially initiated on AT-rich chromatin sites (Figures 3 and 4). The best studied of the AT-hook-containing proteins is the HMGA family of proteins, whose members function in gene transcription, DNA repair, and chromatin remodeling (Goodwin, 1998; Anand and Chada, 2000). Although first generally described to bind DNA at any run of 5–6 AT base pairs, current studies on the HMGA family show that these proteins likely bind to DNA with sequence specificity. HMGA2 was recently shown to bind to a 15-base pair consensus site: 5 AT-rich base pairs, 4–5 GC-rich base pairs, and then 5–6 AT-rich base pairs (Cui and Leng, 2007). It would be intriguing to determine whether ELYS binds to chromatin in a similar sequence-specific restricted manner.

A large body of evidence indicates that gene-poor/AT-rich regions of the genome are positioned at the nuclear periphery and gene-rich/GC-rich regions are positioned in the nuclear interior (Bernardi *et al.*, 1985; Saccone *et al.*, 1993) (Croft *et al.*, 1999; Saccone *et al.*, 2002; Zink *et al.*, 2004; Foster and Bridger, 2005). It is possible that the binding preference of ELYS might aid in the localization of AT-rich chromatin to the nuclear periphery. However, a subset of newly activated genes move to the nuclear periphery after transcriptional activation, both in yeast and in vertebrates and is thought to do so through interaction with nuclear pore proteins (Casolari *et al.*, 2005; Cabal *et al.*, 2006; Brown and Silver, 2007). This movement could involve ELYS in vertebrates or, alternatively, be an ELYS-independent pore localization event.

Nuclear Pore Assembly in S-Phase

Overall, the pore assembly model proposed here would require little alteration to function in the intact nuclear envelope of an S phase cell, where pore number doubles.

Import of ELYS and the Nup107-160 complex into the intact nucleus, followed by their sequential binding to chromatin, could again form a platform on AT-rich sites. POM121 and NDC1, moving laterally through the inner nuclear membrane, could anchor and initiate pore assembly at these platforms. Consistent with this, both nuclear import in general and a nuclear pool of the Nup107-160 complex have been shown to be required for pore assembly in intact nuclei (Walther *et al.*, 2003a; D'Angelo *et al.*, 2006).

One possible extrapolation of our data is that there might be specific sites in the genome capable of initiating nuclear pore assembly. If so, in S-phase the pore initiation sites would double as the DNA is replicated, which would control the doubling in pore number previously observed (Maul *et al.*, 1972). In order for this to occur, however, an intranuclear pool of soluble ELYS/MEL-28 must exist. Indeed, such a pool has already been described, both in HeLa cells (Rasala *et al.*, 2006) and in *C. elegans* (Galy *et al.*, 2006). This intriguing possibility awaits a more precise definition of what constitutes an ELYS-binding sequence on chromatin.

In a recent study, chromatin immunoprecipitation using an Mcm3 antibody led Gillespie *et al.* (2007) to conclude that ELYS and the replication licensing complex, Mcm2-7, are in proximity on chromatin fragments, although the actual size of the average chromatin fragment in that study was not stated (Gillespie *et al.*, 2007). Geminin, a protein that completely blocks Mcm2-7 loading onto chromatin, caused a delay in ELYS and nucleoporin incorporation into reconstituted nuclei. The authors proposed that the Mcm2-7 complex promotes ELYS binding to chromatin. In our study, we found that a GC-binding antibiotic more strongly affected Mcm3 binding to chromatin, whereas an AT-binding antibiotic more strongly inhibited ELYS binding to chromatin (Figure 4B). Further investigation will help to define the potential relationship between replication licensing and nuclear pore assembly.

Summary

In summary, the binding of ELYS to chromatin, minimally via its key AT-hook motif and adjacent second chromatin-binding domain, initiates nuclear pore assembly on AT-rich chromatin tracts. The subsequent recruitment of pore-specific membrane vesicles containing POM121 and NDC1 is dependent on chromatin-bound ELYS and its binding partner, the Nup107-160 complex. This recruitment precedes further pore assembly. Indeed, assembly of the bulk of the remaining soluble pore subunits is dependent on the presence of membranes and is sensitive to GTP γ S. This latter data also argue, in conjunction with the studies of Baur *et al.* (2007), that the majority of postmitotic nuclear pore assembly events occurs within a continuous double lipid bilayer.

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