UNIVERSITY OF CALIFORNIA, SAN DIEGO

Regulation of Assembly of the Vertebrate Nucleus

A dissertation submitted in partial satisfaction of the requirements
for the degree

Doctor of Philosophy in Biology

by

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2005
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This thesis is dedicated to

my grandparents,
Kai-Man and Lai-Fong Wong

and to

my parents,
John and Florence Chan

The heavens declare the glory of God;
the skies proclaim the work of His hands.
Day after day they pour forth speech;
night after night they display knowledge.

Psalm 19:1-2
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ABSTRACT OF THE DISSERTATION

Regulation of Assembly of the Vertebrate Nucleus

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The work in this thesis is divided into three independent chapters. The first chapter demonstrates the role of importin beta in regulating nuclear assembly. The second chapter furthers the understanding of importin beta’s regulatory role through the analysis of potential targets of importin beta in nuclear assembly. Finally, the third chapter identifies and investigates the function of Xenopus gp210, a transmembrane nuclear pore protein.

Toward the end of mitosis in higher eukaryotes, nuclear assembly involves the precise targeting of nucleoporins and membranes to the surface
of chromatin. This process encapsulates the genome in two nuclear membranes perforated with nuclear pore complexes, which allow transport through the nuclear envelope. Nuclear assembly is a complex process, and thus regulatory mechanisms must be in place. In studies reported here, importin beta was found to be a negative regulator of nuclear assembly. Specifically, it negatively regulates nuclear membrane fusion and, separately, the subsequent step of nuclear pore assembly. The small GTPase Ran was demonstrated to be an opposing positive regulator of importin beta in nuclear membrane fusion but, surprisingly, not in nuclear pore assembly.

To identify the regulatory targets of importin beta, proteins it interacted with were investigated. One potential target for importin beta in its block to nuclear membrane fusion is p97, a protein implicated in nuclear membrane fusion. The two proteins proved not to interact. Given the strong nature of the block to nuclear pore assembly by importin beta, it was hypothesized that importin beta would bind to and inhibit many of the nuclear pore subunit interactions involved in assembly. Instead, only a small subset of interactions between nucleoporins was perturbed. Thus, importin beta regulates the assembly of key distinct nucleoporin subunits, but may cooperate with other proteins to regulate complete nuclear pore assembly.
gp210 is a transmembrane nucleoporin, and as such, has been hypothesized to be involved in nuclear pore assembly. To understand the role of gp210 in the nuclear pore, gp210 was studied in the Xenopus egg extract nuclear reconstitution system. Xenopus gp210 was shown not to be involved in nuclear assembly, but may instead be involved in nuclear disassembly.
General Introduction

The nucleus of the cell serves to isolate genomic DNA from the cytoplasm. This allows for the tight regulation of both transcriptional machinery entering the nucleus and RNA exiting the nucleus for translation in the cytoplasm. The nuclear envelope consists of double membranes that surround the DNA. The outer nuclear membrane is contiguous with the ER membrane, thus the ER lumen is continuous with the nuclear envelope lumen. Underlying the double membrane of the nucleus is the nuclear lamina, a meshwork of proteins that lend structural support to the nuclear envelope. The only passageways through the nuclear envelope are via nuclear pore complexes (NPCs). In each cell, several thousand nuclear pore complexes in vertebrates (Maul, 1977), and approximately one hundred nuclear pore complexes in yeast (Winey et al., 1997), allow the active transport of proteins and RNA between the nucleus and cytoplasm.

The bulk of the nuclear pore complex is embedded within the nuclear envelope, and it shows asymmetry as its structure extends into the cytoplasm and nucleoplasm (Figure 1). Eight filaments ~50 nm long extend into the cytoplasm, while at the other end, eight ~100 nm filaments emanate from the
core of the NPC, connecting to a ring structure ~30-50 nm in diameter, to form a basket structure extending into the nucleoplasm. When viewed en face, the nuclear pore complex shows 8-fold symmetry (Akey and Radermacher, 1993; Hinshaw et al., 1992). Each nuclear pore complex, in yeast and vertebrates, is made up of ~30 distinct nuclear pore proteins, termed nucleoporins or Nups. These are present in multiples of 8 copies (8-48 copies) to form a structure with an estimated mass between 60 to 125MDa (Cronshaw et al., 2002; Reichelt et al., 1990) in vertebrates, and between 50 to 70 MDa in yeast (Rout et al., 2000; Rout and Blobel, 1993; Yang et al., 1998). Among the 30 nucleoporins that have been identified in vertebrates, only two are transmembrane proteins: gp210 and POM121, while the rest are soluble proteins. This places the transmembrane nucleoporins in a position to integrate the soluble nucleoporins into a membrane-associated protein structure.

The control of traffic between the nucleus and cytoplasm is one of the major functions of the nuclear pore complex. The importin beta family of transport receptors is involved in carrying proteins and/or RNA in and out of the nuclear pore complex. All members of the family contain a binding domain for Ran, a small GTPase. Importin beta was the earliest identified receptor in the family and functions in nuclear import (Gorlich et al., 1994). In
the classical nuclear import pathway that importin beta participates in, importin beta binds its coreceptor, importin alpha. Importin alpha is capable of simultaneously binding to an NLS- (nuclear localization sequence) containing protein (Adam and Adam, 1994; Gorlich et al., 1994; Radu et al., 1995), although there are exceptions where importin beta acts without importin alpha (Gorlich and Kutay, 1999). This trimeric protein complex consisting of importin beta/importin alpha/NLS-containing protein, is termed an import complex, and makes its way through the nuclear pore complex. Importin beta is capable of interacting with phenylalanine-glycine repeat containing nucleoporins (FG nucleoporins) and a non-FG repeat protein peripherally associated with the nuclear pore, Tpr (Ben-Efraim and Gerace, 2001; Pyhtila and Rexach, 2003; Shah et al., 1998). In vertebrates, the FG nucleoporins are Nup50, Nup62, Nup98, Nup153, Nup214 and Nup358. It is still highly debated whether nuclear import occurs because of the increase in strength of direct interactions between importin beta and nucleoporins as the import complex traverses the NPC from the cytoplasmic to the nucleoplasmic side. Alternate models suggest that the FG nucleoporins in the NPC act collectively as a hydrophobic selective sieve or an entropic barrier, which transport receptors are able to cross but excludes proteins larger than 40kD
(Denning et al., 2003; Macara, 2001; Ribbeck and Gorlich, 2001; Ribbeck and Gorlich, 2002; Rout et al., 2000). Upon entering the nucleus, the import complex is surrounded by a high concentration of the small GTPase, Ran, in the GTP-bound state. The Ran GTP-exchange factor (RanGEF), RCC1, is bound to chromatin and maintains the high RanGTP level in the nucleus. A low level of RanGTP is maintained in the cytoplasm through the conversion of RanGTP to RanGDP by the cytoplasmic Ran GTPase activating protein (RanGAP). In the nucleus, importin beta binds RanGTP, inducing a conformational change in importin beta which causes the release of importin alpha and the NLS-containing protein (Lee et al., 2005). This frees the NLS-containing protein for function in the nucleus. Importin beta and importin alpha then recycle back to the cytoplasm for another round of import. This scenario illustrates how the binding affinity of importin beta for other proteins changes when RanGTP binds, and how importin beta and RanGTP act together as a molecular switch to ensure the directionality of nuclear import.

As a cell enters mitosis to divide into two daughter cells in vertebrates, the original nuclear envelope completely disassembles. The protein constituents of the nuclear pore complexes and the nuclear lamina dissociate into monomers or subcomplexes. The membranes of the nuclear envelope appear
to both retract into the ER and vesiculate (Lenart et al., 2003; Waterman-Storer et al., 1993; Yang et al., 1997). The absence of a nuclear envelope allows the mitotic spindle to tow the once sheltered chromosomes to the two ends of the dividing cell.

Towards the end of mitosis at late anaphase when the chromosomes have separated, the components of the nuclear envelope and nuclear pore complexes start to reassemble on the surface of chromosomes (Bodoor et al., 1999; Yang et al., 1997). A Xenopus in vitro nuclear reconstitution system has allowed the steps in nuclear assembly to be determined by the ordering of nuclear intermediates. In a study by Macaulay and Forbes, was shown that nuclear membranes need to be recruited to the chromatin surface and at least partially fused into double membrane patches, before nucleoporins can be incorporated into nuclear pores (Macaulay and Forbes, 1996). In mitotic tissue culture cells, nucleoporins have been observed to relocate to the nuclear envelope in a sequential manner (Bodoor et al., 1999). By the time the daughter cells are separated, the two newly reassembled nuclei are enclosed by the nuclear envelope, completely perforated with nuclear pore complexes functional for nuclear transport. In mammalian cells, the process of mitosis, from the disassembly of the nucleus to the reformation of two daughter nuclei,
occurs in less than one hour. The intricate process of nuclear assembly thus involves the coming together of membranes and soluble protein components on chromatin in a very narrow time frame.

Positive regulation of nuclear assembly would encourage nuclear membrane fusion and nuclear pore assembly at the surface of chromatin at the end of anaphase. This would need to be countered by negative regulation that prevents nuclear membrane fusion or nuclear pore assembly from occurring at locations other than the chromatin surface, or before chromatin is completely separated to the two ends of the cell. Taken together, tight regulatory processes must be in place to control the spatial and temporal aspects of nuclear assembly reassembly.

Nuclear assembly has been effectively studied using the Xenopus egg extract nuclear reconstitution system. Xenopus eggs are arrested in metaphase of meiosis II, where the nucleus is disassembled and poised to reassemble upon fertilization. Xenopus eggs are thus replete with disassembled nuclear pore components: both soluble nucleoporins dispersed in the cytoplasm and nuclear membranes mixed with other cytoplasmic membranes in sheets and/or vesicles. Once lysed, Xenopus eggs can be fractionated to isolate a cytosolic fraction containing soluble nucleoporins and
other cytoplasmic proteins, and a membrane fraction containing nuclear membrane, ER, and Golgi membrane vesicles. The addition of these two fractions along with a source of DNA, typically sperm chromatin, and a regenerating energy mix allows for the formation of nuclei in the test tube within an hour. These nuclei are functional for nuclear import and DNA replication. This system allows for the addition of chemical inhibitors or dominant negative proteins to assess the effect on nuclear assembly. In addition, the process of nuclear assembly can be visualized without the complications of effects on other cellular processes or cell viability.

The mechanisms for regulating the complex process of nuclear assembly were not known. Nuclear assembly occurs at the chromatin surface at the end of mitosis. What mechanisms exist that are delineated specifically by chromatin? The gradient of RanGTP emanates from the chromatin, where the RanGEF RCC1 resides. This allows for the well established mechanism of nuclear import, whereby importin beta is modulated by RanGTP in the vicinity of DNA. It became clear that this mechanism comes into play in the cell during mitosis as well, when importin beta and RanGTP were found to regulate the formation of the mitotic spindle, by a Ran-dependent release of spindle assembly factors from importin beta only in the vicinity of chromatin
(Gruss et al., 2001; Kalab et al., 1999; Nachury et al., 2001; Wiese et al., 2001). Nuclear assembly occurs on the surface of chromatin, so it was of interest to determine whether importin beta regulates nuclear assembly. In collaboration with Dr. Amnon Harel, I investigated the role of importin beta in nuclear assembly. Our collaborators at the Weizmann Institute of Science, Dr. Michael Elbaum and members of his laboratory, Dr. Ella Zimmerman and Aurelie Lachish-Zalait, visualized nuclear intermediates described in that study with scanning electron microscopy. The results of that study, presented in Chapter 1, show that importin beta negatively regulates nuclear assembly (Harel et al., 2003). The careful dissection of the process of nuclear assembly showed that importin beta inhibits nuclear membrane fusion in a RanGTP-dependent manner, whereas the block to nuclear pore assembly by importin beta is not modulated by RanGTP.

How then does importin beta inhibit nuclear assembly? p97, a AAA-ATPase, was shown to required for nuclear membrane fusion (Hetzer et al., 2001). The role of importin beta in nuclear membrane fusion was found not to be involved in any direct interaction with p97. In a search for targets of importin beta’s block of NPC assembly, importin beta was found to bind a subset of nucleoporins, namely Tpr, the FG nucleoporins, and the Nup107-160
pore subcomplex. A closer look at the effect of importin beta binding to these nucleoporins revealed that importin beta inhibited the Ran-induced interaction between Nup153 and the Nup107-160 pore subcomplex, as well as that between Nup153 and Nup62 pore subcomplexes. These results and their implications are presented in Chapter 2.

The two vertebrate transmembrane nucleoporins, gp210 and POM121, are integral parts of the nuclear pore complex. Since gp210’s discovery, it has been hypothesized that gp210 plays a role in nuclear assembly or anchoring of the nuclear pore in the membrane, due to the unique structure of gp210 and its permanent location in the membranes. In order to clarify the role of the transmembrane protein gp210 in the nuclear pore complex, I investigated Xenopus gp210 within the Xenopus egg extract system. My findings on gp210 constitute Chapter 3.

Collectively, the work presented in this thesis provides insight into the complex process of nuclear assembly, from the involvement and mechanism of importin beta in negatively regulating nuclear assembly to the determination that gp210 is not a key player in nuclear assembly. These findings have advanced the understanding of the mechanism by which importin beta regulates nuclear assembly and clarified the role of gp210.
Figure 1. Schematic of the nuclear envelope: the nuclear pore complex, nuclear lamina and nuclear membranes.

A cross section through the nuclear envelope is represented here.

Two membrane bilayers envelope the genome: the outer nuclear membrane (ONM) and the inner nuclear membrane (INM). The outer nuclear membrane is continuous with the ER, thus the nuclear envelope lumen is contiguous with the ER lumen. The nuclear lamina meshwork lines the nucleoplasmic side of the inner nuclear membrane, thus lending structural integrity to the nuclear envelope.

Nuclear pore complexes are present at the junctions where the outer nuclear membrane and inner nuclear membrane meet. The nuclear pore complex is composed of cytoplasmic filaments extending into the cytoplasm, while the nuclear basket reaches into the nucleoplasm. The bulk of the nuclear pore complex is in the central region where a putative central transporter resides, but it is not well defined (black lines in the middle of the gray pore). One nuclear pore complex is shown here, but each vertebrate nucleus contains thousands of nuclear pore complexes embedded within the nuclear envelope.
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Chapter 1

Importin β Negatively Regulates Nuclear Membrane Fusion and Nuclear Pore Complex Assembly

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Assembly of a eukaryotic nucleus involves three distinct events: membrane recruitment, fusion to form a double nuclear membrane, and nuclear pore complex (NPC) assembly. We report that importin β negatively regulates two of these events, membrane fusion and NPC assembly. When excess importin β is added to a full Xenopus nuclear reconstitution reaction, vesicles are recruited to chromatin but their fusion is blocked. The importin β down-regulation of membrane fusion is Ran-GTP reversible. Indeed, excess RanGTP (RanQ69L) alone stimulates excessive membrane fusion, leading to intranuclear membrane tubules and cytoplasmic annulate lamellae-like structures. We propose that a precise balance of importin β to Ran is required to create a correct double nuclear membrane and simultaneously to repress undesirable fusion events. Interestingly, truncated importin β 45–462 allows membrane fusion but produces nuclei lacking any NPCs. This reveals distinct importin β-regulation of NPC assembly. Excess full-length importin β and β 45–462 act similarly when added to transfused nuclear intermediates, i.e., both block NPC assembly. The importin β NPC block, which maps downstream of GTPyS and BAFT-A-sensitive steps in NPC assembly, is reversible by cytosol. Remarkably, it is not reversible by 25 μM RanGTP, a concentration that easily reverses fusion inhibition. This report, using a full reconstitution system and natural chromatin substrates, significantly expands the repertoire of importin β. Its roles now encompass negative regulation of two of the major events of nuclear assembly: membrane fusion and NPC assembly.

INTRODUCTION

In cells from yeast to mammals, importin α and β act together to ferry classical nuclear localization signal (NLS)-bearing proteins into the nucleus (Gorlich and Kutay, 1999; Stoffler et al., 1999; Darnell and Silver, 2000; Rout et al., 2000; Conti and Lazarradé, 2001; Vasu and Forbes, 2001; Darnell et al., 2002; Weis, 2003). Once in the nucleus the small GT-Pase Ran binds to importin β, displacing importin α and the NLS cargo, thus completing import. In the nucleus, Ran is kept in a GTPyS state by the constant action of its chromatin-bound Ran-GAP, RCC1 (Melchior and Gerace, 1998; Macara, 2001; Dasse, 2002; Kalab et al., 2002; Schwoebel et al., 2002; Steggerda and Fasch, 2002). In contrast, RanGEF is the predominant form found in the cytoplasm due to the cytoplasmic localization of RanGAP.

The horizons for importin α and β were unexpectedly broadened when they were found to play a very different role at mitosis. In metazoans, importin α and β are released to the cytosol by nuclear breakdown, where they act to inhibit proteins essential for mitotic spindle assembly. However, the inhibition of spindle assembly is reversed by Ran in the vicinity of mitotic chromosomes, where RanGTP continues to be produced by chromatin-bound RCC1 (Kalab et al., 1999; Gruss et al., 2001; Nachury et al., 2001; Wiess et al., 2001).

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Dasse, 2002). Thus, a spindle forms only around the mitotic (ER) chromosomes and not elsewhere in the cytoplasm.

At the end of mitosis, in vivo membranes are recruited to the chromatin, likely in the form of endoplasmic reticulum (ER) sheets, and fuse side to side to encompass the DNA with an intact double nuclear membrane (Ellenberg et al., 1997; Yang et al., 1997). It has been shown in vitro that nuclear assembly spontaneously in Xenopus egg extracts: a double nuclear membrane with nuclear pores forms around added chromatin, whether natural sperm chromatin substrate or exogenously added prokaryotic DNA is used (Forbes et al., 1983; Lohka and Masui, 1984; Blow and Laskey, 1986; Newport, 1987; Bauer et al., 1994; Powears et al., 1995; Ullman and Forbes, 1995; Macaulay and Forbes, 1996; Goldberg et al., 1997; Hetzer et al., 2001). In the in vitro situation, nuclear membrane/endoplasmic reticulum-derived vesicles are the source of the future nuclear membrane. Interestingly, the assembly of the nuclear membranes in Xenopus extracts is promoted by RanGTP (Zhang and Clarke, 2000; Hetzer et al., 2000). In this robust in vitro system, even Sepharose beads coated with DNA or Ran recruit membrane vesicles and form nuclear membranes with functional nuclear pore complexes (NPCs) (Heald et al., 1996; Zhang and Clarke, 2000). The subsequent finding that importin β-coated beads could form a nuclear envelope led to the suggestion that importin β positively regulates nuclear assembly in vivo by recruiting membrane vesicles to chromatin through its ability to bind RanGTP on the chromatin and unknown FG nucleoporins (nucleoporins possessing FG repeats; FG Nups) on membranes (Zhang, 2002; for review, see Dasse, 2002).
Although the way in which this would be accomplished is unclear, since importin β in vivo binds either RanGTP or FG nucleoporins, but not both (Bayliss et al., 2000a,b; Ben-Efraim and Gerace, 2001; Allen et al., 2002), the study raised the interesting possibility that importin β could be involved in regulating nuclear assembly.

In vivo experiments involving mutant importin β in Drosophila embryos or interference RNA-targeted destruction of importin α, β, and Ran in Caenorhabditis elegans embryos have also suggested that these proteins could act at some stage in nuclear envelope assembly. Although the multilamination in vivo phenotypes complete the interpretation (Askjaer et al., 2002; Celio et al., 2002; Timms et al., 2002).

Herein, we have determined, using natural chromatin substrate and individual interlamellar domains in nuclear assembly, that importin β negatively regulates 1) the fusion events required to form a double nuclear membrane, and 2) nuclear pore complex assembly into fused nuclear membranes.

MATERIALS AND METHODS

Xenopus egg extracts and the membrane vesicles and cytosolic fractions thereof were prepared as in Heald et al. (2003). Full-length human importin α, importin β, and Ran (5–462) (Kutyay et al., 1997) were expressed in bacteria; purified on Ni²⁺-NTA resin (Qiagen, Valencia, CA), dialyzed into 50mM glycylglycine, pH 7.4, containing 0.5mM EDTA, and concentrated using an Ultrafree microconcentrator (Millipore, Bedford, MA) before use. RanGTP in 50mM GTP was expressed, purified, and loaded with GTP essentially as described in Kutyay et al. (1997).

Nuclei were reconstituted by mixing X. laevis egg membrane vesicles and cytosolic fractions at a 1:3 ratio with an ATP-regenerating system and sperm chromatin (Macay and Forbes, 1996). Protein addition was generally kept to 10% (vol/vol), with the equivalent buffer (50mM glycylglycine, pH 7.4) added if buffer volume was required to be kept to a minimum. To accommodate 20nM importin β plus 20nM RanGTP, the total volume addition was raised to 90% (vol/vol) or 30% buffer.

For every expressed protein, the filtrates from its microconcentrator were tested in parallel and found to have no effect on nuclear assembly. Importins α had no deleterious effects when tested up to 30nM. Additional control proteins tested at 30–150nM included: calmodulin, bovine serum albumin, green fluorescent protein, glutathione S-transferase, GST-Tet repressor, t-His, NLS(4–23)-galactosidase, –His4-tat control fragment (Coon et al., 2001), bHsp90 and 1-235 (Huang et al., 2001), and Nsp1p as 125–255 (Huang et al., 2001). To assay for the presence of nuclear pores and nuclear import, directly labeled Oregon Green-mBlaM488 antibody (Corrie Corporation, CA; Molecular Probes, Eugene, OR) was added as in Heald et al. (2003), and BRET-NSA NLS-FHA transport substrate was added to reconstituted nuclei for 15 min after the start of assembly and fixed 30–60 min later in 3% paraformaldehyde. Nuclear membrane formation was assessed by the ability to exclude tetramethylrhodamine B isothiocyanate (TRITC)-555 kDa dextran or fluorescein isothiocyanate-15kDa dextran (Sigma-Aldrich, St. Louis, MO) after fixation and by continuous rinsing with the lipoprotic dye 5,5-dibromo-5-deoxyfluorescein isothiocyanate (BODIPY FL) (Dainoma, Kodak, Rochester, NY). Nuclei were visualized with an Axiovert 2 microscope (Carl Zeiss, Thornwood, NY).

Ordering of Nuclear Assembly Intermediates

Importin β 45–462 inhibited nuclear intermediates were formed by the addition of 35nM importin α 45–462 to a nuclear reconstitution reaction at t = 0.

After 60 min, the reaction containing importin β 45–462-inhibited nuclei was diluted 1:10 into X. laevis egg cytosol or egg cytosol containing 5nM TRITC fluorophore-labeled 555kDa dextran (BRET-NSA) (Molecular Probes, Eugene, OR) was added to assay for FG nucleoporin incorporation. BRET-NSA-inhibited, membrane-exposed nuclear intermediates were then observed by the addition of 5nM BAFTA at the start of a nuclear reconstitution reaction (Macay and Forbes, 1996). The BAFTA block was released by dilution of 1 volume of the nuclear intermediate reaction into nine volumes of a 1:1 mixture of cytosol and importin β 45–462, RanGTP, or buffer. After 45 min of incubation, nuclei were assayed for presence of nuclear pores with Oregon Green-mBlaM488.

Field Emission Scanning Electron Microscopy (FESEM)

Control and importin β 45–462-inhibited nuclei were observed for 40 min as described above and then prepared essentially as described in Wallsher et al. (2000) and references therein. Samples were critical point dried from ultradry CO₂ (CPD9 Critical Point Drier, Semetek, Southfield, MI), coated with 10–20Å Cr (EMTECH K375, Coors) and examined using a Philips field emission scanning electron microscope (XL30 SEM ESEM).

RESULTS

Importin β Negatively Regulates Nuclear Membrane Fusion

Importin β regulates nuclear import in interphase and spindle assembly in metaphase. To ask whether importin β might act as a global regulator of events throughout the cell cycle, we used a nuclear reassembly system derived from Xenopus egg extracts. In this system, robust assembly of functional nuclei occurs around added chromatin templates.

We reasoned that disturbing the balance of importin β in the extract could reveal the step(s) at which such regulation might take place. Reconstituted importin β was added to a reconstitution reaction consisting of cytosol, membrane vesicles, and natural chromatin substrate. Normally, importin β is present at ~3μM concentration in Xenopus cytosol (Kutyay et al., 1997). In control reactions (Figure 1, control) or reactions to which excess importin α (Figure 1, +Imp α, 15μM) or nine unrelated soluble proteins were added (our unpublished data; see MATERIALS AND METHODS), nuclei assembled normally. When recombinant importin β was added at 5μM, membrane vesicles were also recruited to chromatin and fused to form nuclear membranes (our unpublished data). When, however, importin β was added at 15–25μM, membrane vesicles were recruited to the chromatin in abundance, but failed to fuse (Figure 1, +Imp β). The chromatin surfaces showed a discontinuous stain with the membrane dye DHCC, characteristic of unfused vesicles. Indeed, excess importin β completely blocked nuclear membrane fusion in a manner indistinguishable from the chemical fusion inhibitors GTP-γ-S (a nonhydrolyzable analog of GTP) or NEM (N-ethyl maleimide) (Macay and Forbes, 1996; Hebert et al., 2000).

In previous studies, a truncated form of importin β (aa 45–462) acted as a powerful dominant negative inhibitor of nuclear transport (Kutyay et al., 1997). This form of importin β contains the central or nucleoporin-binding domain, but lacks the Ran- and importin α-binding domains (Figure 2A). When importin β 45–462 was added to the nuclear assembly reaction at t = 0, we found it had no effect on nuclear membrane fusion even at concentrations up to 40μM (Figure 2B). Complete nuclear membranes formed, as was clear from the sharp continuous DHCC fluorescent stain characteristic of fused nuclear membranes and from the ability to exclude both 150kDa and 180kDa dextrans (Figure 2B). Thus, excess full-length importin β blocks nuclear vesicle fusion, but the truncated β 45–462 form does not.

Importin β Acts on Membrane Fusion through Ran

The above-mentioned result implied that for full-length importin β, either its importin α-binding domain or its Ran-binding domain could be involved in the strong block to membrane fusion. Addition of increasing amounts of importin α did not, however, reverse importin β’s inhibitory effect (Figure 1, +Imp β +Imp α). We asked whether the fusion defect could be reversed by Ran. RanGFP, a mutant form unable to hydrolyze GTP, was used because Ran-GTP is the form that normally reverses importin β action within the cell (Kutyay et al., 1997; Macay, 2001; Dasso, 2002). When excess RanGFP (25μM, preloaded with GTP) was added together with full-length importin β (20μM), membrane fusion now
occurred (Figure 3). RanQ69L alone did not block membrane fusion at this high concentration (Figure 3). Indeed, RanQ69L stimulated excess membrane fusion in the form of intranuclear membrane tubules (Figure 3). These results argue that importin β negatively regulates nuclear membrane fusion. It seems to do so, not by interacting with nucleoporins, but because the importin β 45–462 form clearly does not block membrane fusion, but through an interplay with RanGTP itself.

**Importin β Negatively Regulates NPC Assembly**

We turned next to potential regulation of downstream assembly events. Addition of truncated importin β 45–462 does not prevent fusion to form a double nuclear membrane, but the resulting nuclei are small and defective for nuclear import (Figure 4). This could result from a simple inhibition of nuclear import through assembled pores, or from a more severe defect in the NPCs themselves. The exclusion of 18-kDa dextran by such nuclei (Figure 2B) implied the latter might indeed be the case. To test this, the presence of nuclear pore complexes was analyzed using Oregon Green mAb414 antibody, which recognizes FG nucleoporins (Wente et al., 1992). This antibody gave a strong punctate nuclear rim on control nuclei (Figure 4). Remarkably, no mAb414 signal was observed on importin β 45–462–treated nuclei (Figure 4), indicating no accessible FG nucleoporins are present. A full block of mAb414 staining was consistently seen when 5–10 μM importin β 45–462 was added. This suggested that importin β 45–462 causes a defect in NPC assembly itself.

Field emission scanning electron microscopy (FESEM) is a high-resolution and sensitive probe for the presence of nuclear pore complexes and their intermediates (Gedik et al., 1997; Wallter et al., 2002). FESEM was used to analyze

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**Figure 1.** Excess importin β blocks nuclear membrane fusion. Nuclear reconstitution reactions were set up and supplemented at t = 0 with buffer (control), importin β (Imp β), importin α (Imp α), or importin β plus importin α (Imp β + Imp α). Protein additions were at 15 μM. The formation of nuclear membranes was assessed at 1 h with the fluorescent membrane dye EHEC (green). Bar, 10 μm. The enlargements at the right are magnified 3×.

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**Figure 2.** Importin β 45–462 does not block nuclear membrane fusion. (A) Binding characteristics of importin β and importin β 45–462 (Kosznicki et al., 1997). (B) Importin β 45–462 does not block nuclear membrane assembly. Importin β 45–462 (β 45–462, 15 μM) or buffer (control) were added at t = 0 to nuclear reconstitution reactions. After 1 h, the integrity of the nuclear envelope was determined by assaying exclusion of TRITC-150-kDa dextran, fluorescein isothiocyanate-18-kDa dextran, and continuous EHEC stain of the nuclear membrane. Bar, 10 μm. The enlargements at right are magnified 3×.
importin β 45–462–arrested nuclei and indicated that these membrane-enclosed nuclei lack any external sign of NPC structure (Figure 3). Thus, importin β 45–462 blocks NPC assembly at or near its inception.

To test whether the NPC block is reversible, importin β 45–462–arrested nuclei were diluted into fresh cytosol for 45 min and then mixed with Oregon Green-labeled mAb414 to probe for the presence of NPCs. The nuclei were found to contain abundant NPCs, indicating reversal of the defect (Figure 6A). Rescue allowed us to attempt to order the importin β 45–462 block with respect to previously identified chemical inhibitors. Three early steps in nuclear envelope assembly were defined by chemical inhibitors (Romain et al., 1992; Macaulay and Forbes, 1996). GTPγS blocks at two points: at membrane-membrane vesicle fusion and at a very early step in NPC assembly (Romain et al., 1992; Macaulay and Forbes, 1996; Hetzer et al., 2000). BAPTA, a Ca2+ chelator, blocks slightly downstream from this second GTPγS-sensitive step of NPC assembly (Macaulay and Forbes, 1996). BAPTA allows membrane enclosure but prevents any connection between inner and outer nuclear membranes to form an NPC (Macaulay and Forbes, 1996). When importin β 45–462–arrested nuclei intermediates were diluted into cytosol plus GTPγS (our unpublished data) or cytosol plus BAPTA (Figure 6A), nuclear pores formed. This indicates

Figure 4. Importin β 45–462 blocks NPC assembly. Importin β 45–462 (5 μM) or buffer (control) were added at t = 0 to nuclear reconstitution reactions. Import was assayed with TRITC-SV40 NLS-FSA and FG nucleopores were detected with Oregon Green mAb414 antibody. Bar, 10 μm.

Figure 5. Importin β 45–462–arrested nuclei lack NPCs. Typical views of control nuclei and 10 μM importin β 45–462–arrested nuclei are shown, as visualized with PI and SAO. Smaller nuclear pore complexes are highlighted with arrowheads. Smaller single granules remaining on the nuclear surface in the lower panel are ribosomes (Goldberg et al., 1997). Inserts are 1.6× higher magnification. Bar, 200 nm.
that importin β 45–462 acts downstream from BPAPTA to regulate NPC assembly. For the converse experiment, BPAPTA-arrested pore-free nuclei were diluted 1:10 into cytosol plus recombinant importin β 45–462. The BPAPTA block was rescued by fresh cytosol, but no NPCs were formed when importin β 45–462 was included (Figure 6B). These experiments demonstrate a novel importin β 45–462-sensitive step in nuclear pore assembly.

The ability to form membrane-enclosed pore-free nuclear intermediates with BPAPTA allowed us to ask whether full-length importin β itself plays a regulatory role in NPC assembly. Low concentrations of importin β (5 μM) did not interfere with the ability of cytosol to rescue NPC assembly in BPAPTA-blocked nuclei (Figure 7A) However, higher concentrations of importin β (15–25 μM) completely blocked NPC assembly in these prefused nuclear intermediates (Figure 7A). We asked whether an excess of RanGTP could reverse importin β’s inhibitory action on NPC assembly. Most surprisingly, RanQ69L (25 μM) was completely unable to reverse the block (Figure 7B).

Together, our results indicate that importin β acts at two distinct steps in nuclear assembly. In light of this, we would predict that when excess importin β and RanQ69L are present in a reconstitution reaction from t = 0 (as in Figure 5), the block to membrane fusion would be relieved by RanQ69L, but the block to NPC assembly would not. Indeed, when we performed this experiment, the resulting nuclei contained fused membranes (Figure 5, + Imp β + RanQ69L) but did not stain with FG antibodies (Figure 8, + Imp β + RanQ69L), indicating no NPCs were formed. RanQ69L alone did not prevent NPC assembly (Figure 8, + RanQ69L). Interestingly, RanQ69L induced the assembly of additional structures outside the nucleus that stain with anti-FG antibody. Importin β suppressed the FG-staining extranuclear structures (Figure 8, + Imp β + RanQ69L), presumably the well-characterized cytoplasmic stacks of NPC-containing membranes, termed annulate lamellae (Dahawan et al., 1991; Miller and Forbes, 2000, and references therein). Overall, the data demonstrate that importin β blocks the insertion of NPCs into fused nuclear membranes and suggest that the reversal of this blocked step is different than the fusion step.

**DISCUSSION**

Nuclear assembly on natural chromatin substrates in vitro involves vesicle recruitment to the chromatin, vesicle-vesicle fusion, and NPC insertion into the fused nuclear membranes. We have demonstrated that importin β acts at two of these vital steps: nuclear membrane fusion and the subsequent assembly of nuclear pore complexes. Specifically, excess importin β blocks vesicle-vesicle fusion around natural chromatin substrates. This inhibitory action, like importin β’s inhibition of spindle assembly, is reversible by RanGTP. Importin β 45–462, unable to bind Ran, does not block membrane fusion, but interestingly, blocks downstream NPC assembly. This second block occurs near the insertion of NPC formation, as shown by FESEM, and results in nuclei lacking any visible NPC structures. Excess full-length importin β also blocks NPC assembly, if added to prefused nuclear intermediates. Remarkably, the full-length importin β block of NPC assembly is not reversible by 25 μM RanQ69L-GTP. The NPC block maps downstream from BPAPTA- and GTP-βS-sensitive steps in NPC assembly. Our data thus suggest a new model of stepwise nuclear assembly regulated at multiple points by importin β, as summarized in Figure 9.

One of the first visible steps in nuclear assembly in vitro is the recruitment of membrane vesicles to chromatin. A previous study observed nuclear envelope assembly on importin β-coated beads (Zhao et al., 2002). This led to the hypothesis that, in vivo, importin β might positively induce membrane recruitment through its ability to bind RanGTP on the chromatin and to immobilize FG nucleoporins in the membranes, either simultaneously or sequentially (Zhang et al., 2002; for review, see Dasso, 2002). A clear expectation of
this hypothesis would be that importin β 45–462, which cannot bind RanGTP, should block membrane recruitment. We found no evidence for inhibition of membrane recruitment to natural chromatin substrates by importin β 45–462; fully fused nuclear membranes formed in its presence. Thus, although importin β may be involved in membrane recruitment in some way, our experiments indicate it cannot be playing an exclusive role. Because nuclear lamins and LAPs (lamin-associated polypeptides) are known to mediate interaction between membranes and chromatin (Foissner and Geacoc, 1993; Drummond et al., 1999; Gant et al., 1999; Raharjo et al., 2001; Goldm et al., 2002), ample evidence for alternative mechanisms for membrane recruitment exist.

Our data strongly indicate that importin β acts to negatively regulate the vesicle-vesicle fusion step of nuclear assembly. Why would such regulation be required? Ran has previously been shown to promote membrane fusion, working through an unknown partner (Fu and Dasso, 1997; Hetzer et al., 2000; Zhang and Clarke, 2000, 2001). We show that importin β blocks membrane fusion, thus countering Ran in vivo, we would predict that it is the strict ratio of importin β to RanGTP that ensures fusion in the correct location and proportion. We hypothesize that importin β is needed in vivo to carefully regulate membrane fusion where RanGTP is high, such as at the surface of telophase chromosomes. Thus, fusion to form the surrounding nuclear membranes is desirable, but undesirable fusion would need to be repressed. Examples of undesirable fusion include fusion that is spatially undesirable such as the formation of intranuclear membrane tubules, and fusion that is proportionately undesirable such as excess growth of the outer nuclear membrane relative to the inner. Interestingly, we find that when high RanQ69L is added alone to an extract, it does indeed promote the appearance of excess intranuclear membranes and outer nuclear membrane bubbles (Figures 3 and 8; our unpublished data), indicating that the normal regulatory balance is disturbed toward fusion. Combining excess importin β and excess RanQ69L not only reverses the importin β block to fusion, but also reverses the RanQ69L stimulation of intranuclear membranes (Figure 5). In this case, correct nuclear membranes form, suggesting the required balance of importin β and RanGTP has been restored.

These conclusions were reinforced when the presence of nuclear pore complexes in nuclei was assessed with anti-FG antibody. When high RanQ69L was added, excess FG-staining NPCs were observed in the nuclear envelope, in intranuclear tubules, and extranuclearly in presumed amenucleate lamellae. No NPCs were seen if excess importin β was present alone with the Ran (Figure 8). Together, our data suggest that a balance of RanGTP to importin β is necessary not only to promote a correctly positioned nuclear envelope but also to repress intranuclear and cytoplasmic amnulate lamellae. Our experiments were conducted in a full nuclear assembly reaction, using abundant membranes and natural chromatin substrates, a situation that most closely approximates the in vivo situation. Other cases where individual proteins are immobilized on beads, or chromatin is incubated in limiting membranes, may not necessarily recapitulate the correct steps of nuclear assembly. For example, in previous experiments where membranes were very limiting or chemically modified, RanQ69L reportedly blocked membrane tu-
Figure 8. RanQ69L reverses the full-length importin β block to membrane fusion, but not the block to NPC assembly. Nuclear reconstitution reactions containing chromatin, cytosol, and membranes were supplemented at t = 0 with 50% volume buffer, full-length importin β (20 μM), full-length importin β (20 μM) plus RanQ69L (25 μM), or RanQ69L (25 μM) alone. The reactions containing importin β or importin β plus RanQ69L failed to incorporate FG nucleoporins as detected with Oregon Green-mAb414. FG nucleoporins incorporated into nuclear envelopes in reactions supplemented with buffer or RanQ69L. The RanQ69L reactions also showed FG-containing structures forming outside of the nucleus, presumed annulate lamellae. Additionally, the nuclei in the RanQ69L reactions contained areas of the nuclear envelope devoid of FG nucleoporin staining. These corresponded to outer nuclear membrane bubbles, which appear as gaps in the mAb414 stain (this figure) or as bubbles when DIFCC membrane dye is used (our unpublished data). Bar, 10 μm.

Figure 9. A model of novel importin β-regulated steps in nuclear membrane fusion and NPC assembly. Chromatin is depicted as a large black oblong; vesicles and membranes are shown by black lines enclosing green luminal contents. NPCs are depicted as black ovals spanning the inner and outer nuclear membranes. Importin β and the importin β-regulated steps observed in this study are shown in red.

Inhibition can be rescued by fresh cytosol; it is not reversible by RanQ69L (25 μM). This is in marked contrast to the membrane fusion step, which is Ran-reversible at 25 μM. Although the possibility remains that an unusually high RanQ69L concentration might be required to reverse NPC assembly inhibition, experiments where high importin β and high RanQ69L are added together at t = 0 show membrane fusion, but no trace of NPC formation (Figures 3 and 8). At the very least, this result indicates that we are not near the threshold of any potential Ran reversibility.

Importin β is known to bind to a subset of vertebrate FG nucleoporins: Nup358, Nup153, Nup62, as well as the NPC-associated protein Tpr. However, these interactions are all disrupted by high Ran-GTP or GMP-PNP (Shah et al., 1998; Bayliss et al., 2000; Ben-Efraim and Gerace, 2001; Conti and Iazzeppo, 2002; Allen et al., 2002). Because of the lack of importin β binding to NPCs and the absence of NPC assembly described above and the fact that none of the FG Nups are essential for NPC assembly (Finlay and Forbes, 1995; Meier et al., 1995; Walther et al., 2001, 2002), importin β cannot be blocking NPC assembly simply by sequestering free FG nucleoporins.

This suggests that importin β must instead regulate either an essential nucleoporin or unknown NPC assembly factor. An attractive target is the Nup107-160 complex, because nucleoporin purified in the absence of this complex lack nuclear pores (Hazel et al., 2003; Walther et al., 2003; see also Boeger et al., 2003). Other possibilities include gsp210, an integral membrane protein involved in pore assembly (Drummond and Wilson, 2003), and nucleoporins not yet characterized for roles in NPC assembly, such as Nup153 or the WD repeat Nups (Cromshaw et al., 2002). We are currently investigating these and other potential targets.

Importin β likely binds to many nucleoporins in the mature NPC during transport. It may be that importin β plays dual roles with respect to these nucleoporins, binding to them in interphase NPCs during transport, but in mitosis acting to keep the same Nups in a disassembled state. The story could also have additional layers, in the sense that not all Nups that are blocked by importin β need be regulated in the same manner. Certain Nups, such as the FG Nups, might be bound by importin β and prevented from assembly, but the binding could be easily reversible by Ran-GTP. Other Nups could bind importin β and be similarly blocked for NPC assembly, but whether require much more Ran-GTP, or require a completely different regulatory protein for reversal of their importin β block. There are multiple examples of Ran independence in nuclear transport, including the import of
In summary, we have shown using natural chromatin substrates that importin β negatively regulates two distinct steps in nuclear assembly: the vesicle-vesicle fusion required to form double nuclear membranes and the assembly of nuclear pore complexes into fused nuclear membranes.

Note added in proof. Following acceptance of this work, Wallace et al. (2003) reported related results on the NPC assembly step in a study entitled “Ran-GTP mediates nuclear pore complex assembly” (Nature 424, 689–694).

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Acknowledgements, Chapter 1

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Chapter 2

Targets of Importin Beta in Nuclear Membrane Fusion

and Nuclear Pore Complex Assembly

Abstract

Nuclear assembly is an endeavor that requires tight coordination to recruit soluble nucleoporins and nuclear membranes at the correct time and location to form functional nuclear pore complexes embedded in the nuclear envelope. Importin beta and RanGTP have been found to be regulators of nuclear assembly. Importin beta negatively regulates nuclear membrane fusion and nuclear pore assembly, while RanGTP reverses the effect of importin beta on nuclear membrane fusion only. Here, the targets of importin beta in nuclear membrane fusion and nuclear pore assembly are investigated in order to further understand the mechanism of regulation by importin beta. Importin beta binds to membranes in a Ran-sensitive manner, and may thus
block nuclear membrane fusion by inhibiting a protein involved in nuclear
membrane fusion. Here, p97, a protein implicated in such fusion, was
investigated for interaction with importin beta. p97 and importin beta did not
bind to one another, suggesting that they act in separate pathways to regulate
nuclear membrane fusion. To understand importin beta’s negative regulation
of nuclear pore assembly, the effect of importin beta on interactions between
nucleoporins was examined. Xenopus egg extracts contain large quantities of
disassembled nuclear pores where individual pore subunits can be assessed
for importin beta binding. First, a comprehensive analysis of nucleoporins
that bound to importin beta was undertaken. These interactions were probed
by immunoprecipitating importin beta and any associated pore proteins from
Xenopus egg extracts. Recombinant Xenopus importin beta was also used to
pulldown interacting pore proteins from egg extracts. After establishing
which nucleoporins bound importin beta, the effect of importin beta on
interactions within and between nucleoporin subcomplexes was investigated.
Importin beta did not disrupt the interactions within the Nup107-160 or
Nup62/Nup214 nucleoporin subcomplexes, thus interactions within these
nucleoporin subcomplexes are still maintained in the presence of importin
beta. Importantly, when interactions between nucleoporin subcomplexes were
analyzed, importin beta only abrogated the known Ran-induced interaction between Nup153 and the Nup107-160 complex and a newly discovered interaction between Nup153 and Nup62. Importin beta and RanQ69L did not regulate any other interactions tested here. Thus, importin beta does not act by regulating all nucleoporin interactions. Instead, it has been shown here to modulate a few quite specific interactions. Implications of this finding are discussed, along with models for the organization of nucleoporins within the nuclear pore complex.
Introduction

As a cell goes through the last stages of mitosis, considerable activity happens within to ensure the functionality of the two subsequent daughter cells. Parts that had been scattered throughout the cytoplasm to ensure even distribution between the two are now gathered in the correct location and in the right order to form the compartments and machinery of the cell. The extremely complex process of reassembling the cell is clearly illustrated in the assembly of one compartment of the cell, the nucleus, home to the genome and a multitude of supporting factors.

Nuclear Assembly: a family reunion

The reassembly of the nucleus at the end of mitosis involves the coming together of nuclear membranes and proteins of the nuclear pore complex and nuclear lamina. Dispersed membrane vesicles or sheets of membrane continuous with the endoplasmic reticulum arrive at chromatin to form the nuclear envelope, a unique membrane structure that consists of two membrane bilayers. The outer nuclear membrane (one bilayer) is continuous with the endoplasmic reticulum membrane. Thus, the ER lumen is continuous with the nuclear envelope lumen. The inner nuclear membrane (the other
bilayer), however, is distinct in it’s proximity to chromatin and in its resident transmembrane and peripheral proteins. The latter make up the nuclear lamina, the structural support of the nuclear envelope. The 125 megadalton vertebrate nuclear pore complex is composed of multiple copies of ∼30 different nuclear pore proteins, also termed nucleoporins (Nups, Figure 1A). The reassembly of the nuclear pore is a daunting task, as nucleoporins need to find their way to the correct location and assemble in the correct order (Bodoor et al., 1999). During mitosis, the majority of soluble nucleoporins are dispersed in the cytoplasm, while the two transmembrane nucleoporins are found dispersed on ER membranes (Daigle et al., 2001; Yang et al., 1997). Approximately 5-10% of subcomplexes such as the Nup107-160 complex and Nup358 are found at the kinetochores (Belgareh et al., 2001; Harel et al., 2003b; Salina et al., 2003). How do all the other nucleoporins assemble into functional pores within the double nuclear membranes?

**Nucleoporin subcomplexes: family units**

One phenomenon that enables the efficient assembly of the nuclear pore complex is that most nucleoporins are present in subcomplexes (Figure 1A). These subcomplexes result when the nuclear pore is disassembled at mitosis.
Many of these subcomplexes were identified in Xenopus egg extract. Xenopus egg extract is derived from eggs arrested in metaphase II of meiosis. The crushing and immediate separation of cytosol from membranes causes the extract to be poised for interphase, but no nuclear pores form because the protein and membrane components are separated. Thus, nucleoporins in Xenopus egg extract represent the state before incorporation into nuclear pores. The high concentration of proteins in Xenopus egg extract has enabled nucleoporin subcomplexes to be isolated with ease. Nucleoporin subcomplexes that have been isolated so far include the Nup62/Nup58/Nup54/Nup45 complex (Finlay et al., 1991; Hu et al., 1996; Kita et al., 1993), the Nup214/Nup88/Nup62 complex (Bastos et al., 1997; Fornerod et al., 1997; Macaulay et al., 1995), the Nup205/Nup93/Nup188 complex (Grandi et al., 1997; Miller et al., 2000), the Nup98/Gle2 complex (Blevins et al., 2003; Pritchard et al., 1999) and the Nup107-160 complex (Belgareh et al., 2001; Harel et al., 2003b; Vasu et al., 2001).

The packaging of nucleoporins into subcomplexes significantly decreases the complexity of subsequent nuclear pore assembly, but how are the interactions between and within subcomplexes regulated so that they
come together to form functional nuclear pores at the correct time and in the correct place and proportion?

There are several inter-nucleoporin subcomplex interactions that have been identified and can be studied in Xenopus egg extracts. The Nup107-160 complex binds to and can be isolated with recombinant fragments of Nup98 and Nup153 (Vasu et al., 2001), as well as with endogenous Nup153 (Walther et al., 2003b). The Nup205/Nup93/Nup188 complex can be isolated on a column containing wheat germ agglutinin-binding pore proteins, Nup214, Nup98 and Nup62 (Miller et al., 2000). In addition, the Nup205/Nup93 complex binds to Nup53 (Hawryluk-Gara et al., 2005). Thus, the Xenopus egg extract system provides a platform by which nucleoporin subcomplex interactions can be studied.

**Regulation of nuclear assembly: importin beta and RanGTP**

It was only until recently that importin beta and RanGTP were revealed to be regulatory protein factors of nuclear envelope and nuclear pore assembly (Figure 1B; Harel et al., 2003a; Walther et al., 2003). Importin beta was found to negatively regulate both nuclear envelope and nuclear pore assembly (Harel et al., 2003a; Walther et al., 2003b). Surprisingly, importin
beta’s block to nuclear membrane fusion was reversible by RanQ69L, though its block to nuclear pore assembly was not (Figure 1B; Harel et al., 2003a). The Ran-sensitive interactions involved in nuclear membrane fusion and Ran-insensitive interactions involved in nuclear pore assembly pointed to two different downstream effectors of importin beta. Conversely, RanGTP is a positive regulator of both nuclear envelope fusion and nuclear pore assembly. The addition of RanQ69L, a Ran mutant constitutively in the GTP state, to a nuclear reconstitution reaction caused increased membrane fusion, nuclear pore assembly, and the formation of annulate lamellae, cytoplasmic stores of nuclear pore complexes on stacks of membranes (Harel et al., 2003a; Walther et al., 2003b).

The positive and negative regulation of nuclear assembly is crucial for the formation of functional nuclei. The balance between regulators that repress and activate nuclear membrane fusion and nuclear pore assembly ensures the correct amount, location and order of nuclear formation. The lack of positive regulators of nuclear assembly would result in partially fused nuclear membranes that fail to separate nucleoplasmic and cytoplasmic processes of the cell. Conversely, the absence of negative regulators of nuclear assembly would cause uncontrolled nuclear membrane fusion and nuclear
pore assembly, clogging the cell with fused membrane structures that would prevent normal nuclear functions. Thus, the identification of importin beta and RanGTP as regulators of nuclear assembly is just at the iceberg of understanding how the complex process of nuclear assembly is regulated.

**The search for targets of importin beta in nuclear assembly**

I set out to search for the targets that importin beta interacts with in its regulation of nuclear membrane fusion and nuclear pore assembly. Not many proteins have been implicated in nuclear membrane fusion. Among those are the AAA ATPase, p97 along with its cofactors (p47 and Ufd1-Npl4), and a transmembrane nucleoporin, POM121, although the mechanisms by which they are involved remain unknown (Antonin et al., 2005; Hetzer et al., 2001). Could importin beta interact with a factor involved in nuclear membrane fusion, and thus regulate and block the fusion of the nuclear membrane in this manner? Here I address whether p97 is a target of importin beta in the Ran-sensitive block to nuclear membrane fusion.

As for importin beta’s inhibition of nuclear pore assembly, highly plausible targets for importin beta are the nucleoporins. Studies of importin beta’s role in *nuclear transport* have found that it binds to a subset of
nucleoporins in a Ran-sensitive manner (Bednenko et al., 2003; Ben-Efraim and Gerace, 2001; Pyhtila and Rexach, 2003; Shah et al., 1998). It is believed that as importin beta translocates through the nuclear pore during import and out of the nuclear pore during recycling, it interacts with specific, often FG-nucleoporins. Therefore, regulation of nuclear pore assembly might also involve the binding to these FG nucleoporins to prevent assembly, later reversed by RanGTP to allow assembly.

Although our previous study showed that importin beta blocks nuclear pore assembly in a Ran-insensitive manner (Harel et al., 2003a), Ran-sensitive interactions could well be part of importin beta’s inhibition of nuclear pore assembly. Ran-sensitive interactions would be reversed by the addition of RanQ69L, but the Ran-insensitive interaction would still remain, and thus nuclear pore assembly would still be blocked. In each of the previous studies on interactions between nucleoporins and importin beta, only a few nucleoporins were analyzed with different methods and systems (Bednenko et al., 2003; Ben-Efraim and Gerace, 2001; Pyhtila and Rexach, 2003; Shah et al., 1998). Here, a comprehensive analysis of importin beta’s nucleoporin targets was undertaken using the Xenopus egg extract system, which has the
advantage, as detailed earlier, of containing abundant amounts of nuclear pore subcomplexes in a native state.

**The search for nucleoporin interactions disrupted by importin beta**

Most nucleoporins do not go solo in the cytoplasm, residing instead in nucleoporin subcomplexes (Figure 1A). To understand the implication of importin beta’s interaction with nucleoporins, the stability within or between nucleoporin subcomplexes was investigated in the presence of importin beta and RanGTP, its primary modulator. After defining what importin beta binds to, I investigated the effect of importin beta and RanGTP on the interactions between the *members* of individual subcomplexes. I examined the Nup62/Nup214 complex and the Nup107-160 complex, the largest subcomplex, containing nine proteins: Nup160, Nup133, Nup107, Nup96, Nup85, Nup43, Nup37, Seh1 and Sec13 (Harel et al., 2003b; Loiodice et al., 2004; Walther et al., 2003a).

Nucleoporin subcomplexes need to interact with one another as they come together to form the nuclear pore. Lastly, importin beta and RanGTP’s effect on interactions between *nucleoporin subunits* was also investigated here. Specifically, I examined the known interactions of the Nup107-160 complex
with Nup153, and with Nup98, as well as identifying a novel interaction between Nup153 and Nup62.

Thus, this chapter investigates importin beta’s function in nuclear assembly progressively; from the proteins that it binds, to the effect it has on protein interactions within nucleoporin subcomplexes and lastly, its effect on interactions between nucleoporin subunits.
Materials and Methods

Cloning and Sequencing

The sequence of Xenopus importin beta was compiled with overlapping Xenopus EST sequences showing homology to human importin beta. Xenopus importin beta was then cloned from Xenopus RNA by reverse transcription and polymerase chain reaction (PCR) amplification with forward primer 5’-CCCCGATCCATGGAGCTCGTCACCATCCTC-3’ (with BamHI site underlined) and reverse primer 5’-CCCCCGCGCCGCTCAGGCTTGGTTTTTCAG-3’ (with NotI site underlined). The full-length Xenopus importin beta cDNA was cloned into a His tag vector (pET28-Xbfl), which results in a His-tag at the N-terminus of the recombinant Xenopus importin beta. GST-Xenopus importin beta (pGEX6P-Xbfl) was cloned by restriction digesting pET28-Xbfl with BamHI and Not I to release the Xenopus importin beta insert, and ligating the insert into the pGEX6P-3 vector digested with the same restriction enzymes.

The sequence of Xenopus importin beta was confirmed by DNA sequencing of the pET28-Xbfl construct with two forward primers: T7
promoter and an internal primer (Xbfl intF1, 5’ GCTGCAGTGAACACCTGG 3’) and a reverse primer, the T7 terminator primer.

**Protein Expression and Purification**

The His-tagged Xenopus importin beta protein was expressed in *E. coli* by growing the taking an overnight culture of bacteria and growing it up to log phase in 1L LB with kanamycin at 37°C. The culture was then induced with 0.1 mM IPTG at 17°C overnight. The bacterial pellet was resuspended in 10mL binding buffer (0.5 M NaCl, 20 mM Tris, pH 8, 5 mM imidazole) and sonicated twenty-1 second pulses after which the sample was placed on ice for 1 minute in between, for a total of five times. After sonicating, the bacterial resuspension was spun at 10,000rpm for 10 minutes. The resulting supernatant was run over a Ni-agarose column pre-equilibrated with binding buffer and washed with 2 column volumes of binding buffer. The protein was eluted with 5.5mL elution buffer (0.1M NaCl, 30mM Tris, pH 8, 500mM imidazole). The protein was then dialyzed into PBS and stored at -80°C after freezing with liquid nitrogen.

The His-mouse p97 DNA construct (ampicillin-resistant, described in Ye et al., 2003), a gift from Dr. Yihong Ye, was transformed into *E. coli*. A
single colony was then grown in 50mL LB with ampicillin overnight at 37°C. The culture was then expanded from 30mL of the overnight culture into 1L LB with ampicillin. The 1L bacterial culture was grown for 5 hrs at 37°C. No induction of the protein was needed. The bacterial pellet was resuspended in 10mL p97 resuspension buffer (20mM Tris pH 8, 0.5M KCl, 10% glycerol, 1mM ATP, 1mM MgCl2, 1mM PMSF, 1x aprotinin/leupeptin). The resuspended bacterial pellet was sonicated and pelleted, as with the His-tagged protein above. The resulting supernatant was loaded onto Ni-agarose beads equilibrated with the p97 resuspension buffer (see above). The beads were washed 2x 10mL p97 wash buffer (50mM Hepes pH 7.4, 150mM KCl, 10% glycerol, 1mM ATP, 1mM MgCl2, 1mM PMSF, 1x aprotinin/leupeptin, 20mM imidazole). Finally, the protein was eluted with 5-6mL p97 elution buffer (50mM Hepes pH 7.4, 150mM KCl, 10% glycerol, 1mM ATP, 1mM MgCl2, 1mM PMSF, 1x aprotinin/leupeptin, 500mM imidazole), dialyzed overnight in PBS, then concentrated.

GST-tagged Xenopus importin beta was expressed by growing cultures overnight from glycerol stocks (50mL LB with ampicillin and chloramphenicol). The next day, the culture was expanded into 1L LB with ampicillin and chloramphenicol, and grown at 37°C until the OD600 was at
least 0.7. The culture was induced with 0.1 mM IPTG at 17°C overnight. After spinning the bacterial pellet, resuspension in PBS, and sonication (as for the His-tagged proteins above), the supernatant was incubated with glutathione sepharose 4B beads (GSH-sepharose beads, Amersham Biosciences, Sweden) for 1 hr at 4°C. The beads were then washed three times with PBS and eluted with 5mL GSH elution buffer (100mM Tris pH 8, 100mM NaCl, 20mM glutathione in water). The eluted proteins were dialyzed in PBS overnight at 4°C, followed by a 2 hr dialysis into 5% glycerol/PBS. After concentration, the proteins were aliquoted and stored at -80°C after freezing with liquid nitrogen.

**Antibody generation and purification**

Anti-Xenopus importin beta antibody was raised against native His-tagged full-length Xenopus importin beta protein (in pET28a vector). Rabbit 732 was initially injected with 100μg His-Xenopus importin beta full-length protein with 500μL Freund’s complete. Subsequent injections with Freund’s incomplete each contained between 50-200μg His-Xenopus importin beta full-length protein.

Anti-Xenopus importin beta antibody (anti-serum 732) was purified by first depleting away His-tag antibody, followed by affinity purification with
cyanogen-bromide beads to which recombinant GST-Xenopus importin beta had been coupled. 2mL of the serum was diluted with an equal volume of 2x CnBr buffer (1M NaCl, 100mM Tris, pH 8). The diluted serum was put through a His-GFP cyanogen bromide bead column 4 times. A GST-Xenopus importin beta cyanogen-bromide (CnBr) bead column was prepared by washing with 1 column volume PBS, followed by blocking with 2mL 2% BSA/PBS. The resultant His-tag antibody depleted serum was run through the GST-Xenopus importin beta CnBr bead column 3 times. The column was washed with 2 column volumes of 1x CnBr buffer (0.5M NaCl, 50mM Tris, pH 8), then with 2 column volumes PBS. The affinity purified antibody was eluted 2 times with 1mL 0.1M glycine, pH 2.5. The working solution was stored at 4°C, while aliquots were frozen at -20°C. The column was washed 2 times with PBS and stored at 4°C in 10% ethanol for future reuse.

**Nuclear reconstitution and immunofluorescence**

Nuclei were reconstituted using the Xenopus egg extract system. Xenopus egg cytosol and membranes were prepared as previously described (Powers et al., 1995), except for the use of 500 mM KCl in the membrane wash buffer. The regenerating energy mix was made with the ratio of 1 μL of 5
mg/mL creatine phosphokinase (Sigma) to 0.5 μL 1 M phosphocreatine (Sigma) to 0.5 μL 0.2 M adenosine triphosphate (Sigma). For each reaction, 20 μL of Xenopus egg cytosol was mixed with 1 μL energy mix, 2 μL egg lysis buffer (ELB; 250 mM sucrose, 2.5 mM MgCl₂, 50 mM KCl, 10 mM HEPES, pH 7.8) or 2 μL (maximum volume added) recombinant protein, and 1 μL of Xenopus membranes. Immediately after adding the membranes, the reaction was mixed well so that membrane clumps were no longer present. Lastly, 1 μL sperm chromatin (~50,000 units Xenopus sperm chromatin/μL) was added to each reaction. The reaction was incubated at room temperature for 40 min to 1 hour to allow for complete nuclear assembly.

To visualize nuclear pore complexes and nuclear membrane fusion in the same sample without fixing, the reaction was then incubated with directly labeled monoclonal antibody 414 (as described in Harel et al., 2003a) for 30 minutes at room temperature. The extent of nuclear envelope fusion was assessed by staining 2 μL of the reaction with 0.5 μL of the lipophilic membrane dye DHCC (2 μL 1 mg/mL DHCC, 1μl 1mg/mL Hoechst, 300 μL ELB) directly on the slide. Nuclei were visualized on a fluorescence microscope with a 63× objective. Indirect immunofluorescence was also used to visualize nuclear pore proteins in fixed reconstituted nuclei that were first
pelleted onto poly-lysine coverslips before probing with various antibodies (Macaulay and Forbes, 1996).

**Immunoprecipitations**

Endogenous proteins were isolated from Xenopus egg extract by immunoprecipitation. All immunoprecipitations were conducted in PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 1.76 mM KH₂PO₄). Antibodies were crosslinked onto protein A-sepharose beads before incubation with Xenopus egg cytosol. For crosslinking, antibodies (10 μg per reaction, affinity purified or IgG purified) were prebound to protein A-sepharose beads (Amersham Biosciences, Sweden, 20 μL per reaction) for one hour at room temperature. Beads were washed twice with 0.2 M sodium borate, pH 9, followed by crosslinking with 20 mM DMP in 0.2 M sodium borate, pH 9 (5 mg/mL) for 30 minutes at room temperature. The reaction was quenched by washing two times with 0.2 M ethanolamine, pH 9, followed by incubation with 0.2 M ethanolamine, pH 9 for one hour at room temperature. The beads were then mock eluted with 0.1 M glycine, pH 2.5 to remove antibodies that were not crosslinked to the beads. The glycine was removed by washing the beads three times with PBS. Each reaction of crosslinked antibody beads was
incubated with 20 μL Xenopus egg cytosol diluted into 500 μL PBS, along with the recombinant protein or chemical that was to be tested, for two hours at 4°C. Beads were washed three times with PBS, followed by elution with 20 μL 0.1 M glycine pH 2.5, to which 9 μL of 4X SDS-PAGE loading buffer and 2 μL 1 M Tris, pH 8 were added. Samples were then separated by SDS-PAGE and transferred to PVDF membrane for Western blotting.

Anti-Nup62 was raised against Xenopus Nup62 (Rb376). Anti-Nup153 (Rb380) was raised against a recombinant fragment of Xenopus Nup153 corresponding to human Nup153 amino acids 431-732 (Shah et al., 1998). The monoclonal antibody against rat importin beta was purchased from BD Transduction Laboratories. Monoclonal antibody against p97 (Babco) was the kind gift of Dr. Carole Baron, UCSD.

**Pulldowns**

Interactions with importin beta were investigated, in part, using GST-Xenopus importin beta pulldowns. Recombinant GST-Xenopus importin beta was incubated with GSH-sepharose beads without crosslinking. Specifically, GSH-sepharose beads were pre-incubated with GST-tagged protein in PBS (7.5 μg GST or GST-Xenopus importin beta protein/μL beads per reaction) for 1 hr
at 4°C. The beads were washed 1 time in PBS, and then blocked with 20mg/mL ovalbumin/PBS for 30 mins (room temperature for the GST-Xenopus importin beta pulldown, 4°C for the GST-Xenopus importin beta + His-p97 pulldown). Xenopus egg cytosol was then added to the beads with or without RanQ69L and incubated for 2 hours at 4°C (25μL extract in 500μL PBS). The beads were washed with PBS three times and eluted with 20μL 0.1M glycine, pH 2.5, after which 7μL 4xSDS-PAGE sample loading buffer and 2μL 1M Tris, pH 8 was added.

The Nup107-160 complex’s interaction with Nup98 (Vasu et al., 2001) was probed starting with Nup98 (aa470-876) fragment coupled to cyanogen bromide beads. For this, cyanogen bromide beads were activated by washing thoroughly with ~50mL 1 mM HCl. Right before adding the recombinant protein, the activated cyanogen bromide beads were quickly washed with PBS once. In each reaction, 45 μg His-Nup98 (aa470-876) was incubated with 17 μL cyanogen-bromide beads in PBS. In the control reaction, an equal amount of GFP was incubated with cyanogen bromide beads. The proteins were allowed to couple for 2 hours at 4°C. The reaction was washed once in PBS, followed by quenching with 0.1 M Tris, pH 8 for 1 hour at room temperature. The beads were washed two times in PBS before blocking in 20 mg/mL BSA/PBS
for 30 minutes at room temperature. Finally, the protein-coupled beads were incubated in Xenopus egg cytosol diluted 1:50 into 250 µL PBS for 2 hours at 4°C. Recombinant proteins were also added into the reaction at this time.

After incubating in cytosol, the beads were washed five times with PBS before eluting the bound proteins with 35 µL 0.1 M glycine, pH 2.5. SDS-PAGE sample loading buffer (4x, 12µL) and 1M Tris pH 8 (3 µL) were added to the elution. One fifth of each reaction was loaded for SDS-PAGE.

**Immunoblot analysis**

Protein samples were mixed with 4x sample loading buffer, boiled for one minute, and loaded on a SDS-polyacrylamide gel. After running the gel for one hour at 150V, the electrophoresed proteins were transferred to polyvinylidene difluoride (PVDF) membrane (Millipore Corp., Bedford, MA) for one hour at 110V. The membrane was stained with India Ink in PBST (PBS with 0.2% Tween). 5% nonfat powdered milk in PBST was used to block non-specific binding sites on the membrane by incubation for 30 minutes at room temperature. In order to probe for several proteins on one membrane, separate strips were cut, blocked, and probed with primary antibody at the appropriate dilution in 5% milk/PBST for one hour at room temperature. The
strips were washed separately in PBST for 30 minutes, with several changes of PBST in between. Goat anti-mouse or goat anti-rabbit secondary antibodies conjugated to horseradish peroxidase (Jackson ImmunoResearch Laboratories, West Grove, PA) were then incubated with the strips at 1:2000 dilution in 5% milk/PBST for 30 minutes at room temperature. The strips were washed extensively with PBST for one hour at room temperature with several changes in between. The blots were visualized after incubating with chemiluminescent substrate (Super Signal West Pico or Femto Chemiluminescent Substrate, Pierce, Rockford, IL) and exposing to film (Denville Scientific, Metuchen, NJ).

For blots that were reprobed after stripping off antibodies, the following procedure was done. After developing the blots, the blots were incubated with stripping buffer (2% SDS, 62.5 mM Tris-HCl, pH 6.8, 5 mM 2-mercaptoethanol) for 30 minutes at 55°C. The blots were then extensively washed in PBST for at least 3 times 15 minutes. After blocking in 5% milk/PBST for 30 minutes, the blots were reprobed with the appropriate primary antibody and secondary antibody, as described above.
Results

Importin beta has roles in nuclear transport, mitotic spindle assembly and in nuclear assembly. Toward an understanding of the precise mechanism by which importin beta negatively regulates nuclear assembly, Xenopus importin beta was investigated using the Xenopus egg extract nuclear reconstitution system.

Xenopus importin beta is homologous to human importin beta in sequence and function.

In order to study importin beta in a more endogenous environment with the Xenopus nuclear reconstitution system, focus was placed on using the Xenopus homolog of importin beta. All previous experiments with importin beta, both in our studies and all the mitotic spindle studies to date for others, have used recombinant human importin beta added to the Xenopus nuclear reconstitution system (Harel et al., 2003a; Nachury et al., 2001; Walther et al., 2003b; Wiese et al., 2001; Zhang et al., 2002). No Xenopus importin beta clone existed. In a collaborative effort with Brian Sato, a rotation student, Xenopus importin beta was identified, cloned and an
antibody against Xenopus importin beta was generated. Xenopus importin beta was identified by overlapping Xenopus ESTs found by BLAST searches that showed homology to human importin beta. Using these overlapping Xenopus ESTs, primers were designed to clone Xenopus importin beta from Xenopus RNA. Putative Xenopus importin beta cloned into the His-tag pET28a vector was sequenced in both directions from vector primers, and also with an internal primer. When the full-length Xenopus importin beta protein sequence was aligned with human importin beta (Figure 2), it was clear that the two homologs are extremely similar (identities = 94% (828/876), positives = 97% (857/876)).

With the clone of Xenopus importin beta in hand, a polyclonal antibody was generated against full-length recombinant Xenopus importin beta. The antibody (anti-serum 732) recognized a band in Xenopus egg cytosol (Figure 3A, lanes 1+2) that corresponded to that recognized by a monoclonal antibody against rat importin beta (Figure 3A, lanes 3+4). The anti-Xenopus importin beta antibody also recognized endogenous importin beta in the context of nuclei. When in vitro reconstituted nuclei were assembled, spun onto coverslips and probed with the anti-Xenopus importin beta antibody (Figure
3B), the antibody gave a nuclear rim stain, which is known to be indicative of importin beta’s localization at nuclear pores during nuclear transport.

Previous studies have established that human importin beta inhibits nuclear envelope fusion (Harel et al., 2003a) and nuclear pore assembly (Harel et al., 2003a; Walther et al., 2003b). Two possibilities could have explained this. Either human importin beta has a slightly different sequence at key residues and was acting as a dominant negative mutant form of importin beta in the Xenopus system or human beta acts identically to Xenopus importin beta and its excess concentration caused inhibition. To distinguish these possibilities, we put recombinant Xenopus importin beta to the test. The addition of Xenopus importin beta did indeed block nuclear assembly, as seen by the block to nuclear import and the failure of chromatin to decondense fully (Figure 4A). However, higher concentrations of Xenopus importin beta (25 μM or more) compared to human importin beta (15 μM) were needed in nuclear reconstitution reactions to see the same extent of inhibition to nuclear envelope fusion (Figure 4B, compare to Harel et al., 2003a).

Xenopus importin beta prevented nuclear pore assembly as well, both when added directly to a nuclear reconstitution reaction (Figure 4B) and when added to BAPTA nuclei (intermediates which have fused nuclear membranes
yet lack nuclear pore complexes) that have been allowed to rescue in fresh cytosol (C. Lau and D. Forbes, manuscript in preparation). Thus, both Xenopus and human importin beta, in excess, reveal negative regulation of nuclear membrane fusion and pore assembly. Human importin beta may also act as a type of dominant negative mutant at some level in the context of Xenopus egg extract. With Xenopus importin beta mirroring human importin beta in its sequence and function, I proceeded to search for the targets of importin beta in nuclear membrane fusion and pore assembly.

**Targets of importin beta in nuclear membrane fusion**

The involvement of importin beta in hindering nuclear membrane fusion, counterpoint to RanGTP, posed the interesting question of what protein(s) importin beta interferes with to allow this regulation. The protein in question would presumably be involved in nuclear membrane fusion, bind to importin beta in a RanGTP-dependent manner, and could reside on the membranes or in the cytosol.

First, I examined whether importin beta was present on membranes. Upon immunoblotting Xenopus cytosol and membranes with the anti-Xenopus importin beta antibody (anti-serum 732), a distinct importin beta
band was present in cytosol, as well as membranes (Figure 5A, lanes 2 and 1, respectively). In addition, importin beta’s presence on the membranes was regulated by RanQ69L. The addition of RanQ69L to membranes caused importin beta to release from the membranes (top, Figure 5B, lanes 2 and 3). This is in contrast to the transmembrane proteins found on membranes, gp210 and ribophorin, and the soluble protein, p97. In fact, the addition of RanQ69L caused these proteins to increase in amount (Figure 5B, lanes 2 and 3). The increase in transmembrane proteins most likely reflects an increase in membrane fusion and annulate lamellae assembly in the presence of RanQ69L (Harel et al., 2003a; Walther et al., 2003b). Importin beta’s behavior on membranes is in alignment with the Ran-sensitive importin beta block to nuclear membrane fusion. The addition of RanQ69L to membrane could potentially release importin beta from the membrane to allow for membrane fusion.

**Importin beta is present on membranes, but does not interact with p97, a factor required for nuclear membrane fusion.**

An attractive possibility for importin beta’s target on the membrane is p97. p97 has been shown to be involved in fusion events in the Golgi and ER
(Acharya et al., 1995; Latterich et al., 1995), retrotranslocation of proteins out of the ER into the cytosol (Ye et al., 2003), and nuclear membrane fusion (Hetzer et al., 2001). p97 is present on membranes, and is not released from the membranes upon addition of RanQ69L (Figure 5B, lanes 5 and 2, respectively). Both importin beta and p97 have been implicated in nuclear membrane fusion; importin beta as a negative regulator, and p97 as a positive regulator. Does importin beta’s presence on membranes regulate the activity of p97? Or, could it be that importin beta and p97 are involved in distinct pathways?

To ascertain whether importin beta and p97 interact, I first determined whether endogenous importin beta binds endogenous p97. For this, anti-Xenopus importin beta antibody was used to immunoprecipitate importin beta from Xenopus egg cytosol. p97 was not found in the immunoprecipitate (Figure 6A, lane 1). Next, I used recombinant GST-Xenopus importin beta coupled to beads as a bait to see if endogenous p97 from Xenopus egg cytosol could bind. The coupling of recombinant proteins to beads presents a high concentration of protein in one locality, making it effective in isolating even weak protein-protein interactions. p97 did not bind even in the presence of highly concentrated recombinant importin beta on the beads, either in the
absence or presence of RanQ69L (Figure 6B, lanes 3 and 4). Lastly, I added recombinant p97 (4 μM, His-p97) and recombinant importin beta (3 μM, GST-Ximpβ) in concentrations close to the endogenous amounts and incubated them in the presence of Xenopus egg cytosol, to see if there was direct or indirect binding between the two proteins. Upon isolating His-p97, GST-Xenopus importin beta did not bind (Figure 6C, lane 1). These results point to the lack of interaction between importin beta and p97, and point toward the second model proposed above, where importin beta and p97 could act in separate parallel pathways to regulate nuclear membrane fusion.

The mechanism of nuclear membrane fusion is poorly understood, as it is not known how the disparate proteins involved in the process cooperate together. The identification of Ran-sensitive importin beta binding-proteins by mass spectrophotometry may reveal proteins involved downstream of importin beta in nuclear membrane fusion.

**Targets of importin beta in nuclear pore assembly**

Importin beta is involved in the negative regulation of nuclear pore assembly. A likely avenue for importin beta’s effect on nuclear pore assembly is the interaction between importin beta and nucleoporins. Based on the
observation that importin beta prevents the *one* interaction between the Nup107-160 complex and Nup153 and that it also blocks pore assembly (Walther et al., 2003b), it has been assumed that the binding of importin beta to nucleoporins prevents nucleoporin interactions. In this case, the addition of RanQ69L would release importin beta from individual nucleoporins to allow for interactions between nucleoporins, thus getting one step closer to the formation of functional nuclear pores.

Characterized interactions between nucleoporins are far and few in between. The discovery of new interactions between nucleoporins in the presence of RanGTP would greatly aid in the understanding of nuclear pore assembly, and furthermore, would reveal how specific nucleoporin interactions contribute to the structure of the nuclear pore complex. Thus I set out to understand whether importin beta and RanGTP regulate known and novel interactions between nucleoporin subunits.

In order to understand which nucleoporins importin beta could be interfering with, a comprehensive analysis of nucleoporins that bound importin beta was first carried out. A two-pronged approach was used to ensure all importin beta interacting-nucleoporins were isolated. Two different importin beta “baits” were used: endogenous Xenopus importin beta obtained
by immunoprecipitations and recombinant GST-Xenopus importin beta used for pulldowns from Xenopus egg extracts.

**Importin beta binds to FG nucleoporins and members of the Nup107-160 complex.**

The antibody raised to full-length Xenopus importin beta immunoprecipitated importin beta well (Figure 7D and E, lanes 3). Moreover, importin beta coimmunoprecipitated the Nup107-160 complex members Nup160, Nup133, Nup85 and Nup43 (Figure 7A, lane 3), but preimmune antisera did not (lane 2). In addition, importin beta coimmunoprecipitated with the FG nucleoporins Nup153, Nup62 and Nup50 (Figure 7B and E, lanes 3). Among the FG nucleoporins tested, Nup98 did not coimmunoprecipitate with importin beta (Figure 7B, lane 3). However, Nup98, along with Nup214 and Nup358, was later found to bind GST-Xenopus importin beta beads, as high concentrations of recombinant importin beta made it possible to isolate weaker interactions (Figure 8B, lane 3). Tpr, a protein peripherally associated with the nuclear pore basket (and often considered a nucleoporin), bound to importin beta as well (Figure 7E, lane 3). The non-FG nucleoporins Nup205, Nup93 and Nup53 did not show binding to importin beta, either by
immunoprecipitation (Figure 7C, lane 3) or by pulldown with GST-Xenopus importin beta beads (Figure 8C, lane 3). Thus, importin beta binds to FG nucleoporins and members of the Nup107-160 complex, but not all nucleoporins.

**Interactions within the Nup107-160 complex are not disrupted by importin beta or RanGTP.**

I next asked if importin beta affects the interactions *within* nucleoporin subcomplexes. I examined the large Nup107-160 complex to determine whether the members within the complex were affected by importin beta. Anti-Nup43 and Nup133 immunoprecipitations were used to address this issue. The immunoprecipitation by each antibody isolated all the members of the Nup107-160 complex that were probed (Nup160, Nup133, Nup85, Nup43; Figure 9A and B, lanes 4). The addition of 15 μM human importin beta, the concentration at which nuclear pore assembly is blocked, to the immunoprecipitation did not interfere with any of the interactions between Nup160, Nup133, Nup85 or Nup43 (Figure 9A and B, lanes 7). The addition of RanQ69L also had no effects (lanes 5). Thus importin beta does not interfere with interactions within the Nup107-160 complex.
Interactions within the Nup62/Nup214 subcomplex are not disrupted by importin beta or RanGTP.

Nup62 is located in the central region of the nuclear pore in a complex with Nup58, Nup54 and Nup45 (Finlay et al., 1991; Guan et al., 1995), and in another distinct complex with Nup214 and Nup88 located at the cytoplasmic filaments (Bastos et al., 1997; Macaulay et al., 1995). When Nup62 was immunoprecipitated, Nup214 bound strongly (Figure 10, lane 4). The interaction between Nup62 and Nup214 was not perturbed by the addition of RanQ69L, importin beta, or both (Figure 10 lanes 5, 7 and 6, respectively). Thus, importin beta also does not disrupt interactions within the Nup62/Nup214 subcomplex.

Importin beta regulates the interaction between the Nup107-160 complex and Nup153.

Next, I asked whether importin beta interferes with interactions of the Nup107-160 complex with other nucleoporins. One can probe for this by asking whether RanQ69L induces interaction and importin beta prevents interaction. The interaction between the Nup107-160 complex and FG
nucleoporins was investigated first. None of the FG nucleoporins tested 
(Nup358, Nup214, Nup153, Nup62) bound to the Nup107-160 complex 
(Figure 11, lane 4). However, when RanQ69L was added, Nup153 bound to 
the Nup107-160 complex (Figure 11, lane 5). This is in agreement with the 
published result that Nup107 antibody coimmunoprecipitates Nup153 in the 
presence of RanQ69L (Walther et al., 2003b). When importin beta was added 
along with RanQ69L, the interaction of Nup153 with the Nup107-160 complex 
significantly decreased (Figure 11, lane 6). The addition of importin beta alone 
did not change any interaction as compared to control (Figure 11, lane 7). 
From these results, we conclude that importin beta and RanGTP regulate the 
interaction between the Nup107-160 complex and the basket nucleoporin 
Nup153.

When non-FG nucleoporins were investigated, the addition of 
RanQ69L did not induce any interactions between the Nup107-160 complex 
and Nup205, Nup155, Nup93 or Tpr (Figure 12, lanes 5). Thus, of the 
nucleoporins tested here, only Nup153 bound to the Nup107-160 complex in 
the presence of RanGTP.
Importin beta does not regulate the interaction between the Nup107-160 complex and Nup98.

The Nup107-160 complex has also been shown to interact directly with Nup98. Indeed, the Nup107-160 complex can be isolated through its affinity with a fragment of Nup98 (aa 470-876; Vasu et al., 2001). The interaction is apparent in such a Nup98 pulldown, but is not observed by immunoprecipitation. The concentrations of recombinant Nup98 is much higher in pulldowns, thus increasing the affinity for weaker interactions.

When the Nup98 fragment aa 470-876 was used to pulldown interacting proteins, Nup133 and Nup43 showed strong binding; however, Nup160 showed weaker binding, and surprisingly, Nup85 did not show binding at all (Figure 13, lane 6). This pattern was not altered by the presence or absence of importin beta or of RanQ69L (Figure 13, lanes 8 and 7, respectively).

An interesting sidelight of this experiment was that Nup85 was not observed when Nup98 was used to isolate the Nup107-160 complex. We hypothesize that Nup98 may displace Nup85 from the Nup107-160 complex, which may well give us insight into how the nuclear pore forms. This possibility is addressed later in the Discussion. What is clear, however, is that
importin beta and RanGTP do not regulate the binding of Nup98 to the Nup107-160 complex.

No other nucleoporins tested interact with the Nup98 aa 470-876 fragment, including Nup214, Nup153, Nup62, Nup50, Nup93 and Nup53 (Figure 14, lane 6). Moreover, the addition of RanQ69L, importin beta, or a dominant negative fragment of importin beta to the Nup98 pulldown did not significantly induce the interaction with any of these nucleoporins (Figure 14, lanes 7-9, respectively). Thus, importin beta and RanGTP do not regulate the interaction between the Nup107-160 complex and Nup98.

**Importin beta regulates the novel interaction between Nup62 and Nup153.**

In the search for new interactions induced by RanQ69L, immunoprecipitations of Nup62 in the presence of RanQ69L were probed for various nucleoporins. Surprisingly, the addition of RanQ69L caused Nup153 to coimmunoprecipitate with Nup62, an interaction never described before (Figure 15A, lane 2 and Figure 15B, lane 5). This interaction was abrogated with the addition of importin beta (Figure 15B, lane 6). Curiously, the addition of importin beta alone caused a slight amount of Nup153 to bind Nup62 (Figure 15B, lane 7), which was not seen when Nup62 was
immunoprecipitated without importin beta (Figure 15B, lane 4) or in the mock
immunoprecipitation in the presence of importin beta (Figure 15B, lane 3).
None of the other nucleoporins tested; Nup205, Nup98, Nup93, Nup53 or the
Nup107-160 complex proteins; Nup160, Nup133, Nup85, Nup43, showed
interaction with Nup62 even upon addition of RanQ69L (Figure 15B, lane 5).

In order to understand the nature of this novel interaction between
Nup62 and Nup153, reciprocal immunoprecipitations of Nup153 were
analyzed. An antibody against a fragment of Xenopus Nup153 encompassing
parts of the unique N terminus and zinc finger domain (equivalent to human
Nup153 aa 431-732) was used to isolate Nup153 (anti-serum 380, Figure 16A;
Shah et al., 1998). Surprisingly, this antibody immunoprecipitated Nup153
along with Nup62 even without RanQ69L (Figure 16B, lane 4). In addition,
the Nup62 isolated in the Nup153 immunoprecipitation did not interact as
strongly with Nup214 as in the Nup62 immunoprecipitation (Figure 16B, lane
4 versus Figure 10, lane 4). The implication of these results will be presented
in the discussion below. In conclusion, a novel interaction between Nup62
and Nup153 is inhibited by importin beta and allowed to occur when RanGTP
removes importin beta from the nucleoporins.
Discussion

Distinct yet united in their regulation of nuclear membrane fusion:

importin beta and p97.

How does importin beta block nuclear membrane fusion? Importin beta’s association with membranes is abrogated upon incubation with RanQ69L (Figure 5B). Functionally, the addition of importin beta blocks nuclear membrane fusion, while the addition of both importin beta and RanQ69L restores nuclear membrane fusion. The release of importin beta from membranes could be the mechanism by which nuclear membrane fusion occurs.

In the process of understanding importin beta’s targets in nuclear envelope fusion, a factor implicated in nuclear membrane fusion, p97, was investigated. Importin beta was found not to associate with p97 (Figure 6), even when placed together in cytosol that should supply any missing accessory proteins. This likely places importin beta and p97 in separate yet parallel pathways that converge to regulate nuclear membrane fusion (Figure 17). This finding makes room for the discovery of factors that act in these two
pathways and highlights the importance of tightly regulating nuclear membrane fusion.

The regulation of nuclear membrane fusion by several mechanisms is highly plausible, as aberrant nuclear membrane fusion has severe consequences. If nuclear membrane fusion cannot occur, the separation of nuclear versus cytosolic processes will be lost, along with layers of regulatory processes. Regulation is needed to ensure nuclear membrane fusion occurs at the surface of the chromatin, and not elsewhere. The control of membrane fusion will ensure equal amounts of membrane in the inner and outer nuclear membrane. As seen in the formation of the mitotic spindle, multiple checkpoints are present to monitor one process.

**In search for purpose: importin beta on membranes**

What then could importin beta be binding on the membranes to prevent nuclear membrane fusion? As established here, importin beta does not bind p97 (Figure 6). So far, the other protein implicated in nuclear membrane fusion is POM121, a transmembrane nucleoporin (Antonin et al., 2005). It will need to be established whether interaction between POM121 and importin beta occurs and, if so, whether the interaction prevents nuclear membrane
fusion. Specific targets of importin beta on membranes can be identified by removing importin beta from membranes, followed by the incubation of the membranes with an antibody against a specific transmembrane protein to block its binding sites, then assaying whether importin beta can now bind to membranes. Importin beta can be specifically removed from membranes by the addition of RanQ69L, as I have shown (Figure 5B). Antibodies or even protein fragments of transmembrane proteins can be added to this assay to assess whether importin beta interacts directly with that protein. The determination of importin beta’s target on membranes will open up avenues to understand the mechanism of nuclear membrane fusion.

**Importin beta’s targets in nuclear pore assembly**

In this study, a comprehensive study of the targets of Xenopus importin beta was completed. The non-FG nucleoporins tested, i.e., Nup205, Nup155, Nup93 and Nup53, did not bind importin beta. The proteins that bound importin beta include the FG containing nucleoporins, Nup358, Nup214, Nup153, Nup98 and Nup50, members of the Nup107-160 complex, Nup160, Nup133, Nup85 and Nup43, and the pore-associated filament protein Tpr. However, nucleoporins that coimmunoprecipitate with importin beta may not
necessarily reflect direct interactions between them, as nucleoporins present in subcomplexes could coimmunoprecipitate together as long as one member binds importin beta. This is likely the situation for the key Nup107-160 complex. It will be of interest to identify which nucleoporin interacts directly with importin beta using recombinant fragments of nucleoporins, in order to establish the point of control.

A key and unexpected distinction between importin beta’s block to nuclear envelope fusion and nuclear pore assembly is that RanGTP cannot reverse importin beta’s block to nuclear pore assembly. This is conceivable, as once nuclear membranes have fused on the surface of chromatin, the high concentration of RanGTP would be shielded from the cytoplasm. This gives precedence to the existence of another regulator of importin beta.

The analysis of importin beta’s Ran-insensitive interactions were limited by the unavailability of tools to isolate importin beta in the presence of RanQ69L. Specifically, our antibody against Xenopus importin beta no longer binds and immunoprecipitates importin beta when RanQ69L is added (data not shown). It is conceivable that the antibody loses its grip on importin beta in the presence of RanQ69L, as importin beta bound to RanGTP is known to take on a very altered conformation (Lee et al., 2005). In addition, the
recombinant GST-Xenopus importin beta used for the pulldowns here does
not show Ran-sensitivity, even in its interaction with importin alpha. In the
future as tools are developed to isolate importin beta even in the presence of
RanQ69L, it will be possible to isolate importin beta’s Ran-insensitive
interactions. Mass spectrometry of novel importin beta binding proteins will
elucidate candidates for a modulator of importin beta, other than RanGTP,
that may be involved in reversing importin beta’s block to nuclear pore
assembly. This will allow the determination of a novel mechanism by which
importin beta acts.

**Importin beta’s Ran-sensitive interactions with nucleoporins in the context
of nuclear pore assembly**

The involvement of a Ran-insensitive interaction with importin beta in
preventing nuclear pore assembly does not preclude the importance of
importin beta’s Ran-sensitive interactions in the same process. Importin beta
added to egg extract will bind to proteins and interfere with required protein-
protein interactions, whether the interaction is Ran-sensitive or not. The Ran-
sensitivity of importin beta’s interactions with nucleoporins were determined
by reciprocal immunoprecipitation of the nucleoporin in the presence of
RanQ69L, where possible. Of the importin beta-binding nucleoporin immunoprecipitates, all of them show a Ran-sensitive interaction with importin beta (Nup107-160 complex, Figure 9A and B, lanes 4+5; Nup62, Figure 15A, lanes 1+2; Nup153, Figure 16, lanes 4+5; Nup98, data not shown). Thus, this set of importin beta-nucleoporin interactions are broken in the presence of RanQ69L.

This phenomenon presents a simple mechanism for nuclear assembly, whereby nucleoporins are released from importin beta in the vicinity of chromatin, where RanGTP concentrations are high, and association between nucleoporins are thus allowed to occur at the nuclear envelope (Walther et al., 2003b). Is the Ran-sensitive release of importin beta from nucleoporins then the mechanism by which all nucleoporins interact with one another in the vicinity of Ran-rich chromatin? The assumption of importin beta globally disrupting interactions between nucleoporins was thus investigated.

**Nucleoporin subcomplexes: remaining together even in the face of importin beta.**

When importin beta binds to a nucleoporin, importin beta may interfere with interactions with the nucleoporin. The effect of importin beta
on nucleoporin interactions *within* and *between* nucleoporin subcomplexes was investigated here.

The isolation of nucleoporin subcomplexes in the presence of importin beta revealed that the Nup107-160 complex (proteins tested: Nup160, Nup133, Nup85, Nup43) and the Nup62/ Nup214 complex remain intact. There is also evidence that the interaction between Nup62 and Nup54, which represents the Nup62/Nup58/Nup54/Nup45 complex, is not disrupted by the addition of importin beta (V. Delmar and R. Chan, unpublished results). This implies that importin beta does not block nuclear pore assembly by disrupting interactions *within* the Nup107-160 complex, the Nup62/Nup214 complex or the Nup62/Nup58/Nup54/Nup45 complex.

**Interactions between nucleoporin subunits: importin beta modulates some, but not all**

The known interactions of the Nup107-160 complex with Nup153 and Nup98 were investigated in the presence of importin beta. Possible novel Ran-induced interactions with the Nup107-160 complex were also investigated, but the FG nucleoporins, Nup358, Nup214 and Nup62, and non-
FG nucleoporins, Nup205, Nup155, Nup93 and Tpr were all found not to interact with the Nup107-160 complex (Figures 11 and 12).

Nup62 is part of two nucleoporin subcomplexes (Nup62/Nup214 and Nup62/Nup58/ Nup54/Nup45) and binds to importin beta (Figure 15A). Thus, Nup62 was investigated to see if any novel nucleoporin interactions would be induced by RanGTP and countered by importin beta. Surprisingly, it was found that Nup62 interacts with Nup153 in the presence of RanQ69L, and importin beta prevents the interaction (Figure 15B).

Taking one step back to analyze the larger picture of importin beta’s effect on interactions between subcomplexes and their nucleoporin binding partners, it was surprising to find that to date, importin beta only abrogates interactions where Nup153 was involved. These were the interactions induced by RanQ69L between Nup153 and the Nup107-160 complex, and a novel interaction discovered between Nup153 and Nup62 (Figure 18). Why would importin beta and RanQ69L modulate the interactions of Nup153, but not between other nucleoporins tested? Nup153 is a distinctive nucleoporin with a zinc finger domain, where Ran has been shown to bind (Nakielny et al., 1999). It is not known whether conformational changes are induced in Nup153 with the binding of Ran. So, the effect of importin beta (which binds
to the FG domain of Nup153) and RanQ69L (which binds the zinc finger
domain of Nup153) on Nup153 is not as straight forward as RanQ69L simply
releasing importin beta from Nup153. In a possible model, RanQ69L could
induce conformational change in Nup153 to open up a previously masked
binding site for nucleoporins, such as amino acids 1-339 of Nup153. This
would allow the Nup107-160 complex to bind. The addition of importin beta
could remove RanQ69L to mask the nucleoporin binding site, and importin
beta itself could bind to the FG domain of Nup153, preventing other proteins
from binding. In conclusion, the modulation of Nup153’s interactions by
importin beta and Ran may be specific to Nup153 because of its unique
binding properties to both importin beta and Ran.

**Regulation of nuclear assembly: more than meets the eye**

Importin beta is not a global regulator of interactions between
nucleoporins. The lack of modulation of other nucleoporin interactions by
importin beta and Ran points to several things. Firstly, additional factors
other than importin beta and RanGTP could be modulating interactions
between the other nucleoporin subcomplexes. This could include proteins
involved in post-translational modifications, such as kinases/phosphatases or
sumolation enzymes, or the presence of other protein modulators that bind directly, as is the case with importin beta and RanGTP. The search for alternate protein modulators of nucleoporin interactions would involve the identification of proteins that show a consistent pattern of interacting with nucleoporins, and the addition of these proteins will allow one subset of nucleoporins to incorporate into the nuclear envelope, while another group of nucleoporins will not.

Secondly, the proteins required to regulate interactions between the other nucleoporins may not be in the cytosol. Nuclear pores do not assemble in cytosol alone. Minimally, membranes are needed for the formation of nuclear pores. This is clearly seen when the mixing together of cytosol and membranes forms annulate lamellae, cytoplasmic stores of nuclear pore complexes in stacks of membranes. All the nucleoporins analyzed in this study, apart from Tpr, are incorporated into the nuclear pores of annulate lamellae (Meier et al., 1995; Walther et al., 2003b). The two transmembrane nucleoporins, POM121 and gp210, are present in the membranes and may serve as mediators to encourage interactions between soluble nucleoporins. Thus, the nuclear envelope around chromatin acts as a platform for
nucleoporins to be present at high enough concentrations for interaction and positions them at the site of nuclear pore assembly.

In conclusion, this study has focused on a subset of established known interactions within the nuclear pore. I find that importin beta regulates several interactions between nucleoporins, but does not account for the regulation of all the nucleoporin interactions that occur as the nuclear pore complex forms. However, the importin beta regulated interactions, as shown in Chapter 1, are enough to completely abrogate nuclear pore formation.

**At home: nucleoporin interactions can predict the structure of the nuclear pore complex**

The molecular structure of the nuclear pore, i.e., the placement of individual nucleoporins within the pore, is still much a matter of conjecture. Thus, discovery of each new interaction between nucleoporins, especially in vertebrates, where no genetics have been done, provides very valuable keys to potentially arriving at a 3-D structure for the nuclear pore.

The interactions of Nup153 with Nup62 and with the Nup107-160 complex thus pose interesting possibilities for how these interactions would map onto the nuclear pore complex. Nup153 is a highly dynamic protein with
a unique N-terminus, followed by a zinc finger domain and a FG-rich C-terminus (Figure 16A). The N-terminus of Nup153 has been mapped close to the membrane, while the zinc finger domain of Nup153 has been mapped to the nuclear basket ring (Fahrenkrog et al., 2002; Pante et al., 1994; Pante et al., 2000). Immunoelectron microscopy studies using antibodies against domains of Nup153 or full-length Nup153 epitope tagged at the amino- or carboxy-terminus show that the FG-rich C-terminus can reside at the nuclear basket, in the central region of the nuclear pore, or even extend through the pore and out into the cytoplasm (Fahrenkrog et al., 2002).

Nup62 was discovered here to bind Nup153, but where in the context of the pore do they bind to one another? The addition of importin beta to a Nup153 immunoprecipitation disrupts the interaction between Nup62 and Nup153 (Figure 15B, lanes 6+7). In addition, immunoprecipitation of Nup153 using an antibody made against part of the zinc finger domain and the FG-rich C-terminus of Nup153 does not coimmunoprecipitate Nup62 (data not shown). These results point to the possibility of Nup62 binding to the FG-rich C-terminal domain of Nup153. The Nup107-160 complex, on the other hand, has been shown to bind to the N-terminus of Nup153 (aa 1-339; Vasu et al., 2001; Figure 16A).
When the localization of the domains of Nup153 is integrated with the locations of nucleoporins that Nup153 interacts with, an interesting picture of interactions within the pore emerges (Figure 19A). The N-terminus of Nup153 binds the Nup107-160 complex (Vasu et al., 2001), which has been localized to the central scaffold region of the nuclear pore (gray ring, Figure 19A, Fahrenkrog et al., 2002; Krull et al., 2004). The zinc finger domain of Nup153 has been placed at the nuclear basket ring (Fahrenkrog et al., 2002; Pante et al., 1994; Pante et al., 2000). Our data predict the FG-rich C-terminus could make contacts with either the Nup62/Nup58/Nup54/Nup45 complex in the central transporter region of the pore, or with the Nup62/Nup214/Nup88 complex found on the cytoplasmic rim and filaments (Figure 19A). The location of the C-terminus of Nup153 has been shown to be in the central region, as well as the cytoplasmic filaments of the nuclear pore complex by immunoelectron microscopy (Fahrenkrog et al., 2002). The physiological relevance of the interactions between Nup153 and the Nup107-160 complex and between Nup153 and the two Nup62 subcomplexes described here is thus confirmed by the physical locations of the domains of Nup153 and these nucleoporin subcomplexes. This now adds to the molecular understanding of the three-dimensional organization of nucleoporins in the nuclear pore complex.
Brick by brick: changes in the physical organization of the Nup107-160 complex predict nuclear pore structure

The Nup107-160 complex is the largest of all the nucleoporin subcomplexes isolated so far and thus constitutes a fair proportion of the nuclear pore (Belgareh et al., 2001; Cronshaw et al., 2002; Harel et al., 2003b; Loiodice et al., 2004; Vasu et al., 2001; Walther et al., 2003a). The understanding of the organization of proteins within the Nup107-160 complex and where nucleoporins bind this complex will greatly illuminate how individual nucleoporins map to the 3-D structure of the nuclear pore, as visualized by electron microscopy (Akey and Radermacher, 1993; Beck et al., 2004). The physical structure of the Nup84p complex, the Nup107-160 complex homolog in yeast, has been analysed by recombinantly expressing all members of the complex, then visualizing the complex by electron microscopy (Figure 19B; Lutzmann et al., 2002). The yeast Nup84p complex was described as having a “Y-shape”. The yeast homologs of Nup85 and Seh1 form one prong of the Y, while the yeast homolog of Nup160 forms the other prong. The stalk of the Y consists of the yeast homolog of Nup96, followed by Sec13, Nup107 and finally at the end of the stalk, Nup133 (Figure 19B).
The locations of the newly discovered members of the vertebrate Nup107-160 complex, Nup43 and Nup37, which are unique to higher eukaryotes, have not yet been determined in relation to the original complex members (Figure 19B; Loidice et al., 2004). In this present study, the isolation of the Nup107-160 complex using different handles has revealed clues into the organization of the vertebrate Nup107-160 complex in the context of nucleoporin interactions. The immunoprecipitation of Nup43 did not coimmunoprecipitate Nup153 as strongly as with the immunoprecipitation of Nup133 (data not shown). Immunoprecipitating a protein is not merely picking a protein out of the solution. It involves the charging in of the antibody coupled to a monolithic bead. With this picture in mind, the immunoprecipitation of Nup43 most likely caused steric hindrance between Nup153 and the Nup107-160 complex, via Nup43 or another member in the vicinity. Thus, this maps Nup43 on the Nup107-160 complex close to the site of interaction with Nup153 (Figure 19B).

The interaction between the Nup107-160 complex and Nup98 (470-876) revealed clues regarding the structure of the Nup107-160 complex. Nup98 (aa470-876) surprisingly did not bind Nup85, and the binding of Nup160 was very faint (Figure 13, lane 6). Previous studies showed by mass spectrometry
that this fragment of Nup98 bound Nup160, Nup133, Nup107 and Nup96 (Vasu et al., 2001). In addition, this fragment of Nup98 bound less Nup160, as compared to that in Nup107-160 complex isolated by the Nup153 fragment 1-339 (Vasu et al., 2001). Recombinant Nup96 had been shown to bind Nup98 (Rosenblum and Blobel, 1999). Taking the organization of the yeast Nup84p complex into account (Figure 19B), it is plausible that the binding of Nup98 to Nup96 in the complex can cause the two prongs of the Y-shape, Nup85 and Nup160 to fall off, while the rest of the complex members bound to Nup96 on the stalk of the Y-shape remain intact (sec 13, Nup107, Nup133, Figure 19B). The data from the binding characteristics of the Nup107-160 complex members to Nup98 (470-876) in this and previous studies reinforce that the organization between the vertebrate Nup107-160 complex and the yeast Nup84p complex is similar (Figure 19B). All of the binding studies described here add to the understanding of the physical organization of the vertebrate Nup107-160 complex, a critical and large component of the nuclear pore complex.
Summary

In conclusion, the intricate composition of the nuclear pore complex along with the complexities of merging nuclear membrane fusion and nuclear pore assembly highlights the importance of regulatory processes in nuclear pore assembly. Importin beta and RanGTP have been shown here to regulate nuclear pore assembly through the modulation of two distinct interactions between nucleoporin subcomplexes. Importin beta may be modulated by factors other than RanGTP, and future investigations into the nature of this mechanism will deepen our understanding of nuclear assembly.
Figure 1. Nucleoporin subcomplexes and individual nucleoporins that constitute the nuclear pore complex.

A. A schematic of the cross-section of a nuclear pore complex showing the experimentally determined location of nucleoporins. The straight black lines delineate the three main domains of the nuclear pore complex: the cytoplasmic filaments (top), the bulk of the nuclear pore complex that makes contact with the nuclear envelope (middle), and the nuclear basket (bottom). The cytoplasmic filaments of the nuclear pore complex protrude into the cytoplasm (C), while the nuclear basket extends into the nucleoplasm (N). The bulk of the nuclear pore complex is embedded within the double bilayer nuclear envelope (each bilayer represented by a black curved line). The individual nucleoporins (gray boxes) and nucleoporin subcomplexes (black boxes) that make up the nuclear pore complex are placed within the domain of the nuclear pore complex where they are located. Phenylalanine-glycine (FG) repeat-containing nucleoporins are on the right, while nucleoporins without FG repeats are on the left.

B. Working model for nuclear assembly. At the end of mitosis, the nucleus follows a sequence of events to assemble. First, nuclear membranes (green circles) are recruited to chromatin (blue oval). This is followed by the fusion of vesicles or sheets of membranes to form the nuclear envelope (green ovals). Nuclear membrane fusion is inhibited by importin beta and modulated by RanGTP. Nuclear pore complexes then assemble into the fused nuclear membranes. The inset shows a schematic of a nuclear pore complex embedded within the nuclear envelope. Importin beta also negatively regulates nuclear pore assembly, but inhibition is not reversed by RanQ69L (Harel et al., 2003a).
Figure 2. *Xenopus importin beta* shows close homology to human importin beta.

*Xenopus importin beta* was cloned from *Xenopus* RNA using primers designed from overlapping ESTs that showed homology to human importin beta. *Xenopus importin beta* was then sequenced from the cloned His-tagged *Xenopus importin beta*. *Xenopus importin beta* shows very close homology to human importin beta with 94% identities (828/876) and 97% positives (857/876). The amino acid composition, along with the length of the protein, is well conserved between *Xenopus* and human importin beta.
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Figure 3. Anti-Xenopus importin beta antibody recognizes endogenous importin beta.

A. His-tagged Xenopus importin beta was injected into a rabbit to generate polyclonal antibody. The serum from the rabbit was depleted of His-tag antibodies with His-GFP and affinity purified against GST-Xenopus importin beta. The resulting anti-Xenopus importin beta antibody (affinity purified, anti-serum 732) recognizes a distinct band in Xenopus egg cytosol (lanes 1+2) that runs at the same size as the band recognized by a monoclonal antibody against rat importin beta (BD Transduction Laboratories) (lanes 3+4). Increasing amounts of Xenopus egg cytosol (0.25 and 0.5 µL) were run on the gel and probed with the various antibodies.

B. Anti-Xenopus importin beta antibody recognizes endogenous importin beta in reconstituted nuclei. In vitro reconstituted nuclei were assembled, fixed and spun down onto coverslips. The nuclei were probed with anti-Xenopus importin beta antibody, followed by RITC-labeled secondary goat-anti-rabbit antibody. The nuclei were mounted with Vectashield containing DAPI.
Figure 4. Xenopus importin beta prevents nuclear envelope fusion and nuclear pore assembly.

A. The addition of 15 μM His-tagged Xenopus importin beta to a nuclear assembly reaction prevents the nuclear import of GFP-nucleoplasmin (GFP-NP), which supports the lack of nuclear pore assembly as seen in (B). In addition, 15 μM of His-tagged Xenopus importin beta gives a less severe phenotype in terms of the block to nuclear envelope fusion. The bar represents 10 μm.

B. The addition of 25 μM His-tagged Xenopus importin beta to a nuclear assembly reaction blocks nuclear pore assembly, as shown by the absence of FG nucleoporins at the nuclear rim (stained with TAMRA-labeled 414 antibody, which recognizes Nup358, Nup214, Nup153 and Nup62). The lack of a solid membrane rim stain by DHCC shows that nuclear membrane fusion is inhibited. These observations are in accordance with experiments done with recombinant human importin beta in nuclear assembly reactions (Harel et al., 2003). The bar represents 10 μm. The fourth column is a 3x magnification of the membrane stain.
Figure 5. Xenopus importin beta is present on membranes and is removed by RanGTP.

A. Importin beta is present in Xenopus egg cytosol as well as membranes. Equal volumes (0.5 μL) of Xenopus egg membranes (M, lane 1) and cytosol (cyt, lane 2) were separated on a SDS-PAGE gel and probed with anti-Xenopus importin beta antibody.

B. In the presence of RanQ69L, importin beta no longer pellets with Xenopus membranes. Membranes (2 μL) were diluted into 40 mg/mL BSA/ELB blocking buffer and incubated with increasing amounts of His-RanQ69L (5μM and 25 μM RanQ69L, lanes 2+3), or His-GFP (25 μM, lane 4). No recombinant protein was added in the control reaction (lane 1). The reaction was incubated at room temperature for 1 hour, diluted with 100 μL ELB, then spun through a 50 μL sucrose cushion. Membranes that were not diluted nor spun through a sucrose cushion were loaded and blotted to delineate the location of the proteins (M, lane 5).
Figure 6. p97, a factor required for nuclear envelope assembly, does not interact with Xenopus importin beta.

A. In an immunoprecipitation of importin beta from Xenopus egg cytosol (anti-Ximpβ IP, lane 1), endogenous p97 is not coimmunoprecipitated. Cytosol was run in parallel (cyt, lane 2).

B. Recombinant GST-Xenopus importin beta does not bind endogenous p97. GST-Xenopus importin beta beads were incubated with Xenopus egg cytosol in the absence (lane 3) or presence (lane 4) of RanQ69L (15 μM). GST beads were used in the control pulldowns without or with RanQ69L (lanes 1+2, respectively). Cytosol was run in parallel (cyt, lane 5).

C. Recombinant mouse p97 shows no interaction with recombinant Xenopus importin beta in the presence of cytosol. 3 μM GST-Xenopus importin beta (GST-Ximpβ) was incubated with 4 μM His-mouse p97 (His-p97) in Xenopus egg extract diluted 1:20 into PBS, followed by incubation with anti-His antibody beads (lane 1). 3 μM GST (lanes 2+4) and 4 μM His-GFP (lanes 3+4) were added to reactions as controls for the GST and His tag, respectively. The recombinant proteins alone, GST-Xenopus importin beta, His-p97 and His-GFP were separated and probed in parallel to identify where the proteins run (lane 5, 6+7, respectively). The top panel was probed with anti-GST antibody, while the bottom panel was probed with anti-His antibody.
Figure 7. Immunoprecipitation with anti-Xenopus importin beta.

Nucleoporins that bind to endogenous Xenopus importin beta were assessed after immunoprecipitation of importin beta from Xenopus extracts using anti-Xenopus importin beta antibody (β, lane 3). Preimmune antibody immunoprecipitations were done in parallel (PI, lane 2). Cytosol (0.5µL) was run on the same blot and analyzed with the immunoprecipitations (cyt, lane 1). Western blots in A-D were from one experiment, while the Western blot in E was a separate experiment. Proteins that immunoprecipitated with the anti-Xenopus importin beta antibody are highlighted with the diamonds.

A. The Nup107-160 complex members, Nup160, Nup133, Nup85 and Nup43, bound importin beta (lane 3).

B. The FG nucleoporins Nup153 and Nup62 bound to importin beta, whereas Nup98 did not coimmunoprecipitate with importin beta (lane 3).

C. Non-FG nucleoporins; Nup205, Nup155, Nup93 and Nup53 were found not to interact with importin beta (lane 3).

D. Importin beta immunoprecipitates with importin alpha, but not with Ran (lane 3).

E. In a separate immunoprecipitation with Xenopus importin beta (β, lane 3) versus preimmune antibody (PI, lane 2), importin beta specifically binds Nup50 and Tpr. The smear in the preimmune lane when probed with importin beta is due to background from immunoglobulin present in the immunoprecipitation.
Anti-Xenopus importin beta immunoprecipitation

A
Nup107-160 complex
Nup160
Nup133
Nup85
Nup43
1 2 3

B
FG nucleoporins
Nup153
Nup98
Nup62
1 2 3

C
Non-FG nucleoporins
Nup205
Nup93
Nup53
Nup155
1 2 3

D
transport factors
imp β
imp α
Ran
1 2 3

E
imp β
Nup50
Tpr
1 2 3
Figure 8. Pulldown with GST-Xenopus importin beta.

GST-Xenopus importin beta binds similar nucleoporins to those immunoprecipitated with the anti-Xenopus importin beta antibody. GST-Xenopus importin beta bound to GSH-sepharose beads was used to pull down interacting proteins from Xenopus egg cytosol (GST-Xbfl, lane 3). GST pulldowns were done in parallel (GST, lane 2). Cytosol was probed with the same antibodies (0.5μL, cyt, lane 1). The Western blots in A-C were from the same experiment. Proteins interacting with GST-importin beta are highlighted with the diamond.

A. The Nup107-160 complex members Nup160, Nup133 and Nup43 bound to importin beta, but Nup85 did not (lane 3).

B. The FG nucleoporins Nup358, Nup214, Nup153, Nup98, Nup62 and Nup50 and importin beta’s coreceptor, importin alpha, all show interaction with GST-Xenopus importin beta (lane 3).

C. The non-FG nucleoporins (Nup205, Nup93, Nup53, Nup155, Tpr) did not show interaction with GST-Xenopus importin beta (lane 3).
GST-Xenopus importin beta pulldown

A

Nup107-160 complex

Nup160
Nup133
Nup85
Nup43

B

FG nucleoporins

Nup358
Nup214
Nup153

Nup98
Nup62
Nup50

transport factor

imp α

C

Non-FG nucleoporins

Nup205
Nup93
Nup53
Nup155

Tpr

1 2 3 1 2 3
Figure 9. Neither importin beta nor RanGTP disrupt the interactions within members of the critical Nup107-160 complex.

Members of the Nup107-160 complex that were probed (Nup160, Nup133, Nup85, Nup43) maintained interactions with each other even in the presence of importin beta and/or RanQ69L. The Nup107-160 complex members were immunoprecipitated from Xenopus egg extract with anti-Nup43 antibody (A) or Nup133 antibody (B) in the presence of RanQ69L (10 μM, +R, lanes 5), His-human importin beta (15 μM, +hβ, lanes 7) or both (+hβ+R, lanes 6). No recombinant proteins were added in the control reaction (ctl, lanes 4). The mock immunoprecipitations were done with unrelated rabbit antibody in the absence or presence of recombinant proteins (lanes 1-3). The lane with Xenopus egg cytosol alone (0.25 μL, cyt, lanes 8) indicates where proteins would have been detected if bound. RanQ69L and human importin beta recombinant proteins alone were separated to identify crossreactivity of the recombinant proteins with the antibodies (lanes 9+10, respectively). The absence of importin beta in the presence of RanQ69L indicates that the RanQ69L was effective in abrogating the interaction between importin beta and the Nup107-160 complex.
Figure 10. The interaction between Nup62 and Nup214 is maintained in the presence of importin beta and/or RanGTP.

Nup62 was immunoprecipitated from Xenopus egg cytosol in the presence of 10 μM RanQ69L (+R, lane 5), 15 μM human importin beta (+hβ, lane 7), both (+hβ+R, lane 6) or without any recombinant protein (ctl, lane 4). 0.25 μL and 0.5 μL of cytosol were blotted in parallel (cyt, lanes 8+9, respectively), along with recombinant proteins alone to test for cross-reactivity (RanQ69L, R, lane 10; human importin beta, hβ, lane 11). Unrelated rabbit antibody was used to mock immunoprecipitate from cytosol containing 10 μM RanQ69L (lane 2), 15 μM human importin beta (lane 3) or no recombinant protein (lane 1).
Figure 11. Importin beta blocks the RanGTP induced interaction between Nup153 and the Nup107-160 complex.

Nup153 binds the Nup107-160 complex upon addition of RanQ69L. This interaction is disrupted with the addition of importin beta. The Nup107-160 complex was isolated by immunoprecipitating with anti-Nup133 antibody. In the immunoprecipitation reactions, recombinant RanQ69L (10 μM, +R, lane 5), His-human importin beta (+hβ, lane 7, 15 μM) or both (+hβ+R, lane 6) proteins were added to access the effect of RanGTP and importin beta on interaction between the Nup107-160 complex and other proteins. No recombinant proteins were added in the control reaction (ctl, lane 4). Unrelated rabbit antibody was used to mock immunoprecipitate in the absence or presence of recombinant proteins (lanes 1-3). Xenopus egg cytosol (0.25 μL) was separated in parallel to identify the proteins recognized by the various antibodies (cyt, lane 8). The recombinant proteins alone were separated on SDS-PAGE along with the immunoprecipitations to identify which bands cross react with the recombinant proteins (RanQ69L and human importin beta, lanes 9+10, respectively).
Figure 12. RanGTP does not induce interaction between the Nup107-160 complex and non-FG nucleoporins.

The Nup107-160 complex was isolated with anti-Nup133 (A) or anti-Nup43 (B) antibody. The addition of 10μM RanQ69L did not cause interaction between the Nup107-160 complex and the non-FG nucleoporins tested (Nup205, Nup155, Nup93, Tpr; lanes 5). The addition of importin beta alone (15μM, +hβ, lanes 6) or both importin beta and RanQ69L (+hβ+R, lanes 5) did not induce any interaction with the Nup107-160 complex. The mock immunoprecipitations were done with unrelated rabbit antibody in the absence or presence of recombinant proteins (lanes 1-3). Cytosol (0.25μL, cyt, lanes 8) was run in parallel to delineate where the proteins run.
no regulation by importin β or RanGTP

Nup107-160 complex  non-FG Nups
Figure 13. Importin beta and RanGTP do not affect the interaction between Nup98 and the Nup107-160 complex.

The Nup98 (aa470-876) fragment isolates a partial Nup107-160 complex that is not responsive to the addition of importin beta or RanQ69L. A fragment of Nup98 (aa470-876) was coupled to cyanogen-bromide beads (lanes 6-9). GFP coupled to cyanogen bromide beads were processed in parallel (lanes 2-5). Recombinant RanQ69L (25 μM, +R, lanes 3 and 7), His-human importin beta (15 μM, +hβ, lanes 4 and 8), His-human importin beta aa 45-462 (15 μM, +β*, lanes 5 and 9) or no recombinant protein (ctl, lanes 2 and 6) were added to the beads in the presence of diluted Xenopus egg cytosol in PBS (1:50). Xenopus egg cytosol alone was run in parallel (0.5 μL, cyt, lanes 1+10). The recombinant proteins RanQ69L (R, lane 11), human importin beta (hβ, lane 12) and human importin beta aa 45-462 (β*, lane 13) alone were separated on SDS-PAGE along with the immunoprecipitations to identify which bands cross react with the recombinant proteins.
no regulation by importin β or RanGTP

Nup107-160 complex → Nup98
Figure 14. Nup98 does not show interaction with additional nucleoporins.

The Nup98 (aa470-876) fragment showed no interaction with the FG nucleoporins, Nup214, Nup153, Nup62 and Nup50, or non-FG nucleoporins, Nup93 or Nup53 (ctl, no recombinant protein added, lane 6). The addition of RanQ69L (25 μM, +R, lane 7) did not induce any interactions of these nucleoporins with Nup98. His-human importin beta (15 μM, +hβ, lanes 4 and 8) and His-human importin beta aa 45-462 (15 μM, +β*, lanes 5 and 9) were also added to parallel reactions. Pulldowns done with GFP coupled to cyanogen bromide beads served to show the specificity of the Nup98 pulldowns (lanes 2-5). The pulldowns were done in Xenopus egg cytosol diluted 1:50 in PBS. The Xenopus egg cytosol lanes serve to denote each nucleoporin probed (0.5 μL, cyt, lanes 1+10). The recombinant proteins RanQ69L (R, lane 11), human importin beta (hβ, lane 12) and human importin beta aa 45-462 (β*, lane 13) alone were separated on SDS-PAGE along with the immunoprecipitations to identify crossreacting bands from the recombinant proteins.
Figure 15. Importin beta abrogates the RanGTP-induced interaction between Nup62 and Nup153.

A. RanQ69L induces the interaction between Nup62 and Nup153. Nup62 interacts with importin beta (ctl, lane 1, highlighted with the dot). The addition of 10 μM RanQ69L (+R, lane 2) releases importin beta, and causes the binding of Nup153 (highlighted with the diamond). 0.25 μL of Xenopus egg cytosol was loaded to identify the proteins that were probed for (cyt, lane 3).

B. Importin beta blocks the RanQ69L-induced interaction between Nup62 and Nup153. Nup62 was immunoprecipitated in the presence of RanQ69L (+R, lane 5), human importin beta (+hβ, lane 7), both (+hβ+R, lane 6) or no recombinant protein (ctl, lane 4). Unrelated rabbit antibody was used in the mock immunoprecipitations with (+R, 10μM RanQ69L, lane 2; +hβ, 15μM human importin beta, lane 3) or without addition of recombinant proteins (ctl, lane 1). 0.25μL and 0.5μL of cytosol was run and blotted in parallel to identify the endogenous proteins (lanes 8+9). The recombinant proteins alone were run and blotted in parallel (R, RanQ69L, lane 10; hβ, human importin beta, lane 11). The diamond highlights the interaction characteristics between Nup153 and Nup62 when RanQ69L and/or human importin beta were added to the immunoprecipitation.
Figure 16. Nup153 immunoprecipitation confirms a distinct interaction with Nup62.

A. Schematic of Nup153: binding partners and antibody against Xenopus Nup153. Human Nup153 is divided into three domains: a unique N-terminus (aa 1-649, dark gray rectangle), a zinc finger domain (aa 650-895, light gray rectangle), and a FG (phenylalanine-glycine) rich C-terminus (aa 881-1475, black wavy line). Proteins bind Nup153 in different regions, as represented by black lines: the Nup107-160 complex (aa 1-339), Ran (aa 610-895), and importin beta (C terminus FG repeat domain). The epitope (black line) of anti-serum 380 made against Xenopus Nup153 (Y) is illustrated here in alignment with human Nup153. Anti-serum 380 was made against a fragment of Xenopus Nup153 corresponding to human Nup153 aa 431-732. (note: Amino acid numbers correspond to human Nup153, so if interaction with Nup153 was determined in a species other than human, the approximate corresponding amino acids to human Nup153 were used.)

B. Nup153’s interaction with Nup62 is affected by the addition of importin beta, but not by the addition of RanQ69L. Nup153 was immunoprecipitated with antibody made against a fragment of Xenopus Nup153 that corresponds to human Nup153 aa 431-723 (anti-serum 380, IgG). The diamonds highlight Nup153’s interaction characteristics with Nup62, Nup214 and importin beta. 10 μM RanQ69L (+R, lane 5), 15 μM human importin beta (+hβ, lane 7), both (+hβ+R, lane 6) or no recombinant proteins (control, ctl, lane 4) were added to the immunoprecipitation reaction in 1:25 diluted Xenopus egg cytosol in PBS. Mock immunoprecipitations with unrelated rabbit antibody were done in the presence of 10μM RanQ69L (+R, lane 2), 15μM human importin beta (+hβ, lane 3) or no recombinant proteins (ctl, lane 1). 0.25 μL and 0.5 μL Xenopus egg cytosol (cyt, lanes 8+9, respectively) were run in parallel to delineate the endogenous proteins, while recombinant proteins (RanQ69L, R, lane 10; human importin beta, hβ, lane 11) were run to distinguish crossreacting bands.
Figure 17. Model of nuclear assembly: nuclear membrane fusion.

Importin beta and p97 both affect nuclear membrane fusion, but they do not interact with each other to modulate nuclear membrane fusion. Importin beta is a negative regulator of nuclear membrane fusion (Harel et al., 2003a), whereas p97 is required for nuclear membrane fusion (Hetzer et al., 2001). This points to the possibility that importin beta and p97 are involved in two distinct pathways that converge to regulate nuclear membrane fusion.
Figure 18. Model of nuclear assembly: importin beta and RanGTP modulates interactions between a subset of nucleoporins.

At the end of mitosis, soluble nucleoporins need to incorporate into the nuclear pore. In this study, importin beta binding proteins were identified. It was shown that release of importin beta (β, orange circles) by the addition of RanQ69L induced interaction between the Nup107-160 complex and Nup153, and a novel interaction between Nup62 and Nup153. Additional interactions involving other nucleoporins and membranes are required for the nuclear pore to form, as denoted by the gray question marks.
Figure 19. Models of nuclear pore structure based on the physical organization of nucleoporins

A. A working model of Nup153’s interactions with nucleoporin subcomplexes in the context of the nuclear pore. Represented in this model are the three domains of Nup153 in the context of nucleoporins it binds to (this study and Vasu et al., 2001) and the localization of Nup153 as determined by immunoelectron microscopy (Fahrenkrog et al., 2002). The unique N terminal domain of Nup153 (purple rectangle) binds the Nup107-160 complex. The zinc finger domain of Nup153 (mahogany rectangle) localizes to the ring of the nuclear basket by immunoelectron microscopy. The FG-rich C-terminal domain of Nup153 (red wavy line) is postulated to bind to the two Nup62 complexes: the Nup62/Nup58/Nup54/Nup45 complex in the central portion of the nuclear pore complex, and the Nup62/Nup214 complex at the cytoplasmic filaments. The nucleoplasm and cytoplasm are denoted as N and C.

B. A putative model of the organization of nucleoporins within the vertebrate Nup107-160 complex (right) is shown in comparison to the established organization of the related yeast Nup84p complex (left, based on Lutzmann et al., 2002). The vertebrate Nup107-160 complex contains two members that have no homologs in yeast, Nup43 and Nup37. The location of these two proteins in relation to the other members of the complex has not yet been determined.

The interaction of the Nup107-160 complex with Nup98 revealed that Nup98 may interfere with interactions between Nup85, Nup160 and the rest of the Nup107-160 complex. Nup98’s hypothetical site of interaction is shown by the black arrow.

Nup43 may be found in close proximity to Nup153, as the immunoprecipitation of the Nup107-160 complex by anti-Nup43 antibody coimmunoprecipitates less Nup153 compared to anti-Nup133 antibody, even though the same amount of complex is present in both. The anti-Nup43 antibody may sterically hinder interaction between the Nup107-160 complex and Nup153.
Acknowledgements, Chapter 2

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References


Chapter 3

Characterization of the Transmembrane Nuclear Pore Protein, gp210

Abstract

gp210 is a transmembrane protein of the nuclear pore complex. The bulk of this protein of ~210kD is found within the nuclear envelope lumen. A single transmembrane domain connects to with a short cytoplasmic domain. A major question in the field has been the function of gp210 in the nuclear pore complex. Here, Xenopus gp210 has been characterized and investigated using a Xenopus egg extract nuclear reconstitution system. Several Xenopus gp210 tail sequences were found, of which one is physiologically relevant and shows homology across species. The potential role of gp210 in nuclear assembly was investigated. Functional studies were carried out where antibodies against the cytoplasmic domain of Xenopus gp210 or Xenopus gp210 protein fragments were added into nuclear reconstitution reactions and
assessed for effect on nuclear assembly and nuclear function. gp210 was found not to function in nuclear assembly. A new direction for gp210’s function and a model for the progression of gp210 through nuclear assembly are discussed.
Introduction

In the cell, the genomic material is enclosed by a double membrane structure lined with a nuclear lamina, termed the nuclear envelope. Information, in the form of RNA and proteins, constantly traffics between the nucleus and the cytoplasm through nuclear pore complexes. This occurs through the thousands of nuclear pores in vertebrates (Maul, 1977), or approximately one hundred nuclear pores in yeast (Winey et al., 1997) that perforate the nuclear envelope of one nucleus.

The nuclear pore complex: a protein complex in a sea of membrane

Each nuclear pore complex is a proteinaceous structure of 60-125 megadaltons that is integrated with the double membranes of the nuclear envelope. The ability of the nuclear pore complex to embed in the nuclear envelope is hypothesized to be due to the integral membrane proteins that are part of its structure (Figure 1A). There are two known transmembrane proteins that have been biochemically isolated with the vertebrate nuclear pore complex, gp210 and POM121. In the yeast nuclear pore complex, there are three known transmembrane proteins: POM152, POM34, and Ndc1p.
Surprisingly, there is no homology between the vertebrate and yeast nuclear pore complex transmembrane proteins.

**gp210: a transmembrane nucleoporin**

gp210 (glycoprotein of 210 kDa) was first isolated from rat liver nuclear envelopes and initially named gp190 (Gerace et al., 1982). The rat protein, which migrated at 190kDa on an SDS-PAGE protein gel, was resistant to extraction by 2% Triton X-100, 6 M urea and 0.1 N NaOH, thus implicating it as an integral membrane protein. It is a glycoprotein, as the luminal domain binds ConA and Lens culinaris agglutinin (lectins that bind glucosyl and mannosyl residues). It does not contain sialyl or terminal N-acetylglucosaminyl residues, as seen by its inability to bind wheat germ agglutinin (WGA). The complete gene encoding rat gp210, renamed for its predicted molecular weight, was cloned from cDNA (Wozniak et al., 1989). Through biochemical experiments, it was determined that the bulk of gp210 is hidden in the nuclear envelope lumen, followed by a single transmembrane domain and a short cytoplasmic domain of 58 amino acids at the C-terminal end of the protein (Greber et al., 1990). In contrast, POM121, has a short N-terminal domain in the lumen of the nuclear envelope, followed by a single
transmembrane domain and a large C-terminal domain of ~100kDa protruding into the cytoplasm (Soderqvist and Hallberg, 1994).

**gp210: an integral part of the nuclear pore complex**

gp210 is relatively abundant in the nuclear pore. The copy number of gp210 in each nuclear pore is estimated to be around 16, as determined by quantitation of gp210’s SDS-PAGE band intensity from purified nuclear pores of rat liver nuclei and by the assumption that nucleoporins are present in multiples of eight due to the nuclear pore complex’s 8-fold symmetry (Cronshaw et al., 2002). The large amount of gp210 is in comparison to the estimation of 8 copies of POM121 per nuclear pore (Cronshaw et al., 2002).

The exact location of gp210 and POM121 within the nuclear pore complex is not known. Early immunoelectron micrographs show the presence of gp210 on both cytoplasmic (85-90%) and nucleoplasmic (10-15%) regions of nuclear pores in rat liver nuclei (Gerace et al., 1982). However, the antibody used in that study was generated against the full length protein, so it not clear which epitope is being detected. gp210 and POM121 are assumed to make contacts with the rest of the nuclear pore complex, thus placing them in the central region of the nuclear pore complex (Figure 1A).
The search for the function of gp210

The function of gp210 was first investigated by the introduction of antibodies to the lumenal domain of gp210 into the nuclear lumen in tissue culture cells (Greber and Gerace, 1992). This was achieved by stable transfection of the anti-gp210 lumenal antibody into tissue culture cells. A decrease in nuclear import was observed. This study showed that interference with the lumenal domain of gp210 translates to an effect on nuclear import. The importance of gp210 in the functioning of the human body was elucidated by the presence of anti-gp210 autoimmune antibodies in patients with primary biliary cirrhosis (Nickowitz and Worman, 1993). However, the precise function of gp210 within the nuclear pore complex was still unresolved.

gp210 is one of the two vertebrate transmembrane proteins. Its permanent residence in the membranes gives it an added advantage of being in the location where future nuclear pore complexes will assemble. Nuclear assembly can be studied in isolation from other cellular processes using the Xenopus egg extract nuclear reconstitution system. In this system, the steps in nuclear assembly can be clearly visualized and characterized, from the
decondensation of the chromatin to the recruitment of membranes, followed by fusion of the nuclear envelope and the formation of nuclear pore complexes. This is not possible in tissue culture cells, where the disruption of nuclear assembly results in cell death. Rat, mouse and human gp210 sequences were available, and very distantly related Drosophila and C. elegans homologs have also been identified (Berrios et al., 1995; Cohen et al., 2001). In order to study potential roles of gp210 in nuclear assembly using the Xenopus nuclear reconstitution system, it was most relevant to analyze Xenopus gp210.

Thus, this chapter starts with the identification of Xenopus gp210 and the ensuing journey of clarifying assumptions the field has held onto for the past 20 years, whilst crossing paths with competitors who have published results that the work presented below has either disproved or validated. As such, this chapter is a compendium of results that will not be published in its present form, but has generated valuable tools and observations that are spearheading provocative questions to understand the assembly and disassembly of the nucleus.
Materials and Methods

Sequencing and cloning Xenopus gp210

A partial Xenopus gp210 sequence, AW642061, was found in the Xenopus EST database by BLAST searches against rat gp210, and was sequenced from the Xenopus EST clone PBX0113E06 (Research Genetics). The EST clone PBX0113E06 is derived from the Blackshear/Soares Xenopus normalized egg cDNA library cloned into the pT7T3-Pac vector (ampicillin resistant). The size of the EST clone was determined by a double restriction digest with EcoRI and NotI restriction enzymes. T7 and T3 primers were used initially to sequence the insert of PBX0113E06. Forward primers F2 (5’-CCCGAATTCTTCAGTGTGGAGGCAGGCC-3’, with a 5’ BamHI site) and F3 (5’-CCAGGGATCCCTGTCTAC-3’) were designed to confirm sequence of the PBX0113E06 clone downstream of AW642061.

The sequence of PBX0113E06 was used to generate primers for reverse transcription, and primers to PCR fragments of Xenopus gp210 from Xenopus RNA. To clone a fragment of the luminal domain (PCR-xgp210) into pET28a, Xenopus RNA was reverse transcribed with the xgp210RT primer (5’-GTTCCGCTCAGTCTC-3’), followed by PCR with the forward primer
F1xgp210 (5’-CCCGAATTTCGATATATTTTCTGGCTGGCTCCC-3’, with 5’ BamHI site) and the reverse primer xgp210R (5’-CCCCTCGAGGAGGTTTGTATGGAGAGGGTG-3’, with 3’ XhoI site). The entire C terminus tail 1 (x210C1) of Xenopus gp210 was reverse transcribed with the RT EST primer (5’-CCAATGGACAGTTTG-3’), followed by PCR with the forward primer F4 lumen (5’-CCCGAATTTCACCCAGAGAGACCAAC-3’, with 5’ BamHI site) and reverse primer R EST (5’-CCCCTCGAGCAGTAACCTCTG-3’, with 3’ XhoI site). The insert and vector was double digested with BamHI and XhoI, ligated and transformed into bacteria for plasmid maintenance or protein expression.

The Xenopus tail 2 sequence was cloned using sequences from the Xenopus EST BF613564, and encompasses the sequence past the putative transmembrane domain to the stop codon. Xenopus tail 2 was cloned by reverse transcribing with the BF64RT2 primer (5’-CTCAGCAACACACTCGC-3’), followed by PCR with the forward F5 primer (5’-CCCGAATTTCACCCAGAGAGACCAACGTTC-3’, with BamHI site) and reverse primer BF64R2 (5’-CCCCTCGAGGATGGGCTGGCTTTCTA-3’, with XhoI site). The insert and vector (pET28a) was double digested with BamHI and XhoI, followed by ligation and transformation into bacteria for plasmid
maintenance or protein expression. The Xenopus tail 2 sequence was also cloned into the GST vector and the zz-pET28a vector by taking the insert originally cloned into the pET28a vector.

**Protein expression**

A His-tagged Xenopus gp210 lumenal domain fragment was expressed from bacteria containing pET28a-210UPSRT, grown to log phase, then induced with 1mM IPTG for 4.5 hrs at 30°C. The expressed gp210 protein fragment was found to be insoluble, so the protein was purified in urea. The bacterial pellet was sonicated in 0.5M NaCl, 0.02M Tris, pH 8, 5mM imidazole. The sonicated bacteria was spun at 13000 rpm for 15 mins, after which the pellet, containing the insoluble protein, was resuspended in buffer containing urea (8M urea, 0.5M NaCl, 5mM imidazole). The lysate was spun again, after which the supernatant was run over Ni-agarose beads. The Ni-agarose beads were washed with 2 column volumes of 5mM imidazole wash buffer (5mM imidazole, 0.5M NaCl, 7.2M urea), followed by 1 column wash of 15mM imidazole wash buffer (15mM imidazole, 0.5M NaCl, 7.2M urea). Finally the column was washed with 5mLs of 30mM imidazole wash buffer (30mM imidazole, 0.5M NaCl, 7.2M urea). The gp210 lumenal domain protein
fragment was eluted with elution buffer containing urea (100mM imidazole, 0.5M NaCl, 7.2M urea).

Insoluble His-tagged Xenopus gp210 tail 1 protein fragment was expressed by taking a plate of bacterial colonies transfected with the construct, expanding the culture into 500mL LB and growing to log phase. The culture was then induced with 1mM IPTG for 5 hrs at 30°C. The bacterial pellet was resuspended in binding buffer with urea (5mM imidazole, 50mM Tris pH 8, 0.5M NaCl, 7.2M urea). The resuspended bacteria was then sonicated and pelleted. The supernatant was run over a Ni-agarose column. The Ni-agarose was washed sequentially with 1 column volume each of binding buffer containing 5mM, 15mM and then 30mM imidazole. Finally, the His-tagged Xenopus gp210 tail 1 protein fragment was eluted with binding buffer containing 500mM imidazole.

His-tagged Xenopus gp210 tail 2 protein fragment was expressed by first transforming bacteria with the pET28a-210C2stop plasmid. The whole plate of bacterial colonies were then scraped into 1L LB with kanamycin. The culture was grown until the OD600= 0.6. The culture was then induced with 1mM IPTG overnight at room temperature. The protein was insoluble, so the resulting bacterial pellet was resuspended in binding buffer with urea (5mM
imidazole, 50mM Tris, pH 8, 0.5M NaCl, 7.2M urea), sonicated and pelleted. The supernatant was purified on Ni-agarose, and washed with 2 column volumes of urea binding buffer with 5mM imidazole, then 1 column volume of urea binding buffer with 15mM imidazole, and finally with 1 column volume of urea binding buffer with 30mM imidazole. The His-tagged Xenopus gp210 tail 2 protein fragment was eluted with urea binding buffer with 500mM imidazole, then concentrated.

zz-tagged Xenopus gp210 tail 2 protein was expressed for functional studies. Soluble zz-tagged Xenopus gp210 tail 2 protein was expressed by growing the pET28a-zz-210C2stop bacterial culture up to log phase, followed by induction with 1mM IPTG at 37°C for 5 hrs. The bacterial pellet was then resuspended in binding buffer with no urea (5mM imidazole, 10mM sodium phosphate, pH 8, 0.5M NaCl), sonicated and pelleted. The supernatant was purified over Ni-agarose beads, and washed with 1 column volume binding buffer, then 1 column volume of binding buffer with 15mM imidazole, and lastly with 1 column volume of binding buffer with 30mM imidazole. The protein was eluted with binding buffer with 500mM imidazole, dialyzed once in PBS overnight at 4°C, then in PBS/5% glycerol for 2 hrs at 4°C. The protein was then concentrated and frozen in liquid nitrogen.
Generation and purification of antibodies

Denatured His-tagged Xenopus gp210 lumen protein (in 7.2M urea, 0.5M NaCl, 100mM imidazole) was injected into rabbit #592. 130μg was used in the first injection, while 100μg was used in the second, and then 50μg in all subsequent injections. Denatured His-tagged Xenopus gp210 tail 1 protein (200μg) was run on a preparatory gel (1.5mM thick) to isolate the specific band in the gel correlating to His-tagged Xenopus gp210 tail 1.

Denatured His-tagged Xenopus gp210 tail 2 protein (in 7.2M urea, 50mM Tris pH 8, 0.5M NaCl, 500mM imidazole) was injected into rabbit #619. Serum was depleted separately of His-tag antibody by running the serum through a column of His-tagged GFP coupled to cyanogen bromide beads. The subsequent His-tag depleted anti-Xenopus gp210 lumen serum was affinity purified on columns of His-tagged Xenopus lumen protein coupled to cyanogen bromide beads. Anti-Xenopus gp210 tail 2 antibody was affinity purified on cyanogen bromide beads coupled to GST-Xenopus gp210 tail 2 protein. The affinity purified antibody was dialyzed once in PBS, then into 5% glycerol/PBS, followed by concentration in microconcentrators.
The rat gp210 lumen antibody was raised against a fragment of rat gp210 corresponding to amino acids 667-1180. The peptide Xenopus tail 1 long antibody (anti-serum 3860) was a gift from Drs. Sheona Drummond and Katherine Wilson (Drummond and Wilson, 2002). The antibody against POM121 was described elsewhere (Harel et al., 2003b). Nup93 and Nup53 polyclonal antibodies were affinity purified with the recombinant protein they were generated against.

**Nuclear reconstitution**

To assess the role of gp210 in nuclear assembly, nuclear reconstitution reactions were set up with the addition of anti-Xenopus gp210 tail 2 antibody or Xenopus gp210 protein fragments.

The stated concentration of anti-Xenopus gp210 tail 2 antibody, preimmune antibodies, or buffer (2.5μL) were added to Xenopus egg cytosol (20μL), a regenerating energy mix (0.88μL of 4μL creatine kinase + 3.2μL phosphocreatine + 1.6μL ATP), 1μL of Xenopus membranes (non-salt washed), and finally 1μL of 25000 units of sperm chromatin. The reaction was allowed to incubate for 1 hr at room temperature while nuclei assembled, after which GFP-nucleolusmin was added to a 1:10 dilution for assessment of nuclear
import. After mixing, the reaction was incubated for a further 30 mins at room temperature. The nuclei were then fixed for viewing (5μL sample + 2.5 μL fix; fix = 72μL 16% paraformaldehyde + 12.5μL 1M sucrose + 0.2μL 1mg/mL Hoechst) on a Zeiss Axioskop fluorescence microscope with a 63X objective.

Recombinant fragments of Xenopus gp210 tail 2 were added to nuclear reconstitution reactions to assess the effect on nuclear assembly or import. zz-tagged Xenopus gp210 tail 2 or the zz-tag alone (24.5μM) were preincubated with 20μL of cytosol for 15 mins at room temperature. Afterwards, a regenerating energy mix (0.88μL of the energy mix described above), 0.5μL membranes and ~4700 units of sperm chromatin (final) were incubated for 30 mins at room temperature. The nuclei were then incubated with 0.5μL GFP-nucleoplasmin and 0.5μL importin beta aa45-462-TAMRA for 30 mins at room temperature to assay for nuclear import and presence of nuclear pores, respectively.

**Time course of nuclear reconstitution**

To localize gp210 in reforming nuclei over a time course, separate nuclear reconstitution reactions were set up for each time point. In each reaction, 10 μL Xenopus egg extract was incubated with 0.5μL energy mix
(3.6μL phosphocreatine, 4.5μL creatine kinase, 1.8μL ATP), 0.25μL glycogen, 0.5 μL membranes and finally, 0.5μL of 50000 U/μL sperm chromatin. At the time point, the reaction was placed on ice for 10 mins, followed by addition of EGS crosslinker (Ethylene glycolbis(succinimidylsuccinate; Pierce) for 40 mins at room temperature. The nuclei were then spun for 15 mins onto poly-lysine coverslips. The coverslips were fixed and blocked before incubating in primary antibody overnight at 4C. The next day, the coverslips were washed 2x with PBS and incubated in secondary antibody (1:500) for 1 hr at room temperature. The coverslips were then washed 3x for 5 mins in PBS, before mounting in Vectashield with DAPI and sealing the coverslips with nail polish.

**BAPTA rescue**

To assay for a specific effect on nuclear pore assembly, BAPTA (1,2-bis(o-aminophenoxy)ethane-N,N,N’,N’-tetraacetic acid) nuclear intermediates were used. BAPTA addition causes the production of fused nuclear membrane intermediates which contain no nuclear pore complexes (Macaulay and Forbes, 1996). Firstly, BAPTA nuclei were made by incubating 20μL Xenopus cytosol with 1μL regenerating energy mix (1.8μL phosphocreatine, 2.25μL creatine kinase, 0.9μL ATP), 0.5μL glycogen, 1μL Xenopus membranes
and 1µL of 50,000 units sperm chromatin/µL. The reaction was incubated at room temperature for 1 hour. The BAPTA nuclear intermediates (2µL) were then distributed into new tubes containing fresh cytosol (13µL), regenerating energy mix (1µL), and finally either antibody, control buffer (5% glycerol/PBS), or BAPTA. The reactions were incubated for 45 mins at room temperature. The nuclei were then processed for indirect immunofluorescence.

**Immunofluorescence on reconstituted nuclei**

gp210 was localized in reconstituted nuclei by indirect immunofluorescence. One nuclear reconstitution reaction of 10-20 µL is sufficient per coverslip for analysis by indirect immunofluorescence. After nuclear reconstitution reactions were completed, they were gently fixed in 0.8mL EGS/ELB for 40 mins at room temperature. 400µL of sucrose cushion per reaction was prepared (0.75M sucrose in 1xELB). Poly-lysine coverslips were prepared by preparing a solution of 0.1mg/mL polylysine in H2O, placing a coverslip on top of 70µL for at least 15 minutes, then air-drying the coverslip before placing the poly-lysine coated side face up in a karyospinner apparatus. After placing the coverslip in the karyospinner, 400µL of sucrose cushion was layered on top. When the reactions finished incubating in the
EGS fix, each reaction was layered on top of a sucrose cushion in a karyospinner. The karyospinner was spun in the clinical centrifuge at setting #3 for 15 minutes. The coverslips were then washed 3 times with PBS, with the sample face up, in 12-well tissue culture plates filled with PBS. The coverslips were moved from one well into the next, without aspirating the solutions away. This prevented shearing of the fragile reconstituted nuclei. The coverslips were then fixed by placing the sample face down onto 70μL fix (4% paraformaldehyde, 1x PBS) for 10 minutes at room temperature. The coverslips were washed three times, then blocked in 10% fetal calf serum, 1x PBS, 0.1% TX100 for 10 minutes at room temperature. The coverslips were then washed two times with PBS, before incubating with primary antibody. Primary antibody solutions were prepared in the block solution, with 30μL of antibody solution per coverslip. Samples were incubated face down on the drop of antibody solution on parafilm for 1 hr at room temperature. The coverslips were washed two times in PBS, then incubated with 30 μL secondary antibody solution per coverslip, for 1 hour at room temperature. The secondary antibody incubation was done in a foil-covered tissue culture dish to ensure the fluorescently labeled antibody would not be quenched.
Immunofluorescence on tissue culture cells

gp210 was visualized in cells by indirect immunofluorescence. Tissue culture cells processed for immunofluorescence were grown overnight or longer on coverslips to achieve ~70% confluency. Coverslips were rinsed 2x with PBS* (1xPBS, 1mM MgCl₂), followed by a 5 min incubation in fix (4% paraformaldehyde/ PBS*). Coverslips were then washed 2 times in PBS, followed by three washes in 0.2% TX100/PBS to permeabilize the cells. Before incubating with antibody, the cells were blocked by incubation with 5% fetal calf serum/0.2% TX100/PBS for 10 mins. The cells were then incubated in primary antibody for 1 hr at room temperature, followed by 5% fetal calf serum/PBS washes: 3 times briefly, then 3 times 5 minutes. The cells were then incubated in secondary antibody for 1 hr at room temperature. The cells were then washed with 5% fetal calf serum/PBS: 3 times briefly, followed by 3 times 5 minutes. Finally, the cells were mounted with Vectashield with DAPI and viewed on the fluorescence microscope.

Immunoprecipitation of gp210 from membranes

Anti-Xenopus gp210 lumen antibody was prebound to protein A sepharose beads for 1 hr at room temperature (24 µg antibody/40 µL protein A
beads in a total of 500µL PBS). The antibody was crosslinked to the beads with 5 mg/mL DMP in 0.2M sodium borate pH 9 for 30 mins at room temperature. The reaction was then quenched with 0.2M ethanolamine pH 9 for 30 mins at room temperature. The antibody-beads were then mock eluted with 200 µL 0.1M glycine, pH 2.5, and washed 2x with PBS.

Xenopus membranes were solubilized in SDS and TX100. 10 µL of membranes was diluted into 340 µL PBS and 35 µL 10% SDS. The membranes were then subsequently diluted with 7315 µL 1.05% TX100 for a final concentration of 0.05% SDS and 1% TX100.

The antibody-beads and membranes were then incubated together for 2 hours at room temperature. The beads were then washed 2x with RIPA buffer (150 mM NaCl, 1% TX100, 0.5% deoxycholate, 0.1% SDS, 50mM Tris, pH 8) and once with 5mM Tris pH 8. The last wash was spun out, and the immunoprecipitate was eluted with 40 µL 0.1M glycine, pH 2.5, followed by addition of 8µL 1M Tris, pH 8 and 14.7µL 4x sample loading buffer. 14µL of each sample was loaded for SDS-PAGE (~1:4.5 of the total immunoprecipitation).

**Antibody binding membrane assay**
1.375 µg of antibody (preimmune or anti-Xenopus gp210 tail 2) was diluted into 55 µL 2% BSA/PBS, incubated at room temperature for 20 mins. The diluted antibody was spun at 10,000 rpm for 5 minutes to pellet any precipitate. 5 µL of membranes was preincubated with 47.5 µL of 2% BSA/PBS for 20 mins at room temperature. 47.5 µL each of the antibody solution and membrane solution were mixed together and incubated at room temperature for 30 mins, then placed on ice. The total reaction was spun through 30 µL of sucrose cushion at 20,000 rpm for 20 minutes. The pellet was resuspended in 50 µL of sample loading buffer, in each lane, 2.9 µL of sample was loaded.
Results

The two tails: evidence for two distinct Xenopus gp210 cytoplasmic tail sequences

In the search for a Xenopus homolog of gp210, a relatively short Xenopus laevis EST sequence was found by BLAST searching of the NIH GenBank nr database that showed homology to rat gp210. This Xenopus laevis EST sequence, AW642061 (sequenced from the Blackshear/Soares normalized Xenopus egg cDNA library), showed homology to rat gp210 in a short stretch of 167 amino acids upstream of the transmembrane domain (Identities = 78/167 (46%), Positives = 113/167 (66%), Figure 2A). The complete clone (PBX0113E06, Research Genetics) from which the AW642061 EST sequence was derived, contains a 1.2 kb insert. DNA sequencing of this clone revealed sequence downstream of AW642061. Surprisingly, when the additional Xenopus sequence was aligned with rat gp210, the sequence showed no homology to rat gp210 downstream of the transmembrane domain and was also significantly shorter than rat gp210 (Figure 2B). This was unexpected, as it was shown that the C terminus tail of rat gp210 is one
determinant for the correct localization of gp210 to the nuclear pore complex (Wozniak and Blobel, 1992).

As the Xenopus EST database grew, I found an additional Xenopus EST with homology to rat gp210 by BLAST searches. BF613564 showed homology to the previous Xenopus EST sequence (PBX0113E06) in the lumenal and transmembrane sequence, but 9 amino acids after the transmembrane domain, the sequence of BF613564 diverged (data not shown). Upon careful examination of the DNA sequence of BF613564 in all three frames, if a frameshift was introduced, the cytoplasmic tail domain of BF613564 would show close homology to the rat cytoplasmic tail. Subsequent cloning of the cytoplasmic tail domain based on the BF613564 sequence and DNA sequencing confirmed that the BF613564 sequence did not accurately represent the endogenous sequence, as it contained a frameshift. Thus, another distinct Xenopus cytoplasmic tail sequence existed, which I term “tail 2” (Figure 3A and C). In relation to this, the initial Xenopus gp210 cytoplasmic tail sequence identified was termed “tail 1” (Figure 3A and B).

Xenopus gp210 tail 1 and 2 have nine identical amino acids at the beginning of their sequences, but after that, the sequences diverge on the level of DNA and amino acid sequence (data not shown and Figure 3B). The
Xenopus gp210 tail 2 sequence clearly has greater homology to the rat gp210 tail sequence (Figure 3C) in amino acid composition and in its length to the rat tail sequence compared to the tail 1 sequence (Figure 3A).

One tail above them all: tail 2 is the physiologically relevant Xenopus tail sequence.

Two different cytoplasmic tail sequences for Xenopus gp210 were found. Could both of the sequences encode isoforms that have different functions? In order to generate tools to study Xenopus gp210 and to clarify this finding, fragments of the lumenal domain, tail 1 and tail 2 were cloned from Xenopus RNA, expressed and used to generate antibodies (Figure 4 and Figure 3C). A fragment of the lumenal domain of Xenopus gp210 was used to generate polyclonal antibody 592 (blue bar, Figure 4). The sequence of the EST clone PBX0113E06 downstream of the transmembrane domain was used to generate antibodies against tail 1 (anti-serum 568; light green bar, Figure 4). Tail 2 antibodies were generated against a fragment cloned immediately downstream of the transmembrane domain to the stop codon, based on the sequence of EST BF613564 (anti-serum 619; Figure 3C).
Anti-lumen, anti-tail 1 and anti-tail 2 antibodies all recognized one band of 205 kD from Xenopus membranes (Figure 5A+B). Anti-lumen and anti-tail 2 antibodies did not detect major epitopes in cytosol (Figure 5A, lanes 2+6), but anti-tail 1 antibody did detect a band at ~160kD in cytosol (Figure 5A, lane 4). When the antibodies were used to detect their epitope in the context of the cell, differences became apparent. Anti-lumen and anti-tail 2 antibodies gave nuclear rims, indicative of staining at nuclear pores (Figure 5C). On the contrary, anti-tail 1 antibody did not give a rim stain around the nucleus, but instead stained the nucleoplasm (Figure 5C).

The absence of anti-tail 1 staining at the nuclear rim was puzzling, as it seemed to suggest that tail 1 did not localize to the nuclear pores. In order to understand the significance of the two gp210 tails found in Xenopus, homologs of the two tails were searched for in other species. Homologs of Xenopus tail 2 were found in human, rat, Danio reio (zebra fish) and Fugu rubripes (puffer fish) (Figure 6), but none were found for Xenopus tail 1. Of significance is that the serine 1880 residue in rat gp210 found to be phosphorylated during mitosis is conserved over all of the homologs found here (Figure 6, marked with an asterisk; Favreau et al., 1996). The presence of
tail 2 homologs across species points to the functional relevance of tail 2 over tail 1.

The headless tail: analysis of the Xenopus gp210 tail in Drummond and Wilson (2002)

Drummond and Wilson published a paper describing the role of Xenopus gp210 in nuclear assembly at the time when I was working with Xenopus tail 1 and 2. In order to understand the significance and roles of the three different gp210 tail sequences I had access to, I analyzed the sequence of the gp210 presented in their paper (accession number AF533550) and obtained antibodies that they used in their study.

The sequence described in their paper was different than that of Xenopus tail 1. They had also used the PBX0113E06 EST clone sequence to generate primers to clone the cytoplasmic tail of Xenopus gp210. On closer examination, the sequence they cloned had a single nucleotide frameshift compared to the sequence of PBX0113E06 (green underline, Figure 4). This frameshift caused the sequence to read through the stop codon at which tail 1 ends. The sequence from their clone was entered into the database as accession number AF533550. They then used the last 16 amino acids to
generate a peptide antibody (anti-serum 3860; dark green bar, Figure 4). Thus, for the purpose of discussion here, their sequence and antibody is termed “tail 1 long”.

The anti-tail 1 long antibody (anti-serum 3860) from the Drummond and Wilson (2002) study was analyzed in parallel with antibodies against tail 1, tail 2 and the lumen of Xenopus gp210. When Xenopus cells were stained with the antibodies, it was clear that antibodies against tail 1 and tail 1 long did not give nuclear rims, while antibodies against the lumenal domain and tail 2 did (Figure 5C). In addition, anti-tail 1 long recognized a band in membranes that was lower in molecular weight (180kD, Figure 7, lane 4) compared to that recognized by anti-tail 2, anti-rat lumen or anti-lumen antibody (205kD, Figure 7, lanes 1-3, respectively). The 205kD band represents endogenous gp210, as even the rat anti-gp210 lumen antibody recognized this band from Xenopus membranes (Figure 7, lane 2).

The discrepancy of anti-tail 1 long not staining the nuclear rim and recognizing a lower molecular weight band in membranes may be due to antibody recognizing only a small subset of the 205kD endogenous gp210. If anti-tail 1 long did recognize a small amount of the 205kD species, the enrichment of endogenous gp210 by immunoprecipitation should allow the
antibody to detect the 205kD form. Endogenous gp210 was
immunoprecipitated with anti-Xenopus gp210 lumen antibody, then probed
with the various gp210 antibodies (Figure 7, lanes 5-8). Anti-Xenopus gp210
tail 2 antibody, anti-rat gp210 lumen antibody, and anti-Xenopus gp210 lumen
antibody all detected a band at 205kD in the anti-Xenopus gp210 lumen
immunoprecipitate (Figure 7B, lanes 5-7, respectively). However, anti-
Xenopus tail 1 long antibody did not detect a 205kD or a 180kD band in the
anti-Xenopus gp210 lumen immunoprecipitate (Figure 7B, lane 8). This result
has two implications: 1) the 205kD protein isolated by the anti-Xenopus lumen
antibody is not recognized by the anti-tail 1 long antibody, and 2) the 180kD
protein recognized by the anti-tail 1 long antibody is not immunoprecipitated
by the anti-Xenopus lumen antibody. Taken together, biochemical evidence
shows that the tail 1 long sequence is not connected to the lumenal domain of
Xenopus gp210.

**Interference of Xenopus gp210 does not affect nuclear assembly.**

The function of gp210 was then studied with the nuclear reconstitution
system, where functional nuclei are formed in vitro with sperm chromatin,
large amounts of disassembled nuclear pore proteins and nuclear membranes.
To elucidate the function of gp210 in nuclear assembly, the antibody and protein fragment generated against the cytoplasmic tail of Xenopus gp210 (tail 2) was added to the nuclear reconstitution system. Anti-Xenopus tail 2 antibody was first tested for its ability to bind specifically to gp210 in nuclear membranes. Membranes were incubated with anti-tail 2 antibody or preimmune antibody (Figure 8). In the presence or absence of BSA, anti-tail 2 antibody bound to the membranes (Figure 8, lanes 2+4, respectively), while the preimmune antibody did not (Figure 8, lanes 3+5).

Now, with an antibody that bound specifically to endogenous cytoplasmic tail of Xenopus gp210, nuclear reconstitution reactions were done in the presence of this antibody to assay for effects on nuclear assembly (Figure 9A). The addition of anti-Xenopus gp210 tail 2 antibody (6.25 µg, Figure 9B, top two rows) did not affect the import of GFP-nucleoplasmin, signifying that fully functional nuclear pore complexes formed. The controls where an equal amount of preimmune antibody (Figure 9B, middle two rows) or an equal volume of buffer (ELB, Figure 9B, last two rows) were added also did not perturb nuclear assembly. Even when a higher amount of anti-Xenopus gp210 tail 2 antibody (10 µg) was added to the nuclear reconstitution reaction, nuclear import of GFP-nucleoplasmin and staining of nucleoporins
was still comparable to reactions where an equal amount of preimmune antibody or equal amount of buffer was added (Figure 9C, middle and right-most column, respectively).

In order to confirm that gp210 had no role in nuclear pore assembly specifically, nuclear intermediates with fully fused nuclear envelopes lacking nuclear pores were first formed. These nuclear intermediates were formed as in a normal nuclear reconstitution reaction, but with the addition of the chemical BAPTA, a calcium chelator. After the nuclear intermediates formed, dilution of the intermediates into fresh cytosol released the block to nuclear pore assembly, thus the intermediates were “rescued” (Figure 10A). Thus, the addition of antibodies to the intermediates will determine whether hindering gp210 blocks the “rescue” of nuclear pore assembly. When anti-Xenopus gp210 tail 2 antibody was added to the BAPTA nuclear intermediates in the presence of fresh cytosol, the antibody clearly bound (Figure 10B, first row, second column), but the incorporation of FG nucleoporins was not affected (Figure 10B, first row, third column). The amount of FG nucleoporin incorporation was comparable to when preimmune antibody or buffer was added (Figure 10B, second and third row, third column). In comparison, the block to nuclear pore assembly was maintained when BAPTA was added to
the fresh cytosol (Figure 10B, last row, third column). From these experiments where anti-Xenopus gp210 tail 2 antibody was added to disrupt the function of endogenous gp210, it was clear that nuclear assembly, and specifically nuclear pore assembly, were not affected.

The function of gp210 may also impinge on interactions with other proteins. The addition of recombinant Xenopus gp210 tail 2 protein into a nuclear reconstitution reaction would disrupt such interactions, by binding to proteins that normally bind to the cytoplasmic domain of gp210. When zz-tagged Xenopus gp210 tail 2 was added into a nuclear reconstitution reaction (24.5µM, Figure 11, top row), no difference was seen in the amount of nuclear import (middle column) or nucleoporin staining (right-most column) as compared to the addition of the zz-tag alone (24.5µM, bottom row). Thus, from these experiments, the attempts to disrupt the function of gp210 did not prevent nuclei from forming nor functioning in nuclear import.

**A promising role for gp210: nuclear disassembly**

The cytoplasmic tail of Xenopus gp210 was found not to have a role in nuclear assembly or in nuclear import. However, gp210 is localized to and purified as a part of the nuclear pore complex (Cronshaw et al., 2002; Miller
and Forbes, 2000). The cytoplasmic tail of Xenopus gp210 also shows conservation from humans to pufferfish (Figure 6). What function could gp210 be contributing to the nuclear pore complex?

A homolog for gp210 has not yet been found in yeast. This suggests that gp210’s function is specific to vertebrates. One major difference between vertebrates and yeast is the process to separate components into the two daughter cells. Vertebrates achieve this with open mitosis, while closed mitosis occurs in yeast. In the closed mitosis of yeast, the nuclear envelope and the nuclear pore complexes do not breakdown, allowing the chromosomes to segregate to the daughter cells in the sheltered environment within the nuclear envelope. Conversely, in open mitosis of vertebrates, nuclear pore complexes and the nuclear envelope breakdown to expose the genomic material to the cytosol. gp210 could potentially function in the vertebrate-specific process of nuclear disassembly.

The role of gp210 in nuclear disassembly is being investigated in an on-going collaboration with Katharine Ullman’s laboratory at the University of Utah. Their laboratory studies nuclear disassembly using Xenopus egg extracts. Nuclei reconstituted with Xenopus egg extract are first allowed to import a fluorescent nuclear import substrate. Then a stable mutant of cyclin
B is added to the reaction, which forces the system to enter mitosis, thus triggering nuclear disassembly (Liu et al., 2003, Figure 12A). Initial nuclear disassembly experiments where Xenopus gp210 cytoplasmic tail 2 antibodies were added showed that nuclear breakdown was inhibited, although to a lesser extent than that caused by Nup153 antibodies (Figure 12B). This suggests that the antibodies against Xenopus gp210 tail 2 interfere with the process of nuclear disassembly.

**Early localization of Xenopus gp210 to reconstituted nuclei**

gp210 may not play a role in nuclear assembly, but gp210 does assemble into the nuclear pore complex, of which it is an integral part. The Xenopus nuclear reconstitution system is a powerful tool to examine the process of nuclear assembly, as nuclear assembly can be stopped at various time points or inhibitors of nuclear assembly can be added into the reaction and then analyzed for the localization of nucleoporins.

With this system, gp210’s localization through nuclear assembly was observed in parallel with FG nucleoporins (Figure 13). At 15 minutes, anti-gp210 gave a punctate stain along the surface of the decondensing chromatin (Figure 13, third column, second row). FG nucleoporins (Figure 13, second
column, second row) did not show strong staining at the surface of chromatin at 15 minutes. By 30 minutes, both gp210 and FG nucleoporin gave a nuclear rim stain (Figure 13, third row). This result gives precedence to gp210’s early recruitment to the reforming nucleus in comparison to FG nucleoporins.

Xenopus gp210 is present in nuclear intermediates that lack FG nucleoporins

Another method by which the timing of gp210’s recruitment to the nuclear envelope can be determined is through its staining pattern in nuclear intermediates. Nuclear intermediates are formed when nuclear assembly is arrested at specific stages by the addition of chemicals or dominant negative recombinant proteins. BAPTA, a calcium chelator, blocks nuclear pore assembly while allowing the recruitment and fusion of nuclear membranes (Macaulay and Forbes, 1996). It had been determined by transmission electron microscopy that nuclear pore structures were not found in BAPTA nuclei, nor were FG nucleoporins present in BAPTA nuclei by immunofluorescence (Macaulay and Forbes, 1996). A fragment of the importin beta protein (aa 45-462) is also an inhibitor of nuclear pore assembly that allows nuclear membrane fusion to occur (Harel et al., 2003a).
Presumably importin beta (aa45-462) binds proteins involved in nuclear pore assembly that importin beta normally interacts with in a Ran-insensitive manner. When the importin beta (aa45-462) block to nuclear pore assembly was ordered with respect to the block induced by BAPTA, importin beta aa45-462 was found to exert its effect after BAPTA (Harel et al., 2003a). The difference between the two nuclear intermediates is not known, as they both show fused nuclear membranes and lack FG nucleoporins. If gp210 was observed to localize to the reforming nucleus before FG nucleoporins, could gp210 be present in these two nuclear intermediates?

The localization of gp210 within the nuclear intermediates formed with the addition of BAPTA or importin beta aa45-462 was examined. Nuclei were reconstituted in the presence or absence of BAPTA, followed by immunofluorescence of the nuclei spun onto coverslips (Figure 14A+B). In one experiment, nuclei were costained with monoclonal antibody against FG nucleoporins (mAb 414, recognizes Nup358, Nup214, Nup153, Nup62) and either antibody against gp210 or POM121, the two transmembrane nucleoporins (Figure 14A). In the other experiment, nuclei were costained for FG nucleoporins and either Nup93 or Nup53, all soluble nucleoporins (Figure 14B). In the control nuclei, FG nucleoporins, gp210, POM121, Nup93 and
Nup53 stain the nuclear rims. However, in the BAPTA nuclear intermediates, only gp210 gave a punctate, albeit lower in intensity, rim around the chromatin (Figure 14A, third column, second row). POM121, Nup93, Nup53 and the FG nucleoporins were not present on the BAPTA nuclear intermediates (Figure 14A+B, +BAPTA rows). Thus, gp210 is present on BAPTA nuclear intermediates even when POM121, Nup93, Nup53 and the FG nucleoporins; Nup358, Nup214, Nup153 and Nup62 are absent.

Importin beta aa45-462 arrests nuclear assembly at a step that occurs after that blocked by BAPTA. In importin beta aa45-462 nuclear intermediates, nuclear pores are still prevented from incorporating into the fused nuclear envelope (Harel et al., 2003a). gp210 was found to be present in BAPTA nuclei. Does the pattern of gp210 change in the downstream importin beta aa45-462 nuclear intermediate? Nuclear intermediates arrested with importin beta aa45-462 were stained with anti-Xenopus gp210 tail 2 antibody. gp210 was found to stain importin beta aa45-462 nuclear intermediates strongly (imp β 45-462, Figure 15A+B). This is in comparison to the absence of FG nucleoporins in these nuclear intermediates (+imp β 45-462, Figure 15A). When the staining pattern of gp210 was compared between BAPTA and
importin beta aa45-462 nuclear intermediates, it was clear that more gp210 was present in importin beta aa45-462 intermediates (Figure 15A).

The strong anti-Xenopus gp210 tail 2 stain in importin beta aa45-462 nuclear intermediates needed to be verified that it did indeed represent gp210. This was because of the possibility that the gp210 antibody preparation contained a small amount of anti-His antibody, which could cross react with the His-tagged importin beta aa45-462 protein in the reaction. The gp210 antibody was raised to His-tagged Xenopus gp210 tail 2, but was specifically purified to minimize the presence of anti-His antibodies, by capturing anti-His antibodies in the serum with His-GFP, then affinity purifying anti-Xenopus gp210 tail 2 antibody with GST-Xenopus gp210 tail 2. To clarify this issue, importin beta aa45-462 nuclear intermediates were costained with anti-gp210 tail 2 and anti-His antibodies (Figure 15B). The white arrows delineate strong spots of anti-Xenopus gp210 tail 2 staining that do not show strong staining with the anti-His antibody (Figure 15B). The His-tag stain did not colocalize with gp210, thus nullifying the issue of the gp210 antibody’s crossreactivity with the recombinant protein.

Thus, gp210 is present in BAPTA and importin beta aa45-462 intermediates where nuclear membrane fusion has taken place, but FG
nucleoporins have not yet localized to the nuclear membrane. This is also in agreement with the observation made here that gp210 is recruited to chromatin before FG nucleoporins. The distinct pattern of gp210 in the BAPTA and importin beta aa45-462 nuclear intermediates points to a novel difference between the two.
Discussion

Making head and tails of gp210

In this study of Xenopus gp210, I have determined that the Xenopus gp210 cytoplasmic tail 2 sequence has physiological relevance in the context of the nuclear pore complex, compared to the Xenopus cytoplasmic tail sequence (tail 1 long) published by Drummond and Wilson (2002). The functional relevance of the Xenopus cytoplasmic tail 2 sequence has been corroborated by the finding of closely related homologs in human, rat, zebra fish and puffer fish (Figure 6). Antibodies generated against the Xenopus gp210 tail 2 sequence show that this domain is connected to the lumenal domain of gp210, and localizes to punctate structures on the nuclear envelope. In parallel studies with Xenopus tail 1 long and tail 2 antibodies, I showed that when endogenous gp210 is enriched by anti-gp210 lumenal antibody immunoprecipitation and probed with anti-Xenopus tail 1 long or anti-Xenopus tail 2 antibodies, only the tail 2 antibody recognized immunoprecipitated gp210. The tail 1 long antibody did not (Figure 7). In addition, the antibody against tail 1 long did not stain the nuclear rim in cells,
in contrast to strong nuclear rim stains with antibodies against Xenopus gp210 lumen or tail 2 (Figure 5).

Xenopus laevis is tetrapoid, thus it is highly plausible that there is a higher proportion of transcript variants compared to diploid organisms. The transcripts of tail 1, tail 1 long, and tail 2 were all reverse transcribed from Xenopus RNA (this work and Drummond and Wilson, 2002). However, comparison of the epitopes in Xenopus membranes recognized by these antibodies showed that the tail 1 long antibody recognized a band (~180kD) that was of a lower molecular weight than that recognized by the Xenopus gp210 lumen, tail 1 or tail 2, or even by the cross-species rat gp210 lumen antibody (~200kD, Figure 7). This points to the possibility that the tail 1 long epitope in Xenopus membranes is connected to a luminal domain that is altogether different from that detected by the Xenopus and rat gp210 lumen antibodies. Taken together, these results strongly suggest that the tail 1 long sequence published by Drummond and Wilson (accession number AAM94631) does not represent the population of gp210 found at the nuclear pore, nor is it the sequence of the cytoplasmic tail connected to the luminal domain of Xenopus gp210. Thus, for the rest of the discussion, this sequence will be referred to as “Xenopus tail 1 long”, omitting the gp210 designation.
Nuclear assembly: not in the agenda of gp210

Drummond and Wilson (2002) showed that upon addition of excess Xenopus tail 1 long peptide or antibodies, nuclear assembly was inhibited. However, with the analysis of the tail 1 long antibody in this study, the conclusions made in that paper will need to be reevaluated. The blocks to nuclear assembly by the addition of protein fragments and antibodies against Xenopus tail 1 long in that study do not implicate gp210 in nuclear assembly. Xenopus tail 1 long might be part of a transmembrane protein that is different from gp210 that is found at the nuclear pore complex. In order to understand why nuclear assembly is blocked in the experiments described in Drummond and Wilson (2002), the 180kD membrane protein the Xenopus tail 1 long sequence represents will need to be clearly identified. The identity of this protein can be verified by cloning sequences upstream of tail 1 long, generating antibodies against those upstream regions and performing immunoprecipitation experiments to confirm that the sequences detected by the various antibodies are indeed connected. In addition, homologs of Xenopus tail 1 long in other organisms need to be identified to establish if this
protein is functionally conserved (Note: I have searched for homologs of tail 1 long in vertebrates, but have been found any as of yet).

The lack of a role for gp210 in nuclear assembly was shown here when Xenopus gp210 cytoplasmic tail 2 protein fragments and tail 2 antibodies did not inhibit nuclear assembly, nuclear import, or nuclear pore assembly (Figures 9B, 9C, 10B and 11). In addition, very recent work by the Mattaj lab reinforced this conclusion when they showed that nuclei reconstituted in the presence of gp210 antibodies or membranes depleted of gp210 were still capable of recruiting FG nucleoporins (Antonin et al., 2005). Taken together, the results conclusively show that gp210 is not involved in nuclear assembly, and that the previous study by Drummond and Wilson (2002) does not accurately reflect a function for gp210 in nuclear assembly.

**gp210: heading towards nuclear disassembly**

In vivo studies of gp210 knockdowns by RNAi in C. elegans and HeLa cells cause a loss of FG nucleoporins and altered nuclear phenotypes including condensed chromatin, clustered nuclear pore complexes and pore-related nuclear membrane structures (Cohen et al., 2003). These results were interpreted as providing evidence that gp210 is involved in nuclear assembly.
The in vitro studies done in this study show that gp210 is not involved in nuclear assembly. In support of this, the Mattaj lab showed that the use of gp210 immunodepleted membranes in reconstituting nuclei did not abrogate the incorporation of FG nucleoporins to the nuclear envelope (Antonin et al., 2005). In fact, if the gp210 RNAi studies are revisited with the knowledge that gp210 is not involved in nuclear assembly, a very striking interpretation can be made. The “formation” of partial nuclear pore structures could in fact be interpreted as partially disassembled nuclear pores. The loss of FG nucleoporins and condensed chromatin could well be phenotypes of disassembling nuclei. The observation of clustering nuclear pores may point to a role of gp210 in spacing nuclear pores apart, especially since the ideal location for a “ruler” to spread nuclear pores out from one another would be within the lumen of the nuclear envelope where the large lumenal domain of gp210 is present (see Future Directions).

In intact cells, an assembling nucleus and a disassembling nucleus can be distinguished by its DNA content. Thus, in studies of tissue culture cells where gp210 is knocked down, the DNA content of the nuclei with phenotypes will need to be analyzed before attributing the defect to one of nuclear assembly or disassembly.
The Xenopus nuclear reconstitution system is also a robust system in which to study nuclear disassembly. Studies in Katharine Ullman’s laboratory at the University of Utah have shown that Nup153 and the COPI coatamer complex are intimately involved in nuclear disassembly (Liu et al., 2003). In collaboration with the Ullman laboratory, an analysis of gp210 with the nuclear disassembly assay has opened up the possibility that gp210 functions in nuclear disassembly (Figure 12B). Further studies are underway to confirm the role of gp210 in nuclear disassembly. In light of the study here, and even data from past studies suggesting the contrary, evidence is pointing the role of gp210 away from nuclear assembly towards nuclear disassembly.

**Nuclear intermediates: freezing gp210 in its tracks**

During the process of forming reconstituted nuclei, before nuclear pores become functional or even before FG nucleoporins accumulate at nuclear pore complexes, Xenopus gp210 localizes to the nuclear envelope (Figure 13). In support of this, gp210 was also present in nuclear intermediates that had fused nuclear envelopes and no FG nucleoporins (Figures 14 and 15). Of interest, gp210 was found to stain importin beta aa45-462-inhibited nuclear intermediates, which have no visible pore intermediates
(Figure 15). What does this strong staining of gp210 reflect? gp210 is found on membranes, so the increase in gp210 stain could be an indication of increased membranes on the surface of the importin beta aa45-462 nuclear intermediates. This possibility agrees with the observation that importin beta aa45-462 nuclear intermediates do not allow even 9kD dextrans to pass through, and scanning electron micrographs (FESEM) of these intermediates show a smooth surface devoid of nuclear pores (Harel et al., 2003a). In future experiments, the comparison between levels of gp210 and an ER transmembrane protein, ribophorin, in control versus importin beta aa45-462 nuclear intermediates will elucidate whether gp210’s staining is due to an increase in total membranes (in which case ribophorin’s staining will increase as well), or is specific to the recruitment of gp210 to the nuclear surface. The pattern of gp210 in importin beta aa45-462 nuclear intermediates is opening up doors to understanding how importin beta aa45-462 blocks nuclear assembly.

The journey of gp210 through nuclear assembly

The localization of gp210 in the nuclear intermediates examined here revealed how gp210 progressed from one stage of nuclear assembly to the
next. gp210 was present in different patterns in nuclear intermediates that lack FG nucleoporins. With the Xenopus nuclear reconstitution system, these intermediates have been ordered with respect to each other. It has been established that BAPTA’s block to nuclear assembly occurs before importin beta aa45-462’s block (Harel et al., 2003a). Thus, gp210’s staining pattern transitions from a sparse punctate rim stain in BAPTA nuclei to a strong rim and nucleoplasmic stain in importin beta aa45-462 nuclear intermediates (Figure 16). In yet another nuclear intermediate, where nuclear membranes are fused but lack FG nucleoporins, i.e., those formed with Nup107-160 complex depleted extract, gp210 has a faint diffuse stain (Harel et al., 2003b). It is not known exactly where the Nup107-160 complex exerts its effect relative to the BAPTA and importin beta aa45-462. However, a study showed that a faint Nup107 stain is found on BAPTA nuclei (Walther et al., 2003), suggesting that the Nup107-160 complex is present in BAPTA nuclei, and that the depletion of the complex should arrest the nuclear intermediates at an earlier stage than that caused by BAPTA (Figure 16).

Since gp210 stains distinct patterns in all three of these intermediates, the ordering of the intermediates can determine what exactly affects gp210’s pattern, and perhaps elude to the function of gp210. The precise ordering of
the nuclear intermediates could be achieved by taking nuclear intermediates depleted of the Nup107-160 complex and “rescuing” them in fresh cytosol in the presence of BAPTA. In the presence of fresh cytosol alone, the Nup107-160 depleted nuclear intermediates would “rescue” and incorporate nuclear pores. If Nup107-160 depleted nuclear intermediates are rescued in the presence of BAPTA and fail to form nuclear pores, this would indicate that BAPTA acts after the Nup107-160 complex. The converse result would indicate that BAPTA acts before the Nup107-160 complex.

After the ordering of nuclear intermediates is determined, more can be said about the changing pattern of gp210 through nuclear assembly. If the removal of the Nup107-160 complex blocks a step in nuclear assembly before that blocked by BAPTA, gp210 will be moving from a diffuse pattern into a distinct punctate rim. This would point to the Nup107-160 complex (or possibly downstream proteins) gathering gp210 proteins into the structures seen as dots by immunofluorescence. gp210 has the potential for forming multimers in the context of the nuclear pore complex, as a population of gp210 exist in SDS-resistant dimers (Favreau et al., 2001). Thus, in reconstituted nuclei, gp210 serves as a unique marker to understand the progression and mechanism by which the nucleus assembles.
Figure 1. Schematic of the nuclear pore complex and gp210, a transmembrane nucleoporin.

A. Schematic of the nuclear pore complex highlighting the transmembrane nucleoporins, gp210 and POM121. Both gp210 and POM121 are single transmembrane nucleoporins. A large domain of gp210 (in red) resides in the lumen of the nuclear envelope (light gray area), while most of POM121 (in green) extends into the cytoplasm (C, light yellow) and/or nucleoplasm (N, light blue). gp210 and POM121 are assumed to be present in the membrane bound region of the nuclear pore complex due to their transmembrane domains. The other ~30 soluble nucleoporins that make up the nuclear pore complex are represented by the dark gray structure that is embedded in the double bilayer nuclear envelope (concentric black lines). The approximate location of individual or subcomplexes of nucleoporins to the cytoplasmic filaments (top section), core of the nuclear pore complex (middle section) or the nuclear basket (bottom section) is represented here.

B. Schematic of the domains of gp210, a transmembrane nuclear pore protein. The entire rat gp210 sequence was cloned by Greber et al. 1990, and it was determined experimentally that three domains make up gp210. The largest portion of the gp210 protein is the N-terminal lumenal domain (aa 1-1808), followed by a transmembrane domain (aa 1809-1828). The only portion of gp210 that is exposed to the cytoplasm/nucleoplasm is the 58 amino acid cytoplasmic tail (aa 1829-1886). The amino acid numbers denoted correspond to the lumenal domain and cytoplasmic tail domain. The schematic is not drawn to scale, as is the case in the gp210 schematics following.
Figure 2. The lumenal and transmembrane domain of Xenopus gp210 shows homology with rat gp210, but the tail 1 domain of Xenopus gp210 does not.

Xenopus sequences from the EST database (A, AW642061) and the sequenced EST clone PBX0113E06 (B, PBX) show homology to rat gp210. Protein alignments show that the Xenopus sequences are homologous to the lumenal (A, and B, upstream of the transmembrane domain) and transmembrane domains (B, gray line) of rat gp210, but the Xenopus cytoplasmic sequence is not homologous to the cytoplasmic tail of rat gp210 (B, downstream of the transmembrane domain).
A lumenal segment

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B lumenal, transmembrane + cytoplasmic segments

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Figure 3. *Xenopus* gp210 tail 2 shows close homology to rat gp210.

The sequence of *Xenopus* gp210 tail 2 was initially identified in a *Xenopus* EST sequence (BF613564) showing homology to rat gp210. The fragment of *Xenopus* gp210 tail 2 after the transmembrane domain was then cloned and sequenced. Sequence alignments between the cytoplasmic tail of rat, *Xenopus* tail 1 and *Xenopus* 2 are shown here. Identical amino acids are shaded in black and similar amino acids are shaded in gray.

A. *Xenopus* tail 1 does not share sequence homology with *Xenopus* tail 2. A side-by-side comparison of the *Xenopus* tail 1 (X tail1) and *Xenopus* tail 2 (X tail2) sequence differs after the first 9 amino acids following the transmembrane domain. In addition *Xenopus* tail 1 is much shorter in length.

B. An alignment of *Xenopus* tail 1 (X tail1) and the rat cytoplasmic tail (R tail) shows the divergence of sequence composition and length between *Xenopus* tail 1 and rat gp210.

C. *Xenopus* tail 2 shows homology to the cytoplasmic tail of rat gp210. The amino acid sequence and length of the cytoplasmic tail of rat gp210 and *Xenopus* tail 2 are similar, with 35% identical amino acids (20/57, shaded black) and 44% positive amino acids (25/57, shaded gray).
Figure 4. Xenopus gp210 lumen, tail 1 and tail 1 long – map of cloned fragments and antibodies generated.

The Xenopus EST sequence (AW642061) shows homology to rat gp210. The clone that this sequence was derived from (PBX0113E06) was sequenced to yield more sequence from this clone. The DNA sequence is shown here, along with the protein translation in all three forward frames. Fragments of Xenopus gp210 were cloned from this sequence, as shown by the solid colored rectangles: the lumenal domain (blue rectangle) and tail 1 (light green rectangle). The primers used to clone these fragments are shown by the arrows, with the color corresponding to the fragment they were used to clone. The tail 1 long sequence, published by Drummond and Wilson (2002) is shown here with the dark green line, and the epitope of the peptide antibody against tail 1 long described in their study of gp210 (anti-serum 3860) is represented by the dark green rectangle.
Figure 5. Differential specificity of antibodies against Xenopus gp210 lumen, tail 2, tail 1 and tail 1 long.

A. Epitopes of antibodies against Xenopus gp210 lumen, tail 1 and tail 2 in membranes and cytosol. Equal volumes (0.5µL) of membranes (M) and cytosol (cyt) were blotted with anti-Xenopus gp210 lumen (lanes 1+2), tail 1 (lanes 3+4) or tail 2 antibodies (lanes 5+6).

B. A band of 205kD in membranes is recognized by antibodies made against the luminal, tail 1 and tail 2 domains of Xenopus gp210. Equal amounts of Xenopus membranes (0.5µL) were probed with antibodies made against the domains of Xenopus gp210: the luminal domain (affinity purified 592, lane 1), tail 1 (affinity purified 568, lane 2) and tail 2 (affinity purified 619, lane 3).

C. Antibodies generated against Xenopus gp210 lumen and tail 2 give a clear nuclear rim stain, whereas antibodies against tail 1 and tail 1 long do not. Xenopus A6 kidney cells were stained with separately with antibodies against Xenopus gp210 luminal domain (affinity purified 592), tail 1 (affinity purified 568), tail 2 (affinity purified 619) or tail 1 long (affinity purified 3860, from Drummond and Wilson, 2002), followed by incubation with RITC-labeled goat-anti-rabbit secondary antibody. DNA was stained with DAPI. The bar represents 5 microns.
Figure 6. Xenopus tail 2 sequence homologs in human, rat, zebrafish and pufferfish.

Homologs to Xenopus tail 2 are present in human, rat, zebrafish (Danio reio) and pufferfish (Fugu rubripes). Sequences showing homology to the transmembrane domain (highlighted with the black bar) and onwards of rat gp210 are aligned. The C-terminus end of all five sequences show strong conservation, including the conservation of serine 1880 of rat, which was shown to be phosphorylated in mitosis (highlighted with the asterisk). Identical amino acids are shaded in black, similar amino acids are shared in gray.
Figure 7. Xenopus gp210 lumen is connected to Xenopus gp210 tail 2, but not to “Xenopus gp210 tail 1 long”.

The epitopes of the gp210 antibodies were identified by probing Xenopus membranes (lanes 1-4). The arrows highlight the size difference between the band recognized by the anti-Xenopus tail 1 long antibody (180kD) versus the other antibodies against gp210 (200kD).

Xenopus gp210 was immunoprecipitated from Xenopus membranes with anti-Xenopus gp210 lumen antibody. The immunoprecipitate was then probed with antibodies made against different sequences: Xenopus tail 2, rat luminal domain, Xenopus luminal domain or Xenopus tail 1 long (lanes 5-8, respectively). Xenopus gp210 is recognized by antibodies against the luminal domain of Xenopus and rat gp210, and tail 2 of Xenopus gp210. However, antibody against the tail 1 long sequence (Drummond and Wilson, 2002) did not recognize the immunoprecipitate.
Figure 8. Anti-Xenopus gp210 tail 2 antibody binds specifically to membranes.

Anti-Xenopus gp210 tail 2 antibody bound specifically to membranes, while preimmune antibody did not. Diluted Xenopus membranes (1:20) were incubated with anti-Xenopus gp210 tail 2 antibody (tail2, lanes 2+4) or preimmune antibody (PI, lanes 3+5), in the presence or absence of BSA (lanes 2+3, lanes 4+5, respectively). The membranes were then pelleted and analyzed for the specific binding of antibody by blotting with anti-Xenopus gp210 lumen antibody and goat-anti-rabbit secondary antibody. gp210 was recognized by the anti-Xenopus gp210 lumen antibody, while the goat-anti-rabbit secondary antibody lit up antibody that bound to the membranes (IgG). Membranes alone were probed in parallel to identify gp210 and to show the absence of IgG (M, lane 1).
Figure 9. Xenopus gp210 tail 2 antibody does not interfere with nuclear assembly.

A. Schematic of the Xenopus egg extract nuclear reconstitution reaction. Nuclei can be reconstituted with the Xenopus egg extract system. A source of DNA, sperm chromatin, is incubated together with membranes and cytosol fractionated from Xenopus eggs, and a regenerating energy mix. After incubation for an hour, functional nuclei are formed that are capable of nuclear import. The power of this in vitro system is that antibodies or protein fragments can be added into the reaction to see if functional nuclei or perhaps mutant nuclei form. This allows one to assay whether a protein has a role in nuclear assembly.

B. Anti-Xenopus gp210 tail 2 antibody does not interfere with the formation of import-competent nuclei. The addition of 6.25 μg anti-Xenopus gp210 tail 2 antibody to a 25 μL nuclear reconstitution reaction (+anti-Xgp210 tail 2, top two rows) does not interfere with the assembly of nuclei, as seen by the fully decondense DNA and the ability of GFP-nucleoplasmin to actively import into the nuclei. The addition of either the same amount of preimmune antibody (middle two rows) or the same volume of buffer (ELB, bottom two rows) shows no significant difference with the addition of anti-Xenopus gp210 tail 2 antibody. The bar represents 5 microns.
**A**

Nuclear Reconstitution

Sperm chromatin + membranes + cytosol energy + ATP + antibody or + protein fragments

$\Rightarrow$

Functional nuclei

**B**

DNA nuclear import (GFP-NP)

- + anti-Xgp210 tail 2

- + preimmune antibody

- + buffer

Scale bar = 20 μm
Figure 9. Xenopus gp210 tail 2 antibody does not interfere with nuclear assembly.

C. 10 μg of anti-Xenopus gp210 tail 2 antibody was prebound to diluted membranes (1:20) on ice for 30 minutes. Controls included preincubating membranes with an equal amount of preimmune antibody or an equal volume of buffer (ELB). The membranes were then spun through a sucrose cushion and used to reconstitute nuclei. The presence of nuclear pore complexes was assayed with the binding of importin beta aa45-462 to nucleoporins (middle column), and the functionality of the nuclear pore complexes was assayed with the nuclear import of GFP-nucleoplasmin (right-most column). Representative nuclei are shown here, with nuclei from different regions of the slide shown in the insets. The bar represents 10 microns.
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Figure 10. Xenopus gp210 tail 2 antibody does not interfere with nuclear pore assembly after nuclear membrane fusion has occurred.

A. Nuclear pore assembly can be assayed directly in nuclear intermediates where nuclear membrane fusion has occurred. The addition of the calcium chelator BAPTA to a nuclear reconstitution reaction will generate nuclear intermediates that have fused nuclear membranes but contain no nuclear pores. These BAPTA nuclear intermediates then incorporate nuclear pores upon dilution into fresh cytosol. It is into this step that various antibodies and protein fragments can be added to assay for the specific effect on nuclear pore assembly. Thus, the addition of anti-Xenopus gp210 tail 2 antibody into the BAPTA rescue assay will determine whether gp210 has a role in nuclear pore assembly.

B. The addition of anti-Xenopus gp210 tail 2 antibody along with fresh cytosol to BAPTA nuclei (5μg antibody/ 21μL reaction) does not block incorporation of FG nucleoporins (row 1). The addition of preimmune antibody (row 2) or buffer (row 3) also did not prevent FG nucleoporins from incorporating. However, the addition of BAPTA in the BAPTA rescue reaction did block FG nucleoporins from incorporating into the nuclear envelope (row 4). The localization and specificity of the added antibody was detected by staining with goat-anti-rabbit antibody, which will detect both anti-Xenopus gp210 tail 2 antibody and preimmune antibody. Only anti-Xenopus gp210 tail 2 antibody gave a rim stain. The bar represents 10 microns.
A

BAPTA nuclear intermediates:
- fused nuclear membranes
- no nuclear pores

+ cytosol

nuclear pore assembly rescued

+ anti-Xgp210 tail 2 antibody + cytosol

B

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<tr>
<td>+ BAPTA</td>
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Figure 11. The Xenopus gp210 tail 2 fragment does not interfere with nuclear assembly.

The addition of 24.5μM zz-tagged Xenopus gp210 tail 2 (+zz-Xgp210 tail 2) or zz tag alone (+zz) to a nuclear reconstitution reaction did not affect the incorporation of nucleoporins (as detected by TAMRA-importin beta aa45-462, far right column) or nuclear import of GFP-nucleoplasmin (middle column). The bar represents 10 microns.
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Figure 12. Nuclear disassembly assay and the potential role for gp210 in nuclear disassembly.

A. A schematic of the nuclear disassembly assay. Nuclei are reconstituted for an hour with membranes and cytosol from Xenopus egg extracts, along with sperm chromatin and a regenerating energy mix. After nuclei are formed, a RITC-labeled import substrate (NLS-HSA-RITC) is added and allowed to import into the reconstituted nuclei for 30 minutes. Recombinant cyclinA90 is then added into the reaction for 75 minutes to shift the system into mitosis. The nuclei breakdown, and the visual determination of nuclear disassembly is by the condensed chromatin and the loss of nuclear import substrate accumulation (Liu et al., 2003).

To assess the role of a protein in nuclear disassembly, antibodies or protein fragments can be added in the beginning of the nuclear reconstitution reaction and followed through to the addition of cyclinA90. If the protein plays a role in nuclear disassembly, the addition of antibodies against the protein, or protein fragments will prevent nuclear disassembly. A high percentage of intact nuclei will be present if nuclear disassembly is blocked by antibodies or protein fragments of that protein (Liu et al., 2003).

B. gp210 may play a role in nuclear disassembly. The addition of 2.5 mg of antibody to the nuclear disassembly assay shows that anti-Nup153 blocks nuclear disassembly (66% intact nuclei present), while anti-gp210 tail 2 antibody blocks nuclear disassembly to a lesser extent (22% intact nuclei present), although more than that caused by preimmune antibody (5% intact nuclei present).
A

Nuclear Disassembly Assay

- sperm chromatin + membranes cytosol energy + ATP → + RITC-import substrate + cyclinΔ90 → disassembled nuclei

+ antibodies or + protein fragments

- sperm chromatin + membranes cytosol energy + ATP → + RITC-import substrate + cyclinΔ90 → disassembled nuclei OR intact nuclei

B

% intact nuclei present

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<td>% intact nuclei</td>
<td></td>
<td>60</td>
<td>20</td>
</tr>
</tbody>
</table>

Antibodies added to nuclear disassembly assay
Figure 13. Xenopus gp210 incorporates before FG nucleoporins in reconstituted nuclei.

gp210 is recruited to reconstituted nuclei earlier than FG nucleoporins. Nuclei were assembled, and processed for immunofluorescence at 0, 15, 30 and 60 minutes. Nuclei were costained with a monoclonal antibody against FG nucleoporins (mAb414; recognizes Nup358, Nup214, Nup153 and Nup62) and the anti-Xenopus gp210 tail 2 antibody. The nuclei were then costained with goat-anti-mouse (Oregon green labeled) and goat-anti-rabbit (RITC labeled) secondary antibody. The exposure times for FG nucleoporins and gp210 were each normalized to the intensity of the 60 minute sample. The bar represents 5 microns.
Figure 14. BAPTA nuclear intermediates with fused nuclear membranes contain gp210, but not POM121, Nup93, Nup53 or FG nucleoporins.

Nuclei were reconstituted with 7mM BAPTA (+BAPTA) or without (control). After assembling with one hour, nuclei were fixed with EGS and spun onto coverslips. The exposure times for FG nucleoporins (green) or various other nucleoporins (red) were normalized to the control nuclei. The nucleoporin stains (or lack of it) in BAPTA nuclei were taken at the same exposure as the control nuclei.

A. Nuclei were costained with antibodies against FG nucleoporins (mAb414, recognizes Nup358, Nup214, Nup153, Nup62) and either gp210 (anti-Xenopus gp210 tail 2, anti-serum 619) or POM121 (anti-serum 616). The bar represents 5 microns.

B. Control and BAPTA nuclei were stained against FG nucleoporins (mAb 414, recognizes Nu358, Nup214, Nup153, Nup62) and either Nup93 or Nup53 (both affinity purified antibodies). The bar represents 5 microns.
**Figure 15.** Xenopus gp210 is present in nuclear intermediates even when FG nucleoporins are absent.

A. Nuclear intermediates that have fused nuclear membranes but lack nuclear pores can be formed by either adding the chemical BAPTA or a fragment of the importin beta protein (aa 45-462). Nuclei were assembled with Xenopus egg cytosol and membranes, chromatin and energy in the absence (control nuclei) or presence of 7mM BAPTA or 20µM importin beta (aa45-462) recombinant protein. After assembly, the nuclei were fixed and spun onto coverslips. Nuclei were costained against gp210 and FG nucleoporins (anti-Xenopus gp210 tail 2 antibody and monoclonal antibody 414, respectively). Representative nuclei are shown here, with pictures of a nucleus from the same field in the insets in the importin beta (45-462) column. The bar represents 10 microns.

B. The gp210 stain in importin beta aa45-462 nuclear intermediates does not colocalize with the His-tag from recombinant proteins. 10 µM of importin beta aa45-462 was added to a nuclear reconstitution reaction to form importin beta aa45-462 nuclear intermediates. An equal volume of buffer (ELB) was added into the control reaction. After incubation for an hour, the nuclei were spun onto coverslips and costained with anti-Xenopus gp210 tail 2 and anti-His antibodies. Strong spots of anti-Xenopus gp210 tail 2 staining are highlighted with the white arrows, shown in parallel in the anti-His panel to show that the two antibodies do not colocalize in importin beta aa45-462 nuclear intermediates. The bar represents 2 microns.
Figure 16. A model of the localization of gp210 with respect to nuclear assembly.

Nuclear assembly occurs in a sequential manner. Nuclear membranes are recruited to chromatin, followed by the fusion of nuclear membranes. Chemical and protein inhibitors have been shown to block nuclear assembly at various stages. Highlighted here are steps in nuclear assembly where FG nucleoporins (in green) have not incorporated yet, but gp210 (in red and pink, to illustrate differing intensities) has been shown to localize to these intermediates in distinct patterns. It has been determined that the BAPTA block to nuclear pore assembly is an earlier step than that inhibited by importin beta aa45-462. However, the precise step at which nuclei formed with cytosol depleted of the Nup107-160 complex are blocked has not been determined. Here, the localization of gp210 in three nuclear intermediates suggests that gp210 is recruited early to reforming nuclei, and that the pattern of gp210 serves as a unique marker for each of these intermediates.
nuclear membrane recruitment

? \( \Delta \text{Nup107-160} \)

sparse gp210 punctate rim

BAPTA

strong gp210 rim + nucleoplasmic stain

importin \( \beta \) (aa45-462)

strong gp210 rim

nuclear pore assembly
Acknowledgements, Chapter 3

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Future Directions

The unconventional nucleoporin: gp210

gp210 has been an anomaly of the nuclear pore complex. In Chapter 3, I have shown that it is not involved in nuclear assembly, but it may play a role in nuclear disassembly instead. The elusive role of gp210 in the nuclear pore complex is becoming clearer with the preliminary studies described here. However, the questions of how gp210 is retained at the nuclear pore complex, as well as the function of gp210 during interphase still remain.

Incorporation of gp210 into the nuclear pore complex

gp210 has been consistently copurified with the nuclear pore complex under high salt and detergent treatments (Gerace et al., 1982; Miller and Forbes, 2000). However, the bulk of the protein is hidden within the nuclear envelope lumen, while only a short tail domain protrudes into the cytoplasm (Greber et al., 1990), where it presumably makes contact with the rest of the nuclear pore complex. How then does gp210 remain at the nuclear pore complex? The transmembrane domain and the cytoplasmic domain of gp210 have been shown to target it to the nuclear pore complex (Wozniak and Blobel,
1992). Nucleoporin interactions with the recombinant gp210 cytoplasmic tail protein coupled to beads were investigated, but none of the tested nucleoporins bound (data not shown). Perhaps the inability of the gp210 cytoplasmic tail to isolate protein interactions indicates that gp210 does not work alone. gp210 may require a membrane coreceptor to form a binding site for nucleoporins, as supported by the observation that the transmembrane domain of gp210 is a major determinant for NPC localization (Wozniak and Blobel, 1992). This membrane coreceptor may be another nucleoporin, such as POM121, or could even be gp210 itself. gp210 has been shown to exist in SDS-resistant dimers in HeLa cells and nuclear envelopes from rat liver nuclei (Favreau et al., 2001). The coupling of recombinant cytoplasmic gp210 tail to beads may not have mimicked the three-dimensional structure of the endogenous gp210 dimer, so no nucleoporins bound.

Thus protein interactions with gp210 may be found only in the context of membranes. To probe for this, gp210 can be permanently bound to transmembrane proteins (homodimers or heterodimers) it may interact with, by lightly crosslinking Xenopus egg extract membranes with a short reversible crosslinker. Then gp210 can be immunoprecipitated from the crosslinked membranes in detergent using the anti-Xenopus gp210 lumen antibody. The
gp210 membrane immunoprecipitate is then a bait to search for gp210-interacting proteins found in cytosol. This may reveal endogenous interactions of gp210. Mass spectrophotometer analysis of the eluted soluble proteins and reverse-crosslinked transmembrane proteins bound to endogenous gp210 will reveal potential proteins that gp210 is in contact within the nuclear pore complex.

**Fleeting yet constant: the function of gp210 in interphase**

Another dilemma is the high mobility of gp210 within the nuclear envelope (Rabut et al., 2004). The mobility of various nucleoporins was determined by examining the rate of dispersal of GFP-tagged nucleoporins on the nuclear envelope in inverse fluorescence recovery after photobleaching experiments (iFRAP; Rabut et al., 2004). How does the high mobility of gp210 reconcile with the fact that gp210 is biochemically purified with the nuclear pore complex? The total amount of gp210 in the cell could be quite high. This is in agreement with the fact that the abundance of gp210 in the nuclear envelope allowed it to be the first nucleoporin biochemically identified (Gerace et al., 1982). The fast disappearance of GFP-gp210 in the iFRAP experiments (Rabut et al., 2004) and the constant presence of gp210 at the
nuclear pore complex can be resolved if there is rapid exchange between GFP-gp210 and high amounts of endogenous gp210, but at any given time, gp210 molecules will be at the nuclear pore (Figure 1A-D and Figure 2A and B).

What could explain the transitory localization of gp210 in the nuclear pore? At interphase, the nuclear pore complex is not merely a passageway for nuclear import and export. Inner nuclear membrane transmembrane proteins are synthesized on the ER membrane, which is contiguous with the outer nuclear membrane. Nuclear pore complexes allow for these transmembrane proteins to laterally diffuse from the outer nuclear membrane to the inner nuclear membrane. It had been shown that antibodies to the cytoplasmic domain of gp210 or the addition of wheat germ agglutinin (WGA), which binds to nucleoporins POM121, Nup214, Nup98 and Nup62, prevent transmembrane proteins from laterally diffusing to the inner nuclear membrane (Ohba et al., 2004). This demonstrates that a certain level of fluidity within the nuclear pore complex is required to allow transmembrane proteins of the INM to laterally diffuse along the pore membrane. Antibodies are divalent, so the antibodies against gp210 could be restricting the mobility of gp210 by linking multiple copies of gp210 into a rigid array. Thus, the high mobility of gp210 between nuclear pore complexes could explain how
transmembrane proteins are allowed to laterally diffuse through the nuclear pore complex.

From the studies presented above, gp210 does not seem to have a stable structural role in the nuclear pore complex, but may instead function through transitory, though constant, interactions within the nuclear pore complex. One possible function that fits this description is that of spacing nuclear pore complexes apart. The large lumenal domain of gp210 (Greber et al., 1990) and the isolation of gp210 dimers (Favreau et al., 2001) suggest that gp210 in two different NPCs could interact with one another via their lumenal domains to space the two NPCs apart (Figure 1A and Figure 2B). This hypothesis can be tested by determining whether the lumenal domain of gp210 contains a dimerization domain. In addition, the functional aspect of this hypothesis can be determined if the disruption of gp210 alters the spacing between individual NPCs. There is some evidence of pore clustering when gp210 is knocked down (Cohen et al., 2003). The cell line expressing anti-gp210 lumen antibody, which showed decreased nuclear import, can be revisited with transmission immunoelectron microscopy to assay whether nuclear pore spacing is affected (Greber and Gerace, 1992). In addition, transfection of gp210 truncated in the lumenal domain into tissue culture cells will show whether the distribution of
NPCs is altered. Thus, the further investigation of gp210 will open up insights into the nuclear pore complex during interphase.

**Differential regulation of importin beta in nuclear membrane fusion and nuclear pore assembly**

In Chapter 1, importin beta was shown to be a negative regulator of both nuclear membrane fusion and nuclear pore assembly (Harel et al., 2003). Nuclear membrane fusion needs to occur to a certain extent before nuclear pore assembly occurs (Macaulay and Forbes, 1996), presumably to reach a critical concentration of membrane proteins before nuclear pore assembly can be initiated. The block to nuclear membrane fusion by importin beta is reversed by RanGTP, but the block to nuclear pore assembly by importin beta appears, in part, to be reversed by a different modulator. Perhaps, this is because once the envelope is fused, there is no source of RanGTP. Thus, the regulation of nuclear pore assembly by importin beta may be modulated by a factor that is only present or functional after nuclear membranes have fused.

When importin beta was incubated with BAPTA nuclei, which contain fully fused nuclear membranes, importin beta still exerted a block to nuclear pore assembly (Harel et al., 2003). It will be of interest to determine if this
block to nuclear pore assembly by importin beta can be reversed when a slight amount of membrane fusion is allowed to occur by the addition of membranes along with cytosol. In addition, proteins interacting with importin beta in the presence of fused membranes can be isolated by lightly crosslinking a nuclear reconstitution reaction containing BAPTA. The subsequent solubilization of the reaction followed by immunoprecipitation of importin beta will isolate interacting proteins (soluble or membrane proteins) that importin beta is in close association with.

**In vivo investigation of importin beta in nuclear assembly**

Importin beta affects nuclear assembly when investigated in the Xenopus egg extract nuclear reconstitution system, where nuclear assembly is observed in isolation of other cellular processes. Attempts to investigate the role of importin beta in nuclear assembly in vivo has been complicated by various other cellular phenotypes (Walther et al., 2003), since importin beta has roles in nuclear transport and mitotic spindle formation. We injected anti-importin beta antibodies into HeLa cells at anaphase and saw disruption of nuclear assembly (W. Smith and D. Forbes, unpublished results). However, a novel anaphase role for the Nup107-160 complex may be the reason for this
disruption. The in vivo function of importin beta in nuclear assembly can be studied further when technology allows the degradation or expression of importin beta in tissue culture cells at a specific time, such as at the end of anaphase when the mitotic spindle has already formed and nuclear assembly is starting to occur. Perhaps the targeted loss of function or gain of function of importin beta could also occur at a specific location, such as on the surface of chromatin, where nuclear assembly occurs. With the availability of methods described above, the functions of importin beta uncovered through in vitro experiments can then be confirmed with precisely targeted in vivo studies.
Figure 1. A model of gp210 in the nuclear pore complex.

A. gp210 homodimers can form between two nuclear pore complexes to act as a “ruler” between nuclear pore complexes. One gp210 monomer is highlighted in blue.

B. The nuclear envelope is a double membrane bilayer, and as such, the transmembrane gp210 protein could potentially laterally diffuse along the membrane (1) within one nuclear pore, or (2) between two different nuclear pores, by leaving one pore momentarily while traveling in the outer or inner nuclear membrane.

C. As a result of the possible lateral diffusion of gp210, the gp210 monomer highlighted in blue is now part of a different nuclear pore complex, still maintaining contact with a gp210 molecule from a different nuclear pore.

D. The high mobility seen with gp210 can be explained by transient interactions between gp210 dimers. Here, the gp210 monomer highlighted in blue no longer interacts as a dimer, as it did previously in (C). Instead, another gp210 molecule has taken its place to maintain spacing between the nuclear pore complexes.
Figure 2. A model for the fluid mobility of gp210 between nuclear pore complexes.

A. A gp210 monomer is represented in red in a cross-section of the nuclear envelope. gp210 is a transmembrane protein, and thus is capable of laterally diffusing along the inner and outer nuclear membranes. Movement along the outer nuclear membrane is shown here, but it could also occur along the inner nuclear membrane. In this way, one molecule of gp210 could travel from one nuclear pore to another. The cytoplasm is denoted by C, while the nucleoplasm is denoted by N.

B. A gp210 homodimer is represented by the interaction between a red and a blue gp210 monomer (1). Even if the dimers are interacting, they could still conceivably laterally diffuse along the nuclear membranes to travel from one nuclear pore to another (2). The cytoplasm is denoted by C, while the nucleoplasm is denoted by N.
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