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Author
Delmar, Valerie Anne

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Regulation of the Assembly and Function of the Nuclear Pore

A Dissertation submitted in partial satisfaction of the Requirements for the Degree Doctor of Philosophy

in

Biology

by

Valerie Anne Delmar

Committee in charge:

Professor Doulgass Forbes, Chair
Professor Xiang-Dong Fu
Professor Maho Niwa
Professor Jim Wilhelm
Professor Michael Yaffe

2008
The Dissertation of Valerie Anne Delmar is approved, and it is acceptable in quality and form for publication on microfilm:

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Chair

University of California, San Diego

2008
DEDICATION

This work is dedicated to my parents, Amal and Henri Delmar, and to my sister, Monique who have supported me so much through all of these years.
# TABLE OF CONTENTS

Signature Page ................................................................. iii  
Dedication ........................................................................ iv  
Table of Contents ............................................................ v  
List of Figures ................................................................. vi  
List of Tables ................................................................. ix  
Acknowledgements ....................................................... x  
Vita ................................................................................. xii  
Abstract ........................................................................... xiii  
Introduction ...................................................................... 1  

Chapter 1. Topology of yeast Ndc1p: Predictions for the human NDC1/NET3 homologue .................................................. 17  

Chapter 2. Global histone acetylation induces functional genomic reorganization at mammalian nuclear pore complexes ................. 32  

Chapter 3. *Xenopus* importin beta validates human importin beta as a cell cycle negative regulator ................................................ 46  

Chapter 4. Transportin and importin beta regulate both the initial and downstream steps of nuclear pore assembly .......................... 76  

Future Directions ............................................................ 113
### LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td>Model of Nuclear Pore Complex Assembly</td>
<td>19</td>
</tr>
<tr>
<td>1.2</td>
<td>Sequence alignment of NDC1 homologues in human and Drosophila</td>
<td>21</td>
</tr>
<tr>
<td>1.3</td>
<td>Sequence alignment of NDC1 homologues in vertebrates</td>
<td>22</td>
</tr>
<tr>
<td>1.4</td>
<td>NDC1 is conserved in eukaryotes</td>
<td>23</td>
</tr>
<tr>
<td>1.5</td>
<td>NDC1 homologues share similar topological profiles</td>
<td>24</td>
</tr>
<tr>
<td>1.6</td>
<td>The N- and C- termini of NDC1 homologues are predicted to be exposed to the cytoplasm</td>
<td>25</td>
</tr>
<tr>
<td>1.7</td>
<td>The C-terminus of <em>S. cerevisiae</em> Ndc1p is exposed to the cytoplasm as determined by limited proteolysis</td>
<td>26</td>
</tr>
<tr>
<td>1.8</td>
<td>Region containing scNdc1p mutations is conserved throughout evolution</td>
<td>27</td>
</tr>
<tr>
<td>1.9</td>
<td>Model of NDC1 location and orientation at the <em>S. cerevisiae</em> spindle pole body and nuclear pore complex</td>
<td>28</td>
</tr>
<tr>
<td>2.1</td>
<td>TSA treatment of HeLa S3 cells.</td>
<td>34</td>
</tr>
<tr>
<td>2.2</td>
<td>Nup93 binding map and subnuclear localization of associated genomic regions</td>
<td>35</td>
</tr>
<tr>
<td>2.3</td>
<td>Nup93 association with the genome is significantly altered upon loss of histone deacetylation</td>
<td>37</td>
</tr>
</tbody>
</table>
Figure 2.4: Nup93-binding sites are proximal to differentially expressed genes following TSA treatment

Figure 2.5: Distribution of Nup93-binding sites within G bands

Figure 2.6: Model summarizing the factors involved in Nup93-genomic interactions

Figure 3.1: *Xenopus* importin beta shows close homology to human importin beta

Figure 3.2: *Xenopus* importin beta is an authentic negative regulator of the fusion events in nuclear membrane formation

Figure 3.3: *Xenopus* importin beta is an authentic negative regulator of nuclear pore assembly and is reversed by RanGTP

Figure 3.4: Altering importin beta by addition of a His-tag renders importin beta insensitive to RanGTP specifically in its block to nuclear pore assembly

Figure 4.1: Excess transportin blocks nuclear membrane fusion in a Ran-reversible manner

Figure 4.2: Excess transportin blocks nuclear pore assembly in BAPTA arrested nuclei

Figure 4.3: Annulate lamellae pore assembly is regulated by importin beta and transportin in a Ran-mediated manner

Figure 4.4: Nucleoporin targets of importin beta and transportin

Figure 4.5: Importin beta and transportin regulate the initial step in nuclear pore assembly
Figure 4.6: Model of transportin and importin beta in regulation of multiple steps during nuclear assembly

106
<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td>Sequence comparison of the different NDC1 homologues</td>
<td>23</td>
</tr>
<tr>
<td>2.1</td>
<td>Histone methylation enrichments in Nup93-binding sites</td>
<td>39</td>
</tr>
</tbody>
</table>
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Chapter 1, in full, has been reproduced from Lau CK, Delmar VA, Forbes DJ. Topology of yeast Ndc1p: predictions for the human NDC1/NET3 homologue. The Anatomical Record Part A Discoveries in Molecular, Cellular, and Evolutionary Biology, 2006, 288(7):681-94 by copyright permission from Wiley-Liss, Inc. I was the secondary researcher listed in this publication that forms the basis for this chapter.
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Chapter 3, in full has been reproduced from Delmar VA, Chan RC, Forbes DJ. Xenopus importin beta validates human importin beta as a cell cycle negative regulator. BMC Cell Biology, 2008, 9:14 by copyright permission from BioMed Central. I was the co-primary author listed in this publication that forms the basis for this chapter.

Chapter 4, in full, is in manuscript form and ready for publication. I was the co-primary researcher along with Corine Lau and Douglass Forbes in this study that forms the basis for this chapter.
VITA

1998 Bachelor of Science, Massachusetts Institute of Technology, Cambridge

1998-1999 Visiting Scholar, Massachusetts Institute of Technology, Cambridge

1999-2001 Staff Research Associate, University of California, San Francisco

2008 Doctor of Philosophy, University of California, San Diego

PUBLICATIONS

Delmar VA, Lau CK, Chan RC, Forbes DJ. Transportin and importin beta regulate both the initial and downstream steps of nuclear pore assembly. Manuscript in preparation.


FIELDS OF STUDY

Major Field: Cell Biology

Study of nuclear assembly
Professor Douglass J. Forbes
Regulation of the Assembly and Function of the Nuclear Pore

by

Valerie Anne Delmar

Doctor of Philosophy in Biology

University of California, San Diego, 2008

Professor Douglass J. Forbes, Chair

The nucleus is the defining structure of eukaryotic cells. The nuclear envelope acts as a barrier between nucleus and cytoplasm. Nuclear pore complexes perforating the envelope control all traffic into and out of the nucleus, and thus act to regulate transcription, translation, and other essential cellular processes. During mitosis, the nuclear envelope from flies to mammals disassembles into its component parts, with
the nuclear pore breaking into multiple subunits. The pore then reassembles in a step-wise process as the nuclear envelope reforms in late anaphase. The major focus of this thesis has been to better understand the assembly and function of the nuclear pore. It has resulted in three published papers and one paper in preparation.

First, I participated, with postdoc Dr. Corine Lau, in a study of the novel vertebrate transmembrane nucleoporin, Ndc1. I resolved the topology of yeast Ndc1p and identified conserved amino acids to target for future functional studies (Chapter 1).

In a second study, I participated in a collaboration with the laboratory of Dr. Pamela Silver at Harvard Medical School in identifying a new role for the vertebrate nuclear pore in the regulation of transcription. We found that in vertebrates, specific chromosomal regions move to the nuclear pore complexes during transcriptional activation (Chapter 2).

Key work next centered on the mechanism of action of importin beta in negatively regulating nuclear membrane fusion and pore assembly. (The small GTPase, Ran, positively regulates both these processes.) A major unanswered question has been, which specific steps in nuclear pore assembly are regulated by importin beta or RanGTP. I determined, using Xenopus constructs, that importin beta is an authentic regulator of nuclear pore assembly and that, contrary to previously published results, Ran reverses this negative regulation (Chapter 3).

Finally, a fourth study, done with Dr. Corine Lau, established that the distant importin beta relative, transportin, also negatively regulates nuclear membrane and
pore assembly (Chapter 4). I showed that both transportin and importin beta act early to control the initial step of pore assembly: the binding of the pore-targeting protein ELYS to chromatin, which sets in motion the specific targeting of nuclear pores to the nuclear surface.
INTRODUCTION

The nucleus is the defining organelle of eukaryotes and acts to both sequester and protect our genetic material. Two membrane bilayers encircle the genome, forming a barrier between the contents of the nucleus and those of the cytoplasm. Perforating this barrier are nuclear pore complexes, which act as points of control for all traffic into and out of the nucleus. All molecules 20 kDa or less can freely diffuse across the nuclear pores (For review see [1-5]). However, transport receptors are required for the transport of larger cargoes, such as proteins that contain a nuclear import or export signal or, alternately ribonucleoproteins (RNPs) and RNAs which need to be transported (for review see [6-10]). The regulation of nuclear-cytoplasmic transport is essential to cellular function. A permanently open gate could allow indiscriminant gene transcription or the translation of unspliced mRNA. Thus, the need for tight regulation of traffic into and out of the nucleus positions nuclear pores to be one of the most critical structures of the eukaryotic cell.

Mature vertebrate nuclear pore complexes are one of the largest multi-protein structures in the eukaryotic cell. The NPC spans the two bilayers that form the nuclear envelope, which fuse at the periphery of the NPC [11-14]. NPCs are comprised of ~30 different nucleoporins (or Nups) each in 8-32 copies. These Nups are distributed asymmetrically across the nuclear pores [15]. Facing the nucleus, Nup153 and TPR form a structure resembling a basket, and thus termed the nuclear basket [16-18]. On the opposite or cytoplasmic face of the nuclear pore are Nup214 and Nup358 [19], with Nup358 forming cytoplasmic filaments [20]. Three integral membrane proteins
connect the nuclear pore to the nuclear membrane: POM121, gp210, and NDC1 [21-25]. High resolution scanning electron microscopy images show large concentric rings making up the area between the nuclear basket and cytoplasmic filaments [26]. Here the bulk of the nuclear pore mass is found, including the Nup107-160 and Nup93/205 subcomplexes [18]. The Nup107-160 complex is the largest subcomplex found in the nuclear pore. It is comprised of 9 nucleoporins and is vital to pore assembly [27-30]. Spanning the length of the nuclear pore complex are the different Phenylalanine-Glycine (FG) repeat-containing nucleoporins [18, 31, 32]. For review see [33, 34]. These nucleoporins are important for nuclear-cytoplasmic transport as the nuclear transport receptors are thought to interact with FG-repeat domains as they traverse the nuclear pore [35-42].

The components of metazoan nuclei, including the nuclear pores, disassemble at the onset of mitosis allowing for mitotic spindle formation and faithful segregation of the chromosomes, for review see [43]. The nuclear pores break apart into protein sub-complexes, while the nuclear membranes retreat into the ER and/or vesiculate [44-48]. Beginning in late anaphase of mitosis, the nuclei, including the nuclear pores, begin to reassemble in a step-wise process resulting in mature nuclear pores and a closed nuclear envelope [49-51]. Reviewed in [34] [52].

Yeast nuclear pores are structurally similar to their vertebrate counterparts, but smaller, 70MDa vs. 125MDa in mass [11, 32, 53, 54]. Many of the nucleoporins are homologous between yeast and vertebrates, with several notable exceptions. It was long thought there were three integral membrane proteins in yeast, Pom152p, Pom34p,
and Ndc1p, but only two in vertebrates, POM121 and gp210. Interestingly, no homology was found between these vertebrate and yeast integral membrane pore proteins. By a series of iterative BLAST searches our lab uncovered a vertebrate homologue for Ndc1p [23]. This work accompanied by a detailed comparison between the yeast and vertebrate NDC1 homologues and an experimental determination of the topology of Ndc1p was published in the Anatomical Record in 2006. I was the secondary author of this publication and it is reproduced in full in Chapter 1. After this work was submitted, two papers were published demonstrating that vertebrate NDC1 is indeed part of the nuclear pore complex and necessary for its assembly [24, 25].

The role of nuclear pore complexes as the gateway to the nucleus is expanding. In the budding yeast, *Saccharomyces cerevisiae*, the nuclear periphery, including nuclear pores are shown to regulate transcriptional repression [55-60] [56] and activation [61-72]. In collaboration with the laboratory of Dr. Pamela Silver at Harvard Medical School, we now demonstrate that the nuclear pore complex is involved in transcriptional activation in humans [73]. Antibody to the vertebrate nucleoporin, Nup93, was used to identify chromosomal regions that interacted with the nuclear pore during transcriptional activation. This work was published in Genes & Development in March 2008. This publication is reproduced in full in Chapter 2.

A main focus of my thesis research has been on a negative regulator of nuclear assembly, importin beta. The canonical role for importin beta is as a nuclear import receptor. Importin beta interacts with cargo containing a nuclear localization signal
(NLS) via its cofactor, importin alpha [35, 74, 75]. This trimeric import complex is
competent to pass from the cytoplasm to the nucleus through the nuclear pore, via FG-
nuleoporin binding. Once inside the nucleus the import complex encounters
RanGTP. RanGTP binds strongly to importin beta, inducing a conformational change
that disassembles the import complex [76]. Reviewed in [77]. Interestingly, new
roles for importin beta have emerged in the past several years. It has been shown to be
a negative regulator of mitotic spindle assembly, nuclear membrane fusion, and
nuclear pore complex assembly (Reviewed in [78]) as well as, very recently, a positive
regulator of the chromosome loading of human chromokinesin Kid [79].

Much of nuclear pore complex assembly has been studied in a Xenopus laevis
nuclear reconstitution system [28, 29, 49, 80-86, 92]. Because the early Xenopus
embryo must undergo multiple rounds of cell division without protein synthesis, the
Xenopus egg contains enough material to form millions of nuclear pores. To study the
formation of nuclear pores, Xenopus eggs are fractionated by ultracentrifugation into a
membrane-free cytosol component and a membrane vesicle component. When both of
these components are combined in the presence of a chromatin source and an ATP
regeneration system, nuclei spontaneously assemble. These nuclei contain normal
nuclear membranes, a nuclear lamina, and nuclear pores, are competent for nuclear
import, and can undergo a normal round of replication [85-88].

A large portion of my thesis research has focused on the role of importin beta
as a regulator of nuclear assembly. Importin beta is a negative regulator of this
process while and RanGTP is a positive regulator [83, 84]. In a previous study, when
excess importin beta was added to the *Xenopus in vitro* nuclear assembly system, vesicle-vesicle membrane fusion was inhibited around the nucleus [83]. This block to proper nuclear membrane fusion could be reversed by addition of excess RanGTP [83]. This was the first evidence of importin beta regulating nuclear membrane fusion. In the same study, excess importin beta was added to nuclear intermediates, which had fused nuclear membranes but no detectable nuclear pores [83]. If these nuclear intermediates were diluted into fresh *Xenopus* egg cytosol, then nuclear pores could form. However, if these pore-free intermediates were diluted in cytosol in the presence of excess importin beta, nuclear pore formation was blocked [83]. These studies define a role for importin beta as a negative regulator in nuclear pore formation. In the Harel et al., 2003 study, the importin beta block to pore assembly could not be reversed by the addition of excess RanGTP. Notably, the original research demonstrating a role for importin beta as a negative regulator of nuclear assembly tested recombinant human importin beta in a *Xenopus laevis in vitro* system [83, 84]. Chapter 3 of my dissertation addresses whether the block to nuclear assembly shown by excess human importin beta is authentic, or due to a dominant negative mutation resulting from sequence variation between the *Xenopus* and human importin beta proteins [92]. I demonstrated in work done in collaboration with Dr. Rene Chan, a previous graduate student of the laboratory, that *Xenopus* importin beta acts identically to human importin beta in its negative regulation of nuclear membrane fusion and nuclear pore assembly, thus validating importin beta as a bona fide negative cell cycle regulator. During this investigation I also discovered that when
importin beta, either human or *Xenopus*, is fused to a 6-His purification tag it has an altered sensitivity to RanGTP. By removing the purification tag, I showed that the block to pore assembly by excess untagged *Xenopus* or human importin beta could indeed be reversed by the addition of excess RanGTP [92]. These studies, presented in Chapter 3 of this dissertation, have been published in BMC Cell-Biology.

Dr. Corine Lau, a post-doc in the laboratory, discovered a novel role for the transport receptor, transportin, as a negative regulator of pore assembly. Like importin beta, excess transportin blocks both nuclear membrane fusion and nuclear pore assembly in a Ran sensitive manner. Interestingly, we show that both importin beta and transportin interact with the same set of nucleoporins as shown by a GST-pulldown from *Xenopus* egg extract. In chapter 4 I establish that transportin, like importin beta, also negatively regulates annulate lamellae assembly (AL, or stacks of cytoplasmic pores embedded in specialized ER membranes).

Recent work demonstrated that ELYS, a newly discovered nucleoporin, targets nuclear pore assembly to chromatin [89-91] (Rasala et al., submitted). In the absence of ELYS, pores no longer form in the nuclear envelope around chromatin, but can still form in the cytoplasm as annulate lamellae [89, 91]. The binding of ELYS to chromatin at the end of anaphase initiates nuclear pore assembly by recruiting the Nup107-160 complex to chromatin [89, 91] (Rasala et al., submitted). In Chapter 4, I demonstrate that importin beta and transportin negatively regulate the binding of ELYS to chromatin in a Ran-sensitive manner. Thus, importin beta and transportin negatively regulate the first known step of nuclear pore assembly. This work, together
with that in the previous paragraph, is the subject of a fourth paper now in manuscript form.

This work expands our knowledge of the nuclear pore complex: what its components are, what functions it has, and how its assembly is regulated.
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CHAPTER 1

Topology of Yeast Ndc1p: Predictions for the Human NDC1/NET3 Homologue

CORINE K. LAU, VALERIE A. DELMAR, AND DOUGLAS J. FORBES
Section of Cell and Developmental Biology, Division of Biological Sciences, University of California at San Diego, La Jolla, California

ABSTRACT

The nuclear pore complex is the predominant structure in the nuclear envelope that spans the double nuclear membranes of all eukaryotes. Yeasts have one additional organelle that is also embedded in the nuclear envelope: the spindle pole body, which functions as the microtubule organizing center. The only protein known to localize to and be important in the assembly of both of these yeast structures is the integral membrane protein, Ndc1p. However, no homologues of Ndc1p had been characterized in metazoans. Here, we identify and analyze NDC1 homologues that are conserved throughout evolution. We show that the overall topology of these homologues is conserved. Each contains six transmembrane segments in its N-terminal half and has a large soluble C-terminal half of ~300 amino acids. Charge distribution analysis infers that the N- and C-termini are exposed to the cytoplasm. Limited proteolysis of yeast Ndc1p in cellular membranes confirms the orientation of its C-terminus. Although it is not known whether vertebrate NDC1 protein localizes to nuclear pores like its yeast counterpart, the human homologue contains three PG repeats in the C-terminus, a feature of many nuclear pore proteins. Moreover, a small region containing mutations that affect assembly of the nuclear pore in yeast is highly conserved throughout evolution. Lastly, we bring together data from another study to demonstrate that the human homologue of NDC1 is the known inner nuclear membrane protein, NET3. Anat Rec Part A, 288A:681–694, 2005. © 2006 Wiley-Liss, Inc.

Key words: nuclear envelope; nuclear pore; nucleoporin; spindle pole body; pore membrane proteins; NDC1; NET3; topology

The possession of a nucleus distinguishes eukaryotes from prokaryotes. The nucleus not only provides protection for the genome, but also allows spatial and temporal regulation of gene expression. Two nuclear membranes comprise the wall that surrounds the genome, while nuclear pore complexes serve as highly regulated gates orchestrating traffic into and out of the nucleus.

The nuclear membranes consist of an outer nuclear membrane that is contiguous with the endoplasmic reticulum (ER), with which it shares many common membrane components. In contrast, the inner nuclear membrane contains a distinctive set of membrane proteins. Unique to metazoa is the presence of a nuclear lamina, a proteinaceous structure composed of intermediate filament-like proteins termed lamins. The nuclear lamina is a key organizational and structural component of the nucleus.

Through its filamentous network and associated proteins, the lamina provides structural support and elasticity for the nucleus and acts to position the nuclear pore complexes (for reviews, see Holaska et al., 2002; Hutchinson, 2002; Grunbaum et al., 2005; Hetzer et al., 2005). The

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lamina is also implicated in DNA replication and transcription. Mutations in lamin genes are associated with an increased number of muscular degenerative diseases, as well as with the premature aging syndrome progeria (Hutchinson, 2002; Mounekes and Stewart, 2004; Pollex and Heidenreich, 2004; Greenbaum et al., 2005; Smith et al., 2005). Mutations in inner nuclear membrane proteins often lead to similar types of diseases (Schwarz et al., 2003; Greenbaum et al., 2005; Hetzer et al., 2005).

**Nuclear Transport**

The outer and the inner nuclear membranes are fused at nuclear pore complexes. These are the only gateways where nuclear acids and proteins can traffic between the nucleus and the cytoplasm. Molecules smaller than 20–40 kDa can passively diffuse through the nuclear pores. However, trafficking of large macromolecules is mediated by transport receptors and regulated by the small GTPase Ran.

Transport of cellular factors plays an integral role in gene regulation. For example, many transcription factors are restricted to the cytoplasm, until released by a signal, which then allows them to translocate into the nucleus to alter gene expression (for review, see Kau et al., 2004). In addition, many viruses need to gain access to the nucleus through the nuclear pores in order to utilize the host's cellular machinery to replicate their genomes and multiply (for review, see Whitmire et al., 2000).

There are at least 20 nuclear transport receptors in vertebrates and 14 in the budding yeast (for review, see Cho and Blobel, 2001; Strom and Weis, 2001; Harel and Forbes, 2004; Pemberton and Paschal, 2005). Depending on function, these receptors are termed importins or exportins and collectively are called karyopherins. The most well-studied import receptor is importin β. During classical nuclear localization signal (NLS)-mediated import, importin β associates with importin α, an adaptor that recognizes the positively charged NLS present on a cargo protein. A trimeric complex, consisting of importin α, β, and the NLS-containing cargo protein, then translocates through the nuclear pore. The import complex dissociates when it encounters RanGTP in the nucleus, thereby releasing its cargo and completing import. For certain cargos, importin β foregoes the use of importin α and acts alone. A different import receptor, transportin, recognizes a glyco-rich M9 NLS without the use of an adaptor. It is dissociated from its cargo in the nucleus by RanGTP.

Nuclear export receptors, on the other hand, require binding of RanGTP in order to recognize the nuclear export signal (NES) on their NES-bearing cargo protein. The directionality of nuclear transport is determined by the high RanGTP concentration present in the nucleus and low RanGTP concentration in the cytoplasm. For reviews, see Weis, 2001. This RanGTP gradient is maintained by the localization of the RanGTP exchange factor, RCC1, on chromatin. The RanGTP-activating protein RanGAP and its activators, in contrast, concentrate at the cytoplasmic side of the nuclear pores and in the cytoplasm. Although the basics of nuclear transport have been well documented, the precise mechanism of how the transport complex translocates through the nuclear pore is still under debate. At least three hypothetical models of nuclear transport have been proposed and discussed in detail elsewhere (Paschal, 2002; Fahrenkrog and Aebi, 2003; Sundaralingam and Wente, 2003; Weis, 2003; Fahrenkrog et al., 2004; Harel and Forbes, 2004; Peters, 2005, and references therein).

**Nuclear Pore Structure**

In order to understand the vital process of nuclear transport, it is essential to know the structure, composition, and mechanism of assembly of the nuclear pore complexes. The general structure of the massive nuclear pore is conserved in all eukaryotes, as determined by electron microscopic methods (for reviews, see Root and Aitchison, 2001; Fahrenkrog and Aebi, 2003). The nuclear pore consists of a large spoke-ring structure, a central channel structure, eight cytoplasmic filaments, and eight nuclear filaments joined at the end to form a “nucleobasket.” Due to the inherent eightfold symmetry of the nuclear pore, nuclear pore proteins, termed nucleoporins, are present within the pore in multiples of eight (8–48 copies) (Cronshaw et al., 2002). Each vertebrate nuclear pore contains ~30 different pore proteins to give an overall total of 500–1,000 proteins.

To date, only two of the pore proteins are proven to be integral membrane proteins in higher eukaryotes. The remainder are proteins recruited from the cytoplasm to form the bulk of the nuclear pore structure (Cronshaw et al., 2002; for reviews, see Sundaralingam and Wente, 2003; Hetzer et al., 2005; Schwartz, 2005). Approximately a third of the nucleoporins contain phenylalanine-glycine or FG repeats, which are domains that bind transport receptors. The majority of the nucleoporins also contain α-helical repeats and/or β-propeller domains that are thought to provide surfaces or platforms for protein–protein interactions (Schwartz, 2005). The essential and largest vertebrate nuclear pore subcomplex, the Nup107-160 complex, contains nine nucleoporins (Nup160, Nup133, Nup107, Nup94, Nup85, Nup43, Nup37, Sec1, Sec13) (Belgareh et al., 2001; Cronshaw et al., 2002; Harel et al., 2002; Itoh and Aebi, 2002; Walshe et al., 2003; Loucheux et al., 2004). This “linchpin” complex is believed to form a large portion of the central scaffold of the nuclear pore. It may additionally serve to stabilize the curved nuclear membrane encircling the nuclear pores (Dewos et al., 2004). Interestingly, alterations in a number of nucleoporins are linked to human genetic and autoimmune diseases (Cronshaw and Matunis, 2003; for review, see Enari et al., 2004; Kau et al., 2004; Moore, 2005).

**Nuclear Pore Assembly**

The mechanism of nuclear pore assembly has not been well elucidated. Nonetheless, we know that nuclear pores assemble at two distinct phases of the cell cycle in higher eukaryotes. First, nuclear pore number doubles in S-phase. This requires assembly into the preexisting intact double nuclear membranes. In addition, the nuclear envelope and nuclear pores entirely disassemble at the onset of mitosis. The nucleoporins dissociate into 13 or more individual pore subcomplexes, and the nuclear membranes disassemble into membrane vesicles and reattach into the ER (for discussion, see Burke andellenberg, 2002; Liu et al., 2002; Hetzer et al., 2005). Reassembly of nuclear pores at the end of mitosis in metazoans can occur by the soluble nucleoporins incorporating into fused nuclear membrane sheets, similar to that of interphase assembly (Macauley and Forbes, 1996; Harel et al., 2003b). Evidence exists that pore assembly can also be nucleated by nucleoporin binding first to chromatin before the nuclear
membranes completely fuse (Burke and Ellenberg, 2002; Walther et al., 2003a; Hetzer et al., 2005). It should be noted that in organisms such as Saccharomyces cerevisiae, nuclear pore assembly only occurs in preexisting fused nuclear membranes since the nuclear envelope in these organisms does not break down at mitosis.

A powerful in vitro system has shed light on the molecular mechanism of nuclear pore assembly. In an extract of Xenopus laevis eggs, when a source of DNA or chromatin is added, it is possible to reconstitute nuclei in vitro that are fully competent for nuclear transport and DNA replication (Lohka and Masui, 1984; Newport, 1987; Macaulay and Forbes, 1996; Wilson and Wiese, 1996). In nuclear assembly in vitro, first membrane vesicles containing integral membrane proteins are recruited and bound to the surface of chromatin (Fig. 1A). The membrane vesicles then fuse with one another to form patches of double membrane sheets, whereupon nuclear pores are assembled and embedded in the forming double nuclear membranes.

Fig. 1. Model of nuclear pore complex assembly. A: A first step of in vitro nuclear assembly in vertebrates is the recruitment of nuclear envelope vesicle precursors to chromatin. The vesicles then fuse to form double nuclear envelope patches and eventually a closed double nuclear membrane. It is proposed that integral membrane pore proteins are involved in the fusion between the inner and outer nuclear membranes, leading to subsequent formation of the nuclear pore complex. B: The nuclear pore complex (all eukaryotes) and the spindle pole body (yeast) are both embedded in the double nuclear membranes. C: Schematic of the "closed" mitosis of S. cerevisiae. The duplicated spindle pole bodies are on opposite sides of the nuclear membranes and release spindle microtubules.
Instinct regulatory molecules have been implicated to be involved in nuclear pore assembly. RanGTP acts as a positive regulator of nuclear pore assembly, while importin α, in addition to its role in nuclear transport, acts as a negative regulator for both membrane fusion and nuclear pore assembly (Hacker et al., 2000; Harèl et al., 2003a; Walther et al., 2003b). For a full review, see Harel and Forbes, 2004). The Nup107-160 complex is an early determinant required for vertebrate nuclear pore assembly. Depletion of this complex from the extract leads to a complete lack of assembled nuclear pore structures (Harel et al., 2003a; Walther et al., 2003a). Additionally, in such depleted nuclei, the pore integral membrane proteins are no longer localized in the punctate nuclear rim localization expected of nucleoporins, but instead are distributed diffusely over the surface of nuclei (Harel et al., 2003b).

Additional Player(s) in Vertebrate Nuclear Pore Assembly?

Nuclear pore membrane proteins have long been thought to participate in nuclear pore assembly (for discussions, see Yusa and Forbes, 2001; Burks and Ellenberg, 2002; Suntharalingam and Wieske, 2003). However, it has been difficult to study these membrane proteins, which consist of POM121 and gp210 in vertebrates, and Ndc1p, Pom152p, and Pem34p in the budding yeast S. cerevisiae (Rout et al., 2000; Crenshaw et al., 2002; and references therein). Only recently has vertebrate POM121 been shown to be involved in membrane fusion and nuclear pore assembly (Aronnin et al., 2005, and references therein). The role for gp210 remains controversial (Drummond and Wilcox, 2002, Antonin et al., 2005). POM121 and gp210 have no known homologues in yeast. Ndc1p, Pom152p, and Pem34p, conversely, have had no known homologues in higher eukaryotes. Ndc1p (snc1p) is the only essential pore protein in yeast. Deletion of Pom152p and/or Pem34p does not appear to have an adverse effect on the viability of the cells (Wozniak et al., 1994, and data not shown). Interestingly, snc1p localizes to both the nuclear pore and the spindle pole body (SPB; Fig 1B) (Chial et al., 1998). Similar to the nuclear pores, the budding yeast spindle pole bodies are also involved in the nuclear envelope at a site of fusion between the double nuclear membranes (Fig 1B). The SPB in yeast function to nucleate microtubules and to organize the mitotic spindle (for review, see Jasperson and Wines, 2004). It duplicates once per cell cycle during G1. The pair of SPBs then migrate to opposite poles during mitosis (Fig 1C). By electron microscopy, the S. cerevisiae SPB appears as disk-like structures (O'Toole et al., 1999).

Of the ~20 different proteins that comprise the budding yeast SPB, three integral membrane proteins: Ndc1p, Mps2p, and Mps3p. The membrane proteins of the SPB have been implicated to be involved in the insertion of SPB into the double nuclear membranes during SPB duplication. Cells carrying mutations in Ndc1p and Mps2p begin to assemble a partial SPB, but fail to insert it into the nuclear envelope (Wines et al., 1991, 1993; Lau et al., 2004). Certain mutations in Ndc1p also cause defects in nuclear pore assembly (Lau et al., 2004). As nuclear pores and spindle pole bodies are the only large protein structures that span the double nuclear membranes, it is tempting to speculate that their assembly at some level would involve a similar mechanism.

It would be a pleasing symmetry if higher eukaryotes assembled nuclear pores in a comparable manner as in yeast. However, no nuclear pore membrane proteins appear to be common between yeast and metazoa. The only known functional homologue of S. cerevisiae Ndc1p prior to this study was the Cut11+ protein in Schizosaccharomyces pombe, which localizes to SPBs and nuclear pores (West et al., 1996). While Cut11+ is required for SPB duplication, its role at the nuclear pore has yet to be established (West et al., 1998). Despite the essential nature of yeast Ndc1p, its topology remains unknown; thus, models for its involvement in SPB and nuclear pore assembly are limited in detail.

In this study, we identify and analyze metazoan homologues of NDC1 and predict the topology of both yeast and metazoan homologues using charge distribution comparisons. We biochemically determine the yeast Ndc1p topology and examine the sites of mutation in the yeast Ndc1p that cause defects in SPB and nuclear pore assembly for conservation in evolution. Finally, we present evidence that indicates the inner nuclear membrane protein NFT3 is the human homologue of yeast Ndc1p.

MATERIALS AND METHODS

Finding Homologues of S. cerevisiae Ndc1p

The established homologue of NDC1 in S. pombe, Cut11+ (NP_594025), was used to search the genomic database available through the National Center for Biotechnology Information (NCBI) with the Position Specific Iterated Basic Local Alignment Search Tool (PSI-BLAST).

Once a human homologue (CA122160) was identified by exhaustive iteration, it was used to search the NCBI database for additional homologues using PSI-BLAST.

Multiple Sequence Alignments

All multiple sequence alignments were created using the default parameters of the ClustalW program available through the Workbench program of the San Diego Super Computer Center (workbench.sdsc.edu). The Boxshade program (also available through Workbench) was used to shade identical and similar residues using a similarity threshold fraction of 0.6 or 0.7. AlignX (Invirotec, Carlsbad, CA) was used to manipulate the sequence in Figure 2 to ensure transmembrane regions were properly aligned. Percent identity and similarity among different NDC1 homologues were determined by counting the number of identical and similar residues present in a pairwise alignment using the ClustalW program.

The unrooted phylogenetic tree was generated in the Workbench program as a dendrogram based on a ClustalW alignment of NDC1 sequences in H. sapiens, M. musculus, X. laevis, D. rerio, C. elegans, D. melanogaster, S. pombe, N. crassa, and S. cerevisiae.

Membrane Topology

The hydrophobic plots were generated using the Hidden Markov Model Transmembrane prediction program available through Workbench.

The limited proteolysis assay was done as in Shearer and Hampton (2004). Briefly, yeast microsomes expressing Hmg2p with an myc tag in its fourth luminal loop (EHY12544), Hmg2p with an myc tag in its cytoplasmically exposed C-terminus (EHY1973), or Ndc1p with three myc
Fig. 2. Sequence alignment of NDC1 homologues in human and Drosophila. The sequence of S. cerevisiae Ndc1p (NP_013661) is compared with homologues from Drosophila (NP_65119) and human (CAU1Y2) using ClustalW. The alignment was manually optimized to ensure proper alignment of transmembrane (TM) segments. Dark gray shading indicates identical amino acids, whereas light shading depicts similar amino acids. Bold underline marks the six predicted TM domains.

tags at its C-terminus (Chial et al., 1998) were lysed. The prepared microsomes were then subjected to a light trypsin digestion (250 μg/ml) for 10 min in the presence or absence of 1 M Triton X-100. The reactions were loaded on a 12% SDS-PAGE gel and subsequently transferred to a PVDF membrane for 1 h at 100 V. The blot was probed with the polyclonal anti-myc antibody A-14 (Santa Cruz Antibodies, Santa Cruz, CA).

RESULTS
Identification of Metazoan NDC1 Homologues

The unique localization of S. cerevisiae Ndc1p (scNdc1p) and S. pombe Cmt1p (S. pombe NDC1) and their implicated function in organelle insertion into the yeast nuclear envelope prompted us to investigate whether they have homologues in other organisms. Despite their similar
Fig. 3. Sequence alignment of NDC1 homologues in vertebrates. Vertebrate NDC1 homologues are well conserved. The ClustalW multiple alignment program was used to align human (NP_032190), mouse (NP_0592031), X. levis (AAH79784), and zebrafish (AAH35571) NDC1 homologues. Dark gray indicates identical residues while light gray denotes similar residues. Putative TM domains in the human sequence are underlined. The thin underlined region corresponds to the nonessential region found in S. cerevisiae hNdc1p (amino acids 368–466; see also Fig. 2). Note that much of this region is not well conserved even among vertebrates. The double underlines and the boxed regions represent FG repeats typically found in nucleoporins.

roles, scNdc1p and S. pombe NDC1 themselves have only limited homology (14% identical, 35% similarity). Starting with the S. pombe NDC1 sequence using iterative PSI-BLAST searches that can reveal even small regions of homology, we were able to identify metazoan NDC1 homologues. Human and Drosophila melanogaster NDC1 homologues are shown in Figure 2; others are shown in Figure 3. Specifically, the scNdc1p (Fig. 2) and S. pombe NDC1 proteins are 16% identical and 38–39% similar to human NDC1 (hNDC1; Table 1). In turn, Drosophila NDC1 is 19% identical and 44% similar to hNDC1 (Table 1). A low level of overall sequence homology between yeast and metazoan nucleoporins is in fact very common (Cronshaw et al., 2002; Suntharalingam and Wente, 2003). For example, yeast Nup85p, a member of the Nup107-160 complex, is only 13–14% identical to its metazoan homologues.
TABLE 1. Sequence comparison of the different NDC1 homologues

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<td>S. cerevisiae</td>
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![Diagram showing VC113, NPC1L1, and other proteins](image)

Vertebrate NDC1 Homologues

The PSI-BLAST searches identified NDC1 homologues in multiple vertebrates (Fig. 3). These are more closely related to one another: Danio rerio or zebrafish NDC1 bears 53% identity and 75% similarity to human NDC1, while Xenopus laevis NDC1 has 54% identity and 73% similarity to hNDC1 (Fig. 3, Table 1). As expected, mouse NDC1 and hNDC1 are the most similar with 87% identity and 92% similarity (Fig. 3, Table 1). We identified NDC1 homologues in many species including human, mouse, *Xenopus laevis*, zebrafish, Drosophila, Neurospora, and *C. elegans*. A phylogenetic tree for NDC1 is shown in Figure 4.

Conserved Structure for NDC1 Homologues

The sequence homology data above indicate the presence of NDC1 homologues in metazoa. Importantly, hydropathy plots predict that all of the NDC1 homologues identified contain six to seven transmembrane (TM) domains in the N-terminal half of the protein (Fig. 5A). TM domains are denoted by bold underlines in Figures 2 and 3. Interestingly, the predicted TM domains (Fig. 2, TM 1–6) among human, Drosophila, and yeast are more similar than the loops in the N-terminus (Fig. 2, L1–5). A schematic depicting the hNDC1 structure is shown in Figure 5B. The C-terminal half of the NDC1 homologues is not predicted to contain TM domains and thus is more likely to provide a surface for interaction with other soluble proteins. We conclude that even though the primary sequences can differ substantially, the overall structure of NDC1 is conserved throughout evolution.

N- and C-Termini of NDC1 Homologues

![Diagram showing N- and C-Termini](image)

The topology of yeast Ndc1p has never been established. It is not known whether the large C-terminal domain of ~300 amino acids is present in the ER lumen or instead exposed to the cytoplasm. We found in this study from the Hidd Markov Model Transmembrane (HMMT) analysis that the sNdc1p contains six TM domains in the N-terminal half (Fig. 5A). Although a plausible seventh TM domain was predicted previously from a Kyte-Doolittle hydropathy plot (Winey et al., 1993), we did not observe this with HMMT analysis. To determine the topology of sNdc1p, we used two different strategies: a computational method involving amino acid charge prediction and a biochemical method of limited proteolysis (Calvulin et al., 1997; Sakaguchi, 2002; Shearer and Hampton, 2004; Nilsson et al., 2005).

In a multipass membrane protein, the orientation of the first transmembrane domain is key to determining the orientation of the rest of the protein. Proteins with a cluster of positively charged amino acids before their first TM domain follow the “positive-inside” rule, so termed because the N-terminus is found inside the cytoplasm (von Heijne, 1989; Calvulin et al., 1997; Nilsson et al., 2005).

Proteins that possess this first TM domain orientation are classified as signal anchor type II proteins; this is the preferred orientation of all the known inner nuclear membrane proteins discovered (Sakaguchi, 2002; Schirmer et al., 2003).

The presence of four lysines and arginines before the first TM domain of sNdc1p suggests that its N-terminus is of this type and is exposed to the cytoplasm (Fig. 6). Similar analysis of the human, mouse, zebrafish, Drosophila, and *S. pombe* NDC1 protein sequences reveals that they also contain a high number of positively charged residues before their first TM domain (Fig. 6). Upon further analysis of the regions between TM domains, it is clear that sNdc1p contains a greater number of positively charged amino acids in even-numbered loops (Fig. 6, L2 and L4) than in odd-numbered loops (Fig. 6, L1, L3, and L5). We conclude that sNdc1p is typical of a multipass protein in which positively charged regions or loops are present.
Fig. 5. NDC1 homologues share similar topological profiles. A: Human, mouse, zebrafish, Drosophila, S. pombe, and S. cerevisiae protein sequences were analyzed for putative TM domains by the HMMer prediction program. The x-axis represents the position of the amino acid while the y-axis represents the probability that amino acid is a TM segment. Each protein is shown as multiple segments, with the N-terminal half consisting of six or seven putative TM domains, while the C-terminal half does not contain any TM domains. Those peaks marked with dots in Drosophila and S. pombe are unlikely to be TM domains, as discussed in the text and legend to Figure 6. B: Schematic of the proposed structure of human NDC1 (FLJ10407). Computer predicted TM sequences are represented by black boxes. The figure is not drawn to scale.

Favored in the cytoplasm, whereas loops containing non-charged amino acids are favored in the ER (Lunkevich et al., 1997; Sakaguchi, 2002; Nilsson et al., 2005). It is important to note that domains predicted to be in the cytoplasm can also be in the nucleoplasm. Charge prediction indicates a similar topological organization for the human, mouse, and zebrafish NDC1 homologues, with loops L2 and L4 exposed to the cytoplasm (Fig. 4). Specifically, the analysis predicts that budding yeast, human, mouse, and zebrafish NDC1 are all multipass signal anchor type II proteins with both N- and C-terminals exposed to the cytoplasm.

Hydropathy plots had predicted that Drosophila NDC1 and S. pombe NDC1 could possibly contain seven TM domains (Fig. 5A). However, by the above charge analysis, we found that there are positive charges present on both sides of the potential seventh TM region of Drosophila NDC1, as well as surrounding the potential third TM...
Fig. 6. The N- and C-termini of all NDC1 homologues are predicted to be exposed to the cytoplasm. Based on TM sequence prediction, the distributions of positively charged amino acids mapping between each TM segment were analyzed. Black boxes denote TM domains predicted by the HMMFinder prediction program. Numbers represent the number of positively charged amino acids present in each intervening loop region. Light gray boxes indicate regions that are predicted to be cytoplasmic.

region of S. pombe NDC1 (Fig. 6, shaded regions; see also Fig. 5A, peaks marked by dots). It has been experimentally demonstrated that when a TM domain is spanned by charged regions, the potential TM domain is often not inserted into the membrane but is instead left out (Fig. 5A, dots) (Gafvelin et al., 1997). This results in both of the surrounding charged non-TM regions and the potential TM region they flank remaining in the cytoplasm. Such proteins have been referred to as “frustrated” multipass proteins (Gafvelin et al., 1997). If this is the case for Drosophila and S. pombe NDC1, then these “frustrated” multipass proteins will, like their homologues, have six actual TM domains with their C-termini exposed to the cytoplasm. In summary, we predict that both the short N-terminus and the large C-terminal half of human, mouse, zebrafish, S. cerevisiae, and likely Drosophila and S. pombe NDC1, are cytoplasmically exposed.

### Limited Proteolysis Indicates a Cytoplasmic Orientation for C-Terminal Half of S. cerevisiae Ndc1p

To test the topology of sncDlp predicted above, we performed a limited proteolysis assay on yeast microsomes. Microsomes are small membrane vesicles of ER origin and result when the ER becomes vesiculated by cell lysis. Microsomes are known to retain accurately the orientation of the membrane proteins in the membrane, i.e., the lumen of the ER is equivalent to the lumen of the microsomes (Shearer and Hampton, 2004). We prepared microsomes from a myc-tagged sncDlp yeast strain and from two control protein strains. Trypsin was added to digest any parts of the proteins exposed to the cytoplasm in vitro (i.e., on the outside of microsomes). Only the TM domains and any parts of the protein in the ER lumen are protected in such an assay. The tagged portion of the protein is detected by Western blotting with an anti-myc antibody. The potential orientations of sncDlp in microsomes are shown in Figure 7A.

A multipass Hmg2p protein myc-tagged on its luminal region indeed showed a protected fragment of ~20 kD after trypsin digestion (Fig. 7B, lane 3, square). This protected fragment was not detected when the microsomal membranes were solubilized with the Triton X-100 detergent (Fig. 7B, lane 4). In contrast, when an Hmg2p protein myc-tagged on a cytosolic region was digested by trypsin treatment, either in the absence or presence of detergent,
no protected fragment was detected by Western analysis (Fig. 7B, lanes 7 and 8). These controls established the validity of the assay to be used on S. cerevisiae Ndc1p.

Using a strain containing an myc-tag on the C-terminus of scNdc1p, we found that the C-terminal half of scNdc1p was not protected after trypsin digestion (Fig. 7B, lane 11). If the C-terminus were lumenal, it should have given an expected fragment of ~45 kDa, which was not observed (Fig. 7B, dot). This result suggests that the C-terminus of scNdc1p is indeed exposed to the cyto-
plasm, consistent with our amino acid charge prediction (Fig. 6).

NETS Is Human Homologue of NDC1

Localization of a protein is a key component in the determination of its function. It would be interesting to learn whether human NDC1 localized to the nuclear envelope and nuclear pores. Upon closer examination of the literature, we found that the human NDC1 protein sequence FLJ10407 we obtained by PSI-BLAST (Fig. 2) was also identified in a proteomic study as a new nuclear membrane protein (Schirmer et al., 2003). The FLJ10407 sequence codes for a protein the authors named NETS, a nuclear envelope (transmembrane protein that fraction-
ates specifically with the nuclear membranes (Schirmer et al., 2003). NET3 epitope tagged at the N-terminus with hemagglutinin (HA) showed colocalization in the region of the lamins (Schirmer et al., 2003). In addition, NET3 was predicted to be an integral membrane protein with its N-terminus in the cytoplasm, since the N-terminal HA tag was retained (Schirmer et al., 2003). The NET3 localization and HA tag retention results are in complete agreement with our study. We identified PEG14097 to be human NDC1 based on its homology to yeast Ndc1p, which is an integral membrane protein of the yeast nuclear envelope. We also predict hNDC1 contains N- and C-termini that are exposed to the cytoplasm. In conclusion, our sequence analysis indicates that NET3 is hNDC1.

**DISCUSSION**

In this study, we have identified and analyzed the metazoan homologues of the yeast Ndc1p. We demonstrate their homology by sequence analysis and structural domain prediction. Computational and biochemical analysis showed that both the N- and C-termini of the yeast NDC1 are in the cytoplasm. Computational analysis of the metazoan NDC1 homologues agrees with this prediction. Lastly, we believe that the human NDC1 is the equivalent of the nuclear membrane protein, NET3.

Studies in yeast have shown that sNdc1p and S. pombe Ndc1 localize to both the spindle pole bodies and the nuclear pores. As yet, we do not know whether metazoan NDC1 proteins localize to nuclear pores and/or centrosomes, the latter being the functional equivalent of the yeast SPBs, albeit not membrane-bound. Interestingly, NDC1 was not recognized as a nucleoporin in a proteomic study on detergent-extracted rat liver nuclei by Cronshaw et al. (2002). Generation of antibodies to metazoan NDC1 proteins will be needed for further analysis. Additionally, immunoelectron microscopy studies will be extremely useful for pinpointing the precise localization of hNDC1/NET3 within the nuclear membranes, i.e., whether it is a nuclear membrane protein or a nuclear pore membrane protein. Strikingly, the vertebrate NDC1 proteins contain several FG regions (SPFG, TPFG, and TPFG) that are characteristic of nucleoporins (Fig. 3, double underlines and boxed regions; DP, unpublished observations).

**Clues From Yeast**

To gain insights into the potential functional domains of metazoan NDC1 protein, we assess the implications from the analysis of yeast genetic studies. Few alleles of sNdc1p exist that disrupt function, primarily because the NDC1 protein is absolutely crucial to successful mitosis. Of those, only the temperature-sensitive mutant, ndc1-39, disrupts assembly of both the spindle pole body and the nuclear pore (Lau et al., 2004). Interestingly, ndc1-39 protein remains localized to the SPBs and the nuclear pores at the restrictive temperature (Lau et al., 2004). This indicates that the mutated regions must not be required for localization of the protein, but instead affect the assembly of the SPB and/or nuclear pores, likely by disrupting interactions between sNdc1p and soluble nucleoporins or other spindle pole body components. The ndc1-39 allele resulted from a PCR mutagenesis and contains six point mutations (Lau et al., 2004). None of the mutations are in the predicted TM domains and three of them are sufficient to cause the temperature-sensitive mutant phenotype (Figs. 2 and 8, diamonds) (Lau et al., 2004). Mutation E283G falls in a five-residue region (E283XGGD285) that is extremely conserved across at least 16 species from yeast to vertebrates (Fig. 8, bracket). The L283M mutation lies immediately adjacent to this region. We predict that this small region is likely to be important for NDC1 function in higher eukaryotes. Other well-conserved regions are highlighted in Figure 8.

Indeed, valuable lessons can be learned from studying regions of yeast Ndc1p. Deletion analysis in S. cerevisiae (Lau et al., 2004) showed the only nonessential region of the sNdc1p lies between amino acids 399–449 (Figs. 2 and 3, thin underlines). Strikingly, this nonessential region is one of the least conserved regions in vertebrate NDC1 homologues (Fig. 3) and largely absent in Drosohila NDC1 (Fig. 2). Taken together, our analysis points to
VERTEBRATE NDC1 HOMOLOGUES

important regions in yeast Ndc1p that are conserved in metazoan NDC1 proteins and likely to be essential for their functions, as well as to a region less likely to be essential. The mechanism by which SnCdc1p anchors the SPB and nuclear pores during their assembly is currently not well understood. We speculate that yeast Ndc1p interacts with either soluble or transmembrane nucleoporins via its own canonical domain. Either or both of these interactions may help integrate the fusion between the outer and inner nuclear membrane and to stabilize the fenestra when the two membranes fuse (Fig. 9), processes that are required for both spindle pole body and nuclear pore assembly.

Human NDC1/NET3

hnDC1/NET3 maps to a genetic region that is broadly linked to congenital potois, hereditary type I, a form of muscular dystrophy (Schirmer et al., 2003). Although many other genes reside in this chromosomal location, it will be interesting to characterize the loss-of-function phenotype of the vertebrate NDC1 proteins at the cellular level, as well as at the organismal level.

Further studies will determine whether the metazoan NDC1 is a bona fide member of the nuclear pore. Based on the localization of hnDC1/NET3 to the nuclear envelope (Schirmer et al., 2003), we predict the vertebrate NDC1 proteins carry out similar functions as in yeast. If this is the case, then NDC1 will be the first nuclear pore membrane protein that is conserved throughout evolution and thus may be the long-sought-after link between the early membrane fusion events of vertebrate and yeast nuclear pore assembly.

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LITERATURE CITED


Acknowledgements, Chapter 1

This chapter, in full has been reproduced from Lau CK, Delmar VA, Forbes DJ. Topology of yeast Ndc1p: predictions for the human NDC1/NET3 homologue. The Anatomical Record Part A Discoveries in Molecular, Cellular, and Evolutionary Biology, 2006, 288(7):681-94 by copyright permission from Wiley-Liss, Inc. I was the second author listed in this publication that forms the basis for this chapter. We wish to thank Alexander Shearer and Randy Hampton for technical help and the Hmg2p constructs, Laura Kwinn and Victor Nizet for the use of the AlignX software and Mark Winey for the scNdc1p construct.
CHAPTER 2

Global histone acetylation induces functional genomic reorganization at mammalian nuclear pore complexes

Christopher R. Brown,1,3 Caleb J. Kennedy,1,2 Valerie A. Delmar,2 Douglass J. Forbes,2 and Pamela A. Silver1,4

1Department of Systems Biology, Harvard Medical School, Boston, Massachusetts 02115, USA; 2Section of Cell and Developmental Biology, Division of Biological Sciences, University of California at San Diego, La Jolla, California 92037, USA

The nuclear localization of genes is intimately tied to their transcriptional status in Saccharomyces cerevisiae, with populations of both active and silent genes interacting with components of the nuclear envelope. We investigated the relationship between the mammalian nuclear pore and the human genome by generating high-resolution, chromosome-wide binding maps of human nucleoporin 93 (Nup93) in the presence and absence of a potent histone deacetylase inhibitor (HDAC). Here, we report extensive genomic reorganization with respect to the nuclear periphery following HDACI treatment, including the recruitment of promoter regions, euchromatin-rich domains, and differentially expressed genes. In addition to biochemical mapping, we visually demonstrate the physical reorganization of several genomic loci with respect to the nuclear periphery. Our studies show that inhibiting HDACs leads to significant changes in genomic organization, recruiting regions of transcriptional regulation to mammalian nuclear pore complexes.

(Keywords: NPC; nucleoporin; nuclear organization; Nup93; Chip-chip)

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The nucleus is a structurally and functionally complex organelle with a nonuniform interior consisting of distinct chromatin domains and several proteinaceous subcompartments. Chromosomes occupy nonrandom intra-nuclear positions with respect to each other and the nuclear periphery (Colet et al. 1999; Parede and Misteli 2002; Parada et al. 2001, 2004a,b; Tanabe et al. 2002a,b; Cremer et al. 2006). Chromosome positioning is believed to expose genomic loci to functionally distinct regions in the nucleus, generating transcriptional regulatory domains favoring either activation or repression. Distinct subnuclear regions also direct specific transcriptional programs by organizing genomic loci around specialized protein hubs. The nucleolus is one such region, mediating the localized transcription of ribosomal RNA genes encoded on multiple chromosomes. In the budding yeast, Saccharomyces cerevisiae, a nucleolar-proximal region has also been shown to cluster several tRNA genes (Thompson et al. 2003).

Components of the nuclear envelope have been shown to assert a repressive role in transcription. In yeast, the silent mating-type loci and telomeres are regulated by components of the nuclear periphery, including the nuclear pore complex (NPC) (Stavenhagen and Zakian 1994; Thompson et al. 1994; Maitel et al. 1996; Marcaud et al. 1996; Andrulis et al. 1998; Feuerbach et al. 2002). However, several recent studies have reported NPC-proximal transcriptional activation, with the concomitant recruitment of induced genes from the nuclear interior to the periphery (Brickner and Walter 2004; Casalini et al. 2004, 2005; Menon et al. 2005; Cabal et al. 2006; Drupois et al. 2006; Drubin et al. 2006; Schmid et al. 2006; Taddei et al. 2006; Brickner et al. 2007; Larrea et al. 2007; Barsma et al. 2007). NPC association can increase the efficiency of mRNA processing and export through associations with the SAGA and TREX complexes, regulate the absolute levels of gene expression, and establish an epigenetic state that confers transcriptional memory and rapid reactivation of genes (Cabal et al. 2006; Taddei et al. 2006; Brickner et al. 2007). Furthermore, several components of the nuclear transport machinery possess boundary activity, potentially facilitating the presence of both repressive and activating domains at the nuclear periphery (Ishii et al. 2002).

Elements of the nuclear periphery are also involved in transcriptional regulation in Drosophila melanogaster. In Drosophila, the dosage compensation complex (DCC)
required for the twofold increase in gene expression on the peripherally localized male X chromosome, interacts with two nuclear pore components [Mendes et al. 2006]. The deletion of these pore proteins eliminates the hypertranscription of the male X chromosome, implicating *Drosophila* nuclear pores in transcriptional activation.

In support of this, the *Drosophila* proteins Eyi1 and Xmis-2 were recently shown to regulate mR1A expression, export, and the subnuclear positioning of the bagpipe gene cluster [Kurashkova et al. 2007]. Eyi1 and Xmis-2 are components of the *Drosophila* SAGA and TREX complexes, whose homologs in yeast play a role in NPC-associated transcriptional activation [Cabel et al. 2006; Deppeis et al. 2006; Taddei et al. 2006].

Interestsingly, a recent genome-wide study in *Drosophila* probing interactions between the nuclear lamina and the genome uncovered a repressive role for the nuclear periphery (Pickersgill et al. 2006). Lamins are integral components of a protein network that lines the inner surface of the nuclear envelope between NPCs and have been shown to bind chromatin in vivo. These results suggest that distinct peripheral components, NPC, and lamins, may have divergent roles in transcriptional activation and repression, respectively. In addition, a lamin-associated protein, LAF2p, binds to histone deacetylase 3 (HDAC3), a member of a large family of proteins that removes acetyl modifications from histones [Sonech et al. 2005]. Highly acetylated histones are involved in the promotion of transcriptionally active genes, suggesting that the lamin-mediated enrichment of HDAC3 at the nuclear periphery could aid in the maintenance of a transcriptionally repressive environment [Fukuda et al. 2006].

Observations in both mouse and human cells indicate the presence of an equally diverse transcriptional regulatory domain at the nuclear periphery. For example, gene-poor chromosomes are reproducibly located near the nuclear periphery in human lymphocytes and fibroblasts [Croft et al. 1999; Boyle et al. 2001; Tanabe et al. 2002b]. However, a study of the murine β-globin locus during erythroid maturation showed that significant transcriptional activity occurred at the nuclear periphery prior to the locus’s transit to the interior [Rangecy et al. 2006]. While NPCs have yet to be implicated in β-globin activation, evidence has emerged that a murine nucleoprotein, Nap93, is involved in interferon gene regulation [Faria et al. 2006].

The mammalian NPC is thought to contain as many as 30 unique proteins that are present in multiple copies due to the eightfold symmetry of the complex [Conshaw et al. 2002]. Nucleoporin 93 (Nup93) is a mammalian nucleoporin centrally located in the nuclear pore [Rout et al. 2000; Krull et al. 2004]. siRNA-mediated depletion of Nup93 in HeLa cells results in misshapen NPCs lacking some, but not all nucleoporins, while depletion of both Nup93 and the transmembrane nucleoporin Ndc1 led to a complete disruption of NPCs [Mansfield et al. 2006]. Nup93, as well as importin-α and CAS (an exportin), have been shown to interact with the histone acetyltransferase (HAT), CREB-binding protein (CBF) [Ryan et al. 2006]. This interaction is similar to one seen in *S. cerevisiae*, where association of a HAT with the NPC is mediated by interactions with SAGA, a multisubunit complex required for the expression of numerous yeast genes [Green et al. 2003; Rodriguez-Naveiro et al. 2004]. As mentioned previously, NPC-SAGA interactions are thought to play a major role in mediating gene recruitment to the NPC in *S. cerevisiae* (Cabel et al. 2006; Deppeis et al. 2006; Taddei et al. 2006). HDAC inhibitors (HDACIs) globally elevate levels of histone acetylation in the nucleus by inhibiting Class I and II HDACs [Drummond et al. 2005]. HDACI treatment also leads to the enrichment of acetylated histones at the nuclear periphery [Taddei et al. 2001; Gilchrist et al. 2004; Pickersgill et al. 2006]. For example, repressed genes are no longer associated with lamins in *Drosophila* after treating cells with the HDACI (trichostatin A [TSA]) for 24 h [Pickersgill et al. 2006]. In addition, the human GFER gene moves away from the nuclear periphery upon treatment with TSA for 10 h [Zink et al. 2004]. Importantly, several HDACIs are in clinical trials for various forms of cancer due to their ability to induce the expression of repressed genes that lead to growth arrest, differentiation, and apoptosis in transformed cells [Drummond et al. 2005; Glasser 2007].

Despite observations of both transcriptional activation and repression at the nuclear periphery in *S. cerevisiae* and *Drosophila*, it was not always clear which regulatory domains play the most significant role. Using genomic location analysis [Ren et al. 2000], we report Nup93 interactions with human chromosomes 5, 7, and 16. To investigate the effects of a global histone deacetylation on NPC-chromatin interactions, we treated cells with TSA, a reversible HDAC that does not perturb gross nuclear structure [Taddei et al. 2001; Gilchrist et al. 2004; Pickersgill et al. 2006]. Upon treatment with TSA, Nup93 distribution was significantly altered across all three chromosomes, indicative of a large-scale nuclear reorganization even with associated effects on gene expression. Analysis of Nup93-binding sites identified enrichments for several genomic features involved in transcriptional regulation. Together, these features define regions of functional interaction between the nuclear pore and the human genome.

**Results**

**Identification of Nup93-binding sites**

We investigated Nup93-binding sites in the presence and absence of TSA, a potent HDAC inhibitor. We also performed chromatin immunoprecipitation (ChIP) on several other nucleoporins including Nap62, Nap107, and Nap105, isolating significant amounts of DNA (data not shown). Nup93 was chosen for further study due to the robustness of Nup93 ChIPs and the strictly maintained subnuclear localization of Nup93 at the nuclear envelope.
Figure 1. TSA treatment of HeLa S3 cells. Histone acetylation was monitored with an anti-acetylated H4K5 antibody (H4K5ac) in untreated and TSA-treated HeLa S3 cells. Histone acetylation was enriched at the nuclear periphery after 12 h of exposure to 40 ng/mL TSA. H3K9 acetylation is also enriched, although to a lesser extent (Supplemental Fig. 1). The peripheral localization of Nup93 was unchanged following TSA treatment. Bars, 5 μm.

lope (Fig. 1). Immunofluorescence microscopy of HeLa S3 cells following a 12 h, 40 ng/mL TSA treatment revealed a marked increase in histone H4K5 acetylation at the nuclear periphery (Fig. 1), a finding supported by other studies (Taddei et al. 2001; Gilchrist et al. 2004). H3K9 acetylation was also enriched at the nuclear periphery (Supplemental Fig. 1), although to a lesser extent, consistent with previous observations (Gilchrist et al. 2004). Additionally, we found that Nup93 localization was unchanged following TSA treatment (Fig. 1). The subnuclear localization of two other nuclear envelope proteins, lamin B and LAF1, were also unaffected by TSA treatment (Taddei et al. 2001).

Next, we performed genomic location analysis by coupling ChIPs and microarray analysis (ChIP-chip) to map interactions between Nup93 and the human genome. This technique has been used extensively in S. cerevisiae and is emerging as a powerful tool in higher eukaryotes (Ren et al. 2002; Carroll et al. 2005, 2006; Kim et al. 2005). Asynchronously growing HeLa S3 cells were cross-linked, stabilizing both protein-protein and protein-DNA interactions. We then performed ChIPs of Nup93 and purified, amplified, and hybridized the associated DNA to Affymetrix tiled arrays (ChIPk, also referred to as chip5) containing all nonrepetitive regions of human chromosomes 5, 7, and 16. This subset of chromosomes was examined based on preliminary data obtained from Nup93, Nup107, and Nup305 genomic association studies that showed significant interactions with chromosome 7 on ENCODE arrays, including binding at the HOXA locus and CFTR (data not shown). In addition, chromosome 7 has been shown to preferentially reside near the nuclear periphery in human cells (Boyle et al. 2001).

Nup93 was mapped to 207 sites on chromosomes 5, 7, and 16 in untreated cells and 170 sites in TSA-treated cells (Fig. 2A; Supplemental Table 1). The 18% reduction in total Nup93-binding sites was predominantly due to large-scale changes in binding to chromosome 5, where Nup93 association was reduced from 75 sites in untreated cells to 15 sites following TSA treatment (Fig. 2A; Supplemental Table 1). Significant binding sites were classified as either high confidence (145 untreated and 86 TSA sites, 10^{-12} < P < 10^{-3}) or very high confidence (64 untreated and 84 TSA sites, P < 10^{-15}) based upon our statistical analysis (see the Supplemental Material).

Nup93-binding sites were mapped to their nearest neighboring gene, resulting in the assignment of 86 untreated and 90 TSA-treated genes (some genes contained multiple Nup93-binding sites) (Supplemental Table 2). The untreated gene set did not contain any enriched gene ontology groups as reported by Genomatix (Reismuth and Speed 2004). However, the TSA-treated gene set contained two enriched ontology groups: DNA binding activity (P = 0.002) and transcription factor activity (P = 0.02). These ontology groups include several genes that are members of the HOXA transcription factor cluster on chromosome 7, a gene-dense locus with extensive Nup93 association (Fig. 2A).

Mapping of Nup93 sites in untreated and TSA-treated cells across chromosomes 5, 7, and 16 revealed large changes in nuclear pore association (Fig. 2A). Significant reorganization of the q-arm of chromosome 5 occurred with the loss of statistically significant Nup93 binding over a 95-Mb region following TSA treatment (Fig. 2A). A similar change was seen on the q-arm of chromosome 7 where Nup93 association was lost over a 35-Mb region following TSA treatment (Fig. 2B). However, changes of this kind were not observed on chromosome 16, where many Nup93-binding sites, while mostly intergenic in untreated cells, overlapped with coding regions following TSA treatment (Fig. 2B). Interestingly, the subnuclear localization of chromosome territories has been shown to be unaffected by TSA, while local changes in chromatin condensation have been observed (Crote et al. 1999; Santos et al. 2002).

Visualization of Nup93-associated loci

To verify that Nup93-binding sites were located at the nuclear periphery, we performed FISH combined with confocal microscopy to visualize the intranuclear localization of several genomic loci. Four loci with distinct Nup93 association patterns were chosen (Fig. 2B). One locus, associated in untreated cells and absent in TSA-treated cells, was found at the nuclear periphery 65% of the time in untreated cells versus just 30% in TSA-treated cells (probe 86743, P = 1.79 × 10^{-11}) (Fig. 2B). Another locus, associated in TSA-treated cells and absent in untreated cells, was found at the nuclear periphery...
Figure 2. Nup93 binding map and subnuclear localization of associated genomic regions. (A) Nup93-binding sites identified by ChIP-chip are plotted on schematic representations of chromosomes 5, 7, and 16. Nup93-binding sites are denoted by black bars above or below each chromosome for untreated and TSA-treated cells, respectively. Nup93-binding sites directly overlapping coding regions are labeled blue with the associated gene displayed. Chromosomes are not to scale. (B) FISH analysis of Nup93 targets. Four genomic loci were visually mapped in untreated and TSA-treated HeLa S1 cells. From left to right: those loci interact with Nup93 in untreated cells, TSA-treated cells, both conditions, and neither condition. The top images are examples from untreated cells, while the bottom row are examples from TSA-treated cells. In each image, the FISH signal is green, DNA stained with DAPI is blue, and lamin B, a marker of the nuclear periphery, is red. The bar graphs report percent peripheral localization of each locus in untreated and TSA-treated cells. P values comparing the difference in percent peripheral localization for each locus were calculated using the χ² test. BAC probes used to study each genomic region are identified at the bottom of each column. Bars, 5 μm.

...ery just 48% of the time in untreated cells versus 77% in TSA-treated cells [probe 62E1], P = 1.63 x 10⁻⁶] (Fig. 2B). A third locus, associated in both conditions, was found at the nuclear periphery 63% of the time in untreated cells and 64% of the time in TSA-treated cells [probe 451C12, P = 0.46] (Fig. 2B). Finally, a genomic re-
36

System, http://ccrnc.cbi.pku.edu.cn), a Web-based ChIP-
chip annotation system [Ji et al. 2000]. TSA-treated
Nup93-binding sites contained 106 enriched transcription
factor-binding motifs, the top five are shown in Fig-
ure 3D with a complete list included in Supplemental
Table 3. However, we identified only one enriched tran-
scription factor-binding motif in untreated Nup93-bind-
ing sites, ELF1 (P = 8.71 × 10⁻⁶). Importantly, multiple it-
erations of randomly generated data sets failed to iden-
tify any significant motifs using CEAS. The increase in
the number of transcription factor-binding motifs in the
TSA-treated data set likely reflects the shift in Nup93
binding to promoter regions and transcription start sites.
The identified motifs are recognized by a wide array of
transcription factors whose diverse functions include
transcriptional repression, transcriptional activation, G1
progression, and neuronal development.

**Nup93 binding is proximal to differentially expressed genes**

We performed RNA expression analysis that revealed
patterns of expression upon TSA treatment: 753 genes
were induced and 828 were repressed (Supplemental
Table 4). Of these genes, 91 mapped to chromosome 5 (34
induced, 57 repressed), 70 to chromosome 3 (33 induced,
37 repressed), and 29 to chromosome 16 (17 induced, 12
repressed). However, there was no obvious overlap be-
 tween Nup93-binding sites and transcriptionally in-
duced or repressed genes. To probe the relationship fur-
ther, we mapped all untreated and TSA-treated Nup93-
binding sites to the nearest differentially expressed gene
(induced or repressed). While there was no distance-to-gene
relationship was uncovered for untreated Nup93-binding
sites (Fig. 4A), we did uncover a strong correlation in
TSA-treated Nup93-binding sites (Fig. 4B). One example
of the proximal positioning of Nup93-binding sites to
differentially expressed genes following TSA treatment
is shown in Figure 4C. In untreated cells, Nup93-binding
sites were identified in the intergenic region between
two genes, RPA3 and GLLG1, on chromosome 7. In
TSA-treated cells, Nup93 sites were found in both
promoter regions, consistent with the promoter enrich-
ment discussed earlier (Fig. 3B). Interestingly, while
GLLG1 expression was induced following TSA treat-
ment, RPA3 expression was unaffected. Observations
such as these show that differentially expressed genes
can localize to the nuclear periphery, but that proximal
positioning to the NPC is not always coupled with a
transcriptional change.

**Nup93-binding sites are enriched for specific histone
methylation**

Unlabeled and TSA-treated Nup93-binding sites were
enriched for histone methylations associated with tran-
scriptionally silent and active regions, respectively
(Table 1). A recent study mapped 10 unique histone
methylation, the histone variant H2A.Z, the insulating
factor CTCF, and RNA Polymerase II [PolII] over the
Figure 3. Nup93 association with the genome is significantly altered upon loss of histone deacetylation. (A) Venn diagrams showing the overlap between untreated and TSA-treated Nup93-binding sites and nearest genes. (B) Comparison of untreated and TSA-treated Nup93-binding sites with chip-1 distributions reveals significant changes in association across five genomic regions: exons, introns, promoters, downstream elements, and intergenic regions ($P = 0.001, \chi^2$). Untreated sites are enriched for introns when compared with chip-1 ($P = 0.002$). TSA-binding sites are significantly enriched for introns and promoters ($P = 0.005$ and $P = 1.2 \times 10^{-4}$, respectively) and depleted for exons and intergenic regions ($P = 7.7 \times 10^{-3}$ and $P = 5.4 \times 10^{-5}$, respectively) when compared with chip-1 distributions. (C) Distribution of distances from the center of each untreated Nup93-binding site, TSA-treated Nup93-binding site, and random site to the nearest transcription start site. P-values for C were calculated by comparing untreated and TSA-treated Nup93-binding sites to randomly distributed sites using the two-tailed Mann-Whitney U-test. (D) Sequence logos for the five most significantly enriched transcription factor-binding motifs identified in TSA-treated Nup93-binding sites. One-hundred-six motifs were identified in TSA-treated binding sites by CEAS (Supplemental Table 3).

We compared the reported distributions of histone modifications from Barski et al. (2007) with our untreated and TSA-treated Nup93-binding sites and found several significant overlaps (Table 1). Nine of the histone modifications are associated with active genes, five with silent genes, and six showed no bias. Untreated Nup93 sites were enriched for three specific histone modifications associated with silent genes, depleted for two modifications associated with active genes, and depleted for RNA PolIII-binding sites. The opposite was found with TSA-treated Nup93 sites, which were enriched for seven histone modifications associated with active genes, depleted for three modifications associated with silent genes, and enriched in RNA PolII, H3A-Z, and CTCF-binding sites. These results indicate that regions associated with increased transcriptional activity become associated with Nup93 upon treatment with TSA.
Figure 4. Nap93-binding sites are proximal to differentially expressed genes following TSA treatment. Untreated and TSA-treated Nap93-binding sites were mapped to the nearest differentially expressed gene. The log_{10} transformed distance-to-gene value (y-axis) was plotted against the magnitude of differential expression (y-axis). Spearman rank order correlation was used to assess statistical significance. Down-regulated genes (blue) and up-regulated genes (red) are shown in each plot. The black trend lines were calculated by least squares best fit. (A) There was no correlation between distance-to-gene and magnitude of differential expression for untreated Nap93-binding sites ($P = 0.0241$, $P = 0.7198$). (B) Significant correlation was observed when comparing distance-to-gene and magnitude of differential expression for TSA-treated Nap93-binding sites ($P = 0.5056$, $P = 6.28 	imes 10^{-11}$). (C) Nap93-binding sites near two genes on chromosome 7, APA3 and GLCCI1. Untreated and TSA-treated sites overlap in the intergenic space while TSA treatment induces Nap93 binding to the promoter region of both genes, denoted by thin black rectangles. GLCCI1 expression is induced upon TSA treatment, illustrated by the red rectangle in the Expression track.

Nap93 association with Giemsa (G) bands

To investigate chromosome-wide Nap93-binding patterns we compared the distribution of Nap93-binding sites with the G bands of chromosomes 5, 7, and 16. Staining with G dye reveals chromosome-wide banding patterns that are divided into five classes based on their staining intensity (Faucy and Haakelet 2003). The G-negative (Gneg) class consists of nonstaining bands, while the Gpos25, Gpos50, and Gpos75 classes contain progressively darker G-positive bands. The Gpos100 class consists of the darkest staining bands. Areas of heavy G staining represent condensed regions of the genome that have been shown to be gene poor, transcriptionally inactive, AT-rich, and replicated late in S phase. Of all the classes of G bands, Gneg bands have the highest density of genes, the largest average number of CpG islands, and are considered to represent euchromatin.

We identified nonrandom distributions of Nap93 across G bands in both untreated and TSA-treated data sets when compared with chip5 distributions (Fig. 5A). Untreated binding sites were enriched in Gpos50 bands (27% compared with 15% on chip5), while depleted in Gneg/Gpos25 bands (44% compared with 52% on chip5), suggesting a bias toward heterochromatin ($P < 1.4 \times 10^{-13}$). Conversely, TSA-induced binding sites were enriched in Gneg/Gpos25 bands (60% compared with 52% on chip5), while showing significant depletion in both Gpos75 and Gpos100 bands (9% and 8% compared with 16% and 13% on chip5, respectively), indicating a shift to regions of the genome enriched in euchromatin ($P < 4.6 \times 10^{-10}$). (Fig. 5A)

Next, we investigated the distribution of Nap93-binding sites within G bands. All G bands on chromosomes 5, 7, and 16 were normalized to the same length and then partitioned into 10 sections (bins). We assigned each Nap93 site a bin (0-9) based on its relative position between its nearest G-band boundary. For example, a Nap93-binding site at the extreme 5' G-band boundary would be placed in bin 0, while a binding site at the

GENES & DEVELOPMENT
Table 1. Histone methylation enrichment in Nup93-binding sites

<table>
<thead>
<tr>
<th>Gene bias</th>
<th>Data set</th>
<th>Untreated</th>
<th>TSA</th>
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<tr>
<td>Active</td>
<td>H3K4me1</td>
<td>+++</td>
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<tr>
<td></td>
<td>H3K4me2</td>
<td>+++</td>
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<td>H3K4me3</td>
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<td></td>
<td>H3K27me1</td>
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<td>H3K27me3</td>
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<td></td>
<td>H4K20me1</td>
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<tr>
<td>Silent</td>
<td>H4K20me1</td>
<td>++</td>
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</tr>
<tr>
<td>None</td>
<td>H4K20me1</td>
<td>++</td>
<td></td>
</tr>
<tr>
<td>N/A</td>
<td>CTCF</td>
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Genomic locations of several histone methylations as well as the histone variant H4K20me1, RNA PolII, and the insulating factor CTCF were mapped over the whole genome using the ChiP-Seq method (Gardner et al., 2007). Untreated, TSA-treated, and random intervals were mapped to sequence tags specific to each factor by direct overlap. Each histone modification, as well as H4K20me1, RNA PolII, and CTCF, was associated with a discrete tag frequency distribution for untreated and TSA-treated Nup93-binding sites, which was compared individually with an expected distribution (random sites) using a χ² goodness-of-fit statistical test. Gene biases were reported in Gardner et al. (2007). Tag biases for each factor are reported as either highly enriched (+++, P ≤ 1 × 10⁻⁹), moderately enriched (+++, 1 × 10⁻⁶ ≤ P ≤ 1 × 10⁻⁴), slightly enriched (+, 1 × 10⁻⁴ ≤ P ≤ 6 × 10⁻⁶) or depleted (−, P = 0.05) in untreated or TSA-treated Nup93-binding sites. Blank cells indicate the lack of a statistically significant bias (P > 0.05).

Figure 5. Distribution of Nup93 binding sites within G bands. [A] Nup93 is enriched within Gp050 bands and depleted in Gp050 bands in untreated conditions when compared with chip5 (P = 1.4 × 10⁻²⁷, χ²). However, a similar comparison with chip6 distributions shows that TSA-treated Nup93-binding sites are enriched within Gp0525 bands and depleted within Gp0510 bands (P = 4.6 × 10⁻⁵, χ²). P-values were calculated by comparing the observed G-band distribution to chip6 G-band distributions using the χ² test. All binding distributions across G bands. All G bands were normalized by dividing each into 10 equal sections (bins). We assigned each Nup93 site a bin (0-9) based on its relative position between its nearest G-band boundaries. Each bin shown represents the relative position of several Nup93-binding sites (between 10 and 40 sites per bin). Intervals from randomized data sets were uniformly distributed across G bands, which reflected the positive positional enrichment for chip6. However, both untreated and TSA-treated Nup93-binding sites were enriched in specific regions as shown by the peaks in blue and orange, respectively (P = 3.1 × 10⁻¹⁰ and P = 2.1 × 10⁻⁶, respectively, χ²). Furthermore, untreated and TSA-treated sites were positioned nonrandomly relative to each other, with significant TSA enrichment between two peaks of untreated enrichment (P = 5.4 × 10⁻⁹, χ²).

Discussion
Our results show that the mammalian nuclear pore interacts with the human genome. To probe the functionality of this association we increased global levels of histone acetylation by treating cells with the HDAC inhibitor, TSA. Upon drug treatment, interactions between the nuclear pore and chromatin changed significantly. We observed changes in localized regions of transcriptional importance in addition to chromosome-wide changes in NPC association (Fig. 6).

Local chromatin changes after NPC association
TSA treatment induces the global hyperacetylation of histones by reversibly inhibiting Class I and II HDACs. The resulting histone acetylation, which is enriched at the nuclear periphery in TSA-treated cells, decreases extreme 3' G-band boundary was placed in bin 9. As a result, each bin shown in Figure SB represents the relative position of several Nup93-binding sites (between 10 and 40 sites per bin). A randomized data set failed to produce any enrichment across the normalized G bands in both untreated and TSA-treated data sets, with two very strong peaks of enrichment in the untreated data set flanking a large region of enrichment found solely in the TSA-treated data set (Fig. 5B). These observations are indicative of a nuclear reorganization event induced by the inhibition of histone deacetylation.

GENES & DEVELOPMENT

Brown et al.
chromosome condensation through the recruitment of chromatin remodeling factors such as the SWI/SNF complex (Agalioti et al. 2002). Several other proteins, including CBP and the general transcription initiation factor, TFIID, are also recruited to histones through interactions with acetyl-lysine residues (Agalioti et al. 2002; Fukuda et al. 2006). Acetylated histones are typically enriched in the promoter regions of active genes and we found that Nup93 was highly enriched in these regions following TSA treatment. In addition, we identified >100 transcription factor-binding motifs in the TSA-treated Nup93-binding sites, a finding indicative of the functional repositioning of chromatin. Further evidence for a role of the mammalian nuclear transport machinery in transcriptional regulation comes from the transport protein CAS, the mammalian homolog of the yeast exportin, Cse1. A recent study has shown that CAS associates with 903 as the promoter regions of several pro-apoptotic genes and contributes to their transcriptional activation (Tanaka et al. 2007).

Histone methylation also plays an important role in transcriptional regulation. Several H3K4 methylations are associated with actively expressed genes, while the H3K79me3 modification is associated with silent genes (Barks et al. 2007). Accordingly, Nup93-binding sites in TSA-treated cells are significantly enriched in H3K4me1−3 and depleted in H3K27me3 (Table 1). The opposite histone methylation patterns are observed for untreated Nup93-binding sites, which are random with respect to H3K4me1−3 and enriched in H3K27me3.

Transcriptional regulation at the NPC

TSA-treated Nup93-binding sites were enriched in promoter regions and were closer to transcription start sites than their untreated counterparts. We also show that following TSA treatment, Nup93-binding sites are enriched in RNA Pol II-associated regions and are closer to genes exhibiting differential expression. These regions include genes that are both highly expressed and strongly repressed following drug treatment. The presence of both active and repressed genes at nuclear pores has been observed previously in S. cerevisiae (Casolari et al. 2004). The role of the NPC as a boundary element may help explain these observations (Ishii et al. 2003). Interestingly, TSA-treated Nup93-binding sites are enriched in CTCF-associated regions. CTCF, a protein that recognizes cis-acting transcriptional insulator elements and marks histone modification boundaries (Hiyama et al. 2006; Barks et al. 2007), co-localizes with the histone variant H3A, as well as two components of the nuclear envelope, lamin A/C and importin α3 (Yu et al. 2004). These associations suggest that CTCF interacts with the nuclear periphery and that regions of CTCF-mediated regulation are repositioned proximal to nuclear pores following TSA treatment.

The balance of HAT and HDAC activity is essential for the maintenance of boundaries between active and repressed chromatin (Kimura et al. 2002; Saka et al. 2002). Indeed, boundary elements recruit both HATs and HDACs in order to prevent spreading of silent and active chromatin, respectively (West et al. 2004; Yu et al. 2004). Mutations in HATs and HDACs can significantly shift the borders of functional regions in the genome. For example, deletion of a subunit of the Rpd3 HDAC complex in yeast leads to aberrant transcriptional initiation (Carreau et al. 2005). The mammalian nuclear periphery contains at least one HAT and HDAC, CBP and HDAC3, respectively. CBP interacts with the NPC, while HDAC3 associates with the lamin-binding protein LAP3. These proteins, along with CTCF, could be important components of boundary elements at the mammalian nuclear periphery.

Evidence from work in S. cerevisiae has shown that recently activated genes remain at the nuclear periphery for hours after they have been repressed (Britten et al. 2007). This localization differs from their preactivated positioning, which appears to be random with respect to the nuclear periphery (Drubin et al. 2006). This novel form of transcriptional memory is functional, it was
shown that the recently repressed GAL1 gene was activated faster than the long-term repression. In yeast, Nup93 and the histone variant H2A.Z were involved in this process. Interestingly, we found that TSA-treated Nup93-binding sites were enriched in H2A.Z-binding sites. The presence of H2A.Z at sites of NPC-chromatin interaction could mean a similar form of transcriptional memory is present in human nuclei. Alternatively, H2A.Z site enrichment could reflect the role of this histone variant in peripherally localized boundary activity as H2A.Z has been shown to cooperate with CTCF in HeLa cells and block the spread of telomeric heterochromatin in yeast (Meneghini et al. 2003; Yusufzai et al. 2004).

Chromosome-wide changes in nuclear organization

Large-scale changes in NPC-chromosome interactions were observed after treatment with TSA. Stretches of chromosomes 5 and 7 were no longer associated, while chromosome 16 retained the majority of its associated regions (Fig. 2A). These changes appear to be part of an extensive nuclear reorganization event in response to the global increase in histone acetylation.

The NPC has been implicated in boundary activity in S. cerevisiae and chromosome looping is thought to occur at the nuclear periphery in Drosophila (Genasimova et al. 2000; Ishii et al. 2002). In addition, evidence has emerged that two distal localized genes on murine chromosome 7 can be localized to the same nuclear compartment upon activation (Osborne et al. 2004). It is possible that CTCF-mediated insulator activity near mammalian NPCs could generate similar loops that functionally isolate regions of activation or repression at the nuclear periphery.

Chromosome G-band patterns represent regions of genetic enrichment in either heterochromatin or euchromatin. We show that Nup93-binding sites are more likely found in heterochromatin in untreated cells, while favoring euchromatin following TSA treatment. The significant and opposing histone modifications and G-band enrichments found in Nup93-binding sites reflect a global reorganization event that exchanges silent for active chromatin at NPCs.

In summary, we show that the human genome interacts with Nup93, a component of the mammalian nuclear pore. Nup93 is associated with regions of transcriptional repression and enriched heterochromatin content in HeLa cells. Upon global histone acetylation mediated by the IEC10 inhibitor, TSA, Nup93 associates with regions important for transcriptional regulation. Our results highlight significance for both the general mechanisms of gene expression in mammalian cells and the global effects of histone modifications on nuclear organization.

Materials and methods

Cell culture and drug treatment

HeLa S3 cells were grown in DMEM supplemented with 10% FBS. Exponentially growing cells were incubated in the presence of 40 ng/ml TSA (Sigma) for 12 h. Cells were grown to near confluence before fixation and harvesting for CHIP-chip, indirect immunofluorescence, FISH, and expression profiling.

Immunofluorescence

HeLa S3 cells were grown to near confluence before fixation and immunolabeling. Cells were prepared for labeling as described previously (Brown et al. 1998). Primary antibodies used include anti-lamin B diluted 1:50 (Santa Cruz Biotechnology), anti-Nup93 diluted 1:200 (D. Forbes), anti-acetylated H4K5 di-1:100 (Abcam), and anti-acetylated H4K8-10-dimethyl 1:100 (Upstate Biotechnology). Other antibodies used for preliminary ENCODE studies include anti-Nup93 (BD Transduction Laboratories), anti-Nup107, and anti-Nup133 (generous gifts from V. Cordes). The secondary antibody used to detect polyclonal primaries was goat anti-rabbit Alexa594 diluted 1:1000 (Molecular Probes). Images were acquired on a Nikon E800 epifluorescence microscope equipped with a Radiance 2000 confocal laser scanning system (Bio-Rad).

FISH

Bacterial artificial chromosomes (BACs) were purified using a Large-Construct Kit (Qiagen). DIG-labeled BACs were cohybridized with a T3/T7 RNA polymerase transcription Kit (Roche). For each sample, 40–160 ng of DIG-labeled probe were combined with 40 ng of unlabeled COT1 DNA (Roche) and 20 ng of salmon sperm DNA (Ambion), a portion of which was used for hybridization. Cells were prepared and probed hybridized as described previously (Solovov et al. 2005). The probe signal was detected using an anti-DIG antibody (Roche) and Vectashield containing DAPI (Vector Laboratories) was added to the slides and the coverslips were sealed with nail polish. More than 200 cells were counted for each experimental condition. FISH signal was counted as peripheral if it either overlapped with or was immediately adjacent to the nuclear periphery signal.

CHIP-chip

CHIP was performed essentially as described (Brookeley et al. 2005) with the following differences. Chromatin was prepared from four independently grown batches of cells—two untreated and two treated with 10 ng/ml TSA for 12 h before fixation to generate replicates for each condition. Cleared chromatin was incubated overnight with polyclonal Nup93 antibody at 4°C. After incubation, a fresh batch of 50:50 protein A–G-Sepharose beads were added to the chromatin/antibody solution and incubated overnight. Beads were washed five times for 10 min each, followed by DNA elution overnight, proteinase K treatment, phenol extraction, and RNAse treatment. Isolated DNA was amplified isothermally using random primers and Klenow polymerase [Invitrogen] for 3 h, yielding ~4 ng of DNA per CHIP. DNA was prepared and hybridized to Affymetrix GeneChip Human Tiling 2.0R Chip (chr17): arrays using fragmentation, hybridization, scanning, and scanning procedures described previously (Kennedy et al. 2003). A sample of chromatin was set aside before immunoprecipitation and used to represent the input DNA.

Expression profiling

Total RNA was extracted with Trizol and purified with an RNAeasy Mini Kit (Qiagen) with an added DNase step (RNAeasy DNase-free, Qiagen). Fifteen micrograms of RNA from each
sample were sent to the DFCI Microarray facility for labeling and hybridization to Affymetrix Human Genome U133 Plus 2.0 expression arrays. Raw hybridization intensity CEL (files from three biological replicates were processed using the RMA method [Bolstad et al., 2003], Irizarry et al. 2003a,b). Significant differentially expressed genes were considered those with average log_{2} transformed fold-difference ratios (TARs) untreated ≤-1.2 or less than or equal to -1.2 and P < 0.01 (two-tailed Student’s t-test).

Analysis of genomic binding data

All genomic track data (sequence conservation scores, SNPs, RiboSeq genes, and Genomic idiograms) were downloaded July 2006 from the University of California at Santa Cruz genomic bioinformatics Web site (http://hgdownload.cse.ucsc.edu/ goldenPath for the May 2004 assembly. To maintain consistency in the track variables, these originally downloaded data were used for all subsequent analyses. Duplicate Nup93 ChIP samples were submitted to the Dana-Farber Cancer Institute’s Affymetrix Core facility for hybridization. Enrichments in ChIPs compared with input were calculated from raw intensity (CEI) files using a nonparametric statistical method implemented in Affymetrix Tiling Analysis Software (TAS) (Choi et al. 2006). Binding significances (expressed as probe F-values) were used to detect statistical dependencies between Nup93’s binding enrichments and sequence conservation (see the Supplemental Material) for a technical description of the method, including statistical significance assessment and false discovery.

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Brown et al.


GENES & DEVELOPMENT
44

Genomic association with mammalian NPCs


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This chapter, in full has been reproduced from Brown CR, Kennedy CJ, Delmar VA, Forbes DJ, Silver PA. Global histone acetylation induces functional genomic reorganization at mammalian nuclear pore complexes. Genes & Development, 2008, 22: 627-639 by copyright permission from Cold Spring Harbor Laboratory Press. Supplemental material accompanying this paper can be found at http://www.genesdev.org/cgi/content/full/22/5/627/DC1. I was the tertiary author listed in this publication that forms the basis for this chapter. We would like to thank Alex Brodsky for help developing mammalian ChIP-chip; Beth Rasala for providing generous amounts of antibodies; Pamela Hollasch, Maura Berkeley, and Ed Fox at the Dana-Farber Cancer Institute’s Affymetrix Core Facility for microarray hybridization and scanning; Jennifer Waters and the Harvard Nikon Imaging Center at Harvard Medical School for help with confocal imaging; and J. Casolari, J. Hurt, and A. McKee for comments on the manuscript.
CHAPTER 3

_Xenopus_ importin beta validates human importin beta as a cell cycle negative regulator

Valerie A. Delmar, Rene C. Chan, Douglass J. Forbes

Abstract

Background

Human importin beta has been used in all _Xenopus laevis in vitro_ nuclear assembly and spindle assembly studies. This disconnect between species raised the question for us as to whether importin beta was an authentic negative regulator of cell cycle events, or a dominant negative regulator due to a difference between the human and _Xenopus_ importin beta sequences. No _Xenopus_ importin beta gene was yet identified at the time of those studies. Thus, we first cloned, identified, and tested the _Xenopus_ importin beta gene to address this important mechanistic difference. If human importin beta is an authentic negative regulator then we would expect human and _Xenopus_ importin beta to have identical negative regulatory effects on nuclear membrane fusion and pore assembly. If human importin beta acts instead as a dominant negative mutant inhibitor, we should then see no inhibitory effect when we added the _Xenopus_ homologue.

Results

We found that _Xenopus_ importin beta acts identically to its human counterpart. It negatively regulates both nuclear membrane fusion and pore assembly. Human
importin beta inhibition was previously found to be reversible by Ran for mitotic spindle assembly and nuclear membrane fusion, but not nuclear pore assembly. During the present study, we observed that this differing reversibility varied depending on the presence or absence of a tag on importin beta. Indeed, when untagged importin beta, either human or Xenopus, was used, inhibition of nuclear pore assembly proved to be Ran-reversible.

Conclusions

We conclude that importin beta, human or Xenopus, is an authentic negative regulator of nuclear assembly and, presumably, spindle assembly. A difference in the Ran sensitivity between tagged and untagged importin beta in pore assembly gives us mechanistic insight into nuclear pore formation.

Background

Vertebrate nuclear assembly is a complex process involving the sequential recruitment of specific proteins and membranes to chromatin. At the end of mitosis, membrane vesicles and/or ER membrane sheets arrive at the chromatin surface to fuse and form a unique structure consisting of two complete, encircling membrane bilayers [1, 2]. As soon as regions of double membrane form at the chromatin surface, nuclear pore complexes form within those regions perforating the membranes. Nuclear pore complexes span the bilayers and control virtually all traffic between the nucleus and cytoplasm [3, 4]. The 125-megadalton vertebrate nuclear pore is composed of multiple copies of ~30 different nucleoporins, only three of which are integral
membrane proteins [5]. The majority of nucleoporins are recruited from soluble cytoplasmic subunits. The assembly of these nucleoporins into the 500-1000 protein complex is a daunting task, as nucleoporins must sequentially and precisely assemble in the correct order and location [6-8]. Determining the choreographed molecular mechanism by which nucleoporins assemble into functional pores within the double nuclear membranes is a matter of intense research.

The nuclear import factor, importin beta, and its regulatory counterpart, the small GTPase Ran, were revealed to be two key regulatory factors controlling this choreography, both for nuclear membrane fusion and separately for nuclear pore assembly [9-13]. Addition of excess human importin beta to a *Xenopus* nuclear reconstitution system disrupts the endogenous ratio between importin beta and RanGTP. This disruption blocks proper nuclear membrane fusion and the subsequent step of nuclear pore assembly [9, 10]. The block to nuclear membrane fusion was found to be reversible by the positive regulator, RanGTP, but the block to pore assembly, oddly, was not [9, 10]. There is, however, much precedence for positive Ran effects on nuclear pore assembly: The addition of RanQ69L, a Ran mutant constitutively in the GTP-bound state, to the *Xenopus* reconstitution system causes greatly increased nuclear pore assembly and ectopic formation of additional pores in cytoplasmic membranes or annulate lamellae [9, 10, 14-17]. These studies led to the hypothesis that importin beta acts in the cell cycle to negatively regulate nuclear pore formation and that it does so by binding to nucleoporins, preventing them from interacting with one another. When such importin beta/nucleoporin complexes enter
the vicinity of high RanGTP, importin beta preferentially binds RanGTP, releasing its hold on the nucleoporins. A high concentration of RanGTP is produced only around chromatin, due to the chromosomal localization of the RanGEF, RCC1 [18-21]. The freed nucleoporins are then able to interact with one another in the correct location and the correct ratio to form nuclear pores at the chromatin periphery [9, 10, 22].

Prior to the discovery of its role as a negative regulator of nuclear membrane fusion and pore assembly, importin beta was elegantly shown by a number of groups to be a negative regulator of mitotic spindle assembly in *Xenopus laevis* egg extract [23-29], mammalian cell lines [25, 30], *Drosophila Melanogaster* [31], and *Caenorhabditis elegans* [32] ( Reviewed in [11, 12, 33, 34]). In this arena, mitotic spindle assembly factors (SAFs) such as TPX2, NuMa, and XCTK2 are found to be imported into the nucleus by importin beta and localize there throughout interphase in *Xenopus* egg extract [27, 28, 35-37] and mammalian cell lines [35, 38] ( Reviewed in [39-41]). This sequestration effectively prevents the SAFs from interfering with interphase microtubule formation in the cytoplasm. At mitosis when the nuclear envelope breaks down, the SAFs are released from the nucleus and come under importin beta regulation. Binding of importin beta inhibits the SAFs throughout the cell, except in the vicinity of the RanGTP-rich chromosomes. There, importin beta preferentially binds to RanGTP, releasing its hold on the spindle assembly factors and allowing them to initiate mitotic spindle formation around the chromosomes.

These nuclear and spindle assembly studies on the regulatory role of importin beta were performed in interphase and mitotic assembly systems derived from
Xenopus eggs [23, 26-28, 35, 42-50]. In a Xenopus interphase egg extract, nuclei normally assemble spontaneously around added chromatin or DNA [51-60]. In contrast, in a Xenopus mitotic egg extract, spindles spontaneously form around the added chromatin [61, 62]. Thus, these in vitro systems are powerful tools for studying both nuclear and mitotic spindle assembly.

Upon further analysis, we realized that the recombinant importin beta used in all the Xenopus studies of nuclear and spindle assembly was, in actuality, human importin beta [9, 10, 25, 27-30, 37, 63-68]. (Xenopus importin beta had neither been identified nor cloned and thus was not available for the studies). The use of recombinant human importin beta in the Xenopus system led to a further key question: Is importin beta an authentic negative regulator of cellular function, or does human importin beta act as a dominant negative mutant as a result of sequence variation between the human and Xenopus proteins?

To address this question, in this study we identified, cloned, and tested recombinant Xenopus importin beta for its role in nuclear membrane fusion and nuclear pore assembly. We found Xenopus importin beta to act identically to human importin beta, i.e., it acts as a negative regulator of both nuclear membrane fusion and pore assembly, finally validating the conclusion that importin beta is an authentic negative regulator of cell cycle steps. However, in examining tagged importin betas, which include the form that has been used in all the previous studies, we found evidence that the tag renders importin beta mutant in its response to Ran, but does so specifically with respect to pore assembly. This impairment of importin beta raises
interesting hypotheses as to why nuclear pore assembly is unique, which will be discussed here.

**Results**

**Identification and cloning of Xenopus laevis importin beta.**

To address whether human importin beta acts as an authentic negative regulator of nuclear membrane fusion, pore assembly, and spindle assembly, or as a dominant negative mutant inhibitor due to inherent species sequence differences, we set out to identify and clone *Xenopus* importin beta. Overlapping *Xenopus* EST sequences showing homology to human importin beta were compiled from gene fragments present in the *Xenopus* EST database. A full-length *Xenopus* importin beta sequence was then cloned from total *Xenopus* RNA by reverse transcription and PCR. The resulting full-length *Xenopus* importin beta cDNA was cloned into an N-terminal His tag vector, pET28a, for both protein expression and sequencing. The corresponding nucleotide sequence was submitted to GenBank, Accession number EU286786. Sequence alignment revealed that *Xenopus* importin beta is 94% identical to human importin beta; however, 48 amino acids varied between the species, although often in a conserved manner (Figure 1). These 48 amino acids give scope for the hypothesis that potential “mutant” amino acids could cause a dominant negative phenotype with human importin beta.

To further eliminate any potential differences from endogenous *Xenopus* importin beta, we wished to use recombinant *Xenopus* importin beta free of purification tags. For this, the *Xenopus* importin beta clone was subcloned into a
vector that introduced a cleavable GST tag. After the GST- importin beta was expressed and purified, the GST tag was removed by Precision Protease and the resulting untagged *Xenopus* protein was used in nuclear assembly studies.

*Xenopus importin beta negatively regulates membrane fusion in a Ran-sensitive manner.*

With the *Xenopus* importin beta clone in hand, we set out first to ask whether it blocked nuclear membrane fusion when in excess. If no importin beta is added to a *Xenopus laevis in vitro* system, after one hour smooth fused membranes are formed and can be visualized with the membrane dye DHCC, as we also observed here (Figure 2, Control) [9, 69]. However, when we added excess untagged *Xenopus* importin beta at the beginning of a nuclear reconstitution reaction, nuclear membrane formation was blocked, as shown by the presence of fuzzy unfused membranes (Figure 2, +X-β). This inhibition of fusion was reversed by addition of RanQ69L-GTP, a form of Ran stably associated with GTP, as it cannot hydrolyze GTP (Figure 2, +X-β + Ran) [16]. These results thus indicated that *Xenopus* importin beta acts identically to human importin beta in negatively regulating nuclear membrane fusion, and does so in a Ran-sensitive manner.
**Xenopus importin beta negatively regulates nuclear pore assembly and is reversed by Ran.**

We next tested *Xenopus* importin beta for inhibition of nuclear pore assembly. We had previously shown that human importin beta blocks nuclear pore formation, but cannot be reversed by Ran [9]. To investigate the effect of *Xenopus* importin beta on pore assembly, we first needed to bypass the inhibition of nuclear membrane fusion and look only at the nuclear pore assembly step. It has long been known that when the Ca\(^{++}\) chelator BAPTA is added to a *Xenopus* nuclear reconstitution reaction at t=0’, nuclei result that have a fused nuclear envelope, but no nuclear pores [9, 58, 70]. These “BAPTA pore-free nuclei,” in consequence, do not stain with antibody directed against nucleoporins containing Phenylalanine-Glycine (FG) repeats (Figure 3, left panels) [9, 58]. Upon dilution of the BAPTA nuclei into *Xenopus* cytosol free of BAPTA, nuclear pores form normally, as previously described and shown here (Figure 3, cytosol + buffer) [9]. This ability of BAPTA pore-free nuclei to be rescued provides a convenient system for investigating solely the effect of *Xenopus* importin beta on pore assembly [58]. Here we found that, when BAPTA nuclei were diluted into cytosol containing *Xenopus* importin beta, the nuclei were not able to form nuclear pores (Figure 3, +X-β), identical to the block seen with human importin beta [9]. Thus, we conclude that importin beta, either *Xenopus* or human, is indeed an authentic negative regulator of nuclear pore assembly.

Strikingly, when BAPTA nuclei were diluted into cytosol containing *Xenopus* importin beta and RanQ69L, the BAPTA defect was rescued by Ran, i.e., FG-
Tagging importin beta causes insensitivity to Ran in its block to nuclear pore assembly.

We considered the differing Ran reversibility results seen with human and Xenopus importin beta. Two possibilities existed: 1) either human importin beta differs from Xenopus importin beta with respect to its sensitivity to Ran, because of an inherent sequence difference in the importin beta coding sequence, or, 2) the His-tag present on the human importin beta used in all previous in vitro studies alters its sensitivity to Ran in a detrimental manner, but only with respect to pore assembly. To distinguish between these two mechanistic explanations, the BAPTA rescue experiment was next performed using tagged Xenopus importin beta, where an N-terminal His-tag was introduced. We found that tagged Xenopus importin beta acted identically to tagged human beta, i.e., it was not reversible by Ran (Figure 4A). Thus, the second model of tag-induced insensitivity to Ran appeared correct.

As a final test, however, an untagged form of human importin beta was cloned and used in a rescue experiment. We found that untagged human importin beta blocked the ability of nuclear pores to form when BAPTA-arrested nuclei were diluted...
into fresh cytosol (Figure 4B, +h-β). However, now RanQ69L rescued the pore assembly defect, albeit not as strongly as with the untagged Xenopus importin beta homologue (Figure 4B, compare +h-β +Ran with +X-β + Ran). Therefore, the first model of human importin beta acting as a dominant negative due to sequence variation is also plausible. Taken together, the data indicate that, specifically with respect to importin beta’s block to pore assembly, wild-type human importin beta is less sensitive to Ran than Xenopus importin beta, and the presence of a His-tag on human importin beta renders it insensitive to Ran.

Discussion

In this study we validate importin beta as a negative regulator of cell cycle events, including nuclear membrane fusion and pore assembly. As all importin beta studies on nuclear and mitotic spindle formation using the Xenopus in vitro system to date have involved the addition of human importin beta, we asked whether the effects of importin beta were due to an inter-species sequence variation causing the human protein to act as a dominant negative mutant form. Instead we clearly show in experiments with Xenopus importin beta that this wild type protein acts as a true negative regulator.

Interestingly, during the course of this study we uncovered a mechanistic explanation for the Ran-insensitive importin beta block to pore assembly previously observed [9]. Tagging importin beta at the N- (Xenopus) or C- (human) terminus was discovered to block importin beta’s sensitivity to RanGTP (up to 100µM of added Ran, data not shown) in Xenopus in vitro studies, but only in the realm of nuclear pore
assembly. Both spindle assembly and nuclear membrane assembly are blocked by importin beta, but readily reversed by RanGTP [9]. We showed that, upon removal of the tag, RanGTP now also reversed the block to pore assembly engendered by \textit{Xenopus} importin beta and partially reverses the block by human importin beta.

Importin beta normally undergoes a significant conformational change upon RanGTP binding [71-80]. It is therefore not inconceivable that even a small tag, such as the six histidine tag, could increase rigidity or cause an inability for importin beta to fully change conformation and thus be unable to release its binding partners correctly in response to RanGTP. What is surprising is that the tagged-importin beta insensitivity to RanGTP is only seen with respect to its role as a negative regulator of nuclear pore assembly. All other studies on the dynamics of importin beta and RanGTP in mitotic spindle assembly and nuclear membrane fusion have not shown an unresponsiveness of tagged-importin beta to RanGTP [9, 10]. One explanation for this might derive from the known association of importin beta with multiple FG-nucleoporins, suggesting that multiple sequential steps in pore assembly could potentially be regulated by importin beta [74, 81-84]. The cumulative effect of an impaired importin beta being incompletely released by Ran at \textit{each} step of pore assembly could explain the observed irreversibility of tagged importin beta’s block specifically on nuclear pore assembly.

A second explanation for why importin beta’s regulation of nuclear pore complex assembly differs from nuclear membrane fusion and spindle assembly with respect to Ran reversibility may involve how the targets of regulation interact with
importin beta. What mechanistically might differ between spindle assembly factor (SAF) binding and nucleoporin (Nup) binding to importin beta? One study suggested a region of importin beta (aa 71-876) bound to SAFs and blocked spindle assembly when added to a mitotic extract, whereas amino acids 1-380 of importin beta had a lesser effect on spindle assembly [27], albeit other interpretations are also possible [38]. Notably, importin beta has two known binding sites for nucleoporins, aa 1-396 near the N-terminus and aa 304-876 near the C-terminus [83]. Importantly, the N-terminal Nup binding site of importin beta partially overlaps with the binding site for RanGTP [12, 72, 73, 82, 83, 85, 86]. An intriguing possibility is that this N-terminal Nup binding site could be responsible for tagged importin beta’s insensitivity to RanGTP with respect to pore assembly, as this site appears not to play a significant role in the regulation of mitotic spindle assembly.

There are as yet no identified molecular targets of importin beta with respect to nuclear membrane fusion that can be similarly analyzed. However, when an importin beta fragment (aa 45-462) containing the N-terminal Nup binding site, but lacking the importin alpha, RanGTP, and C-terminal Nup binding sites, is added, nuclear membrane fusion goes forward [9]. Thus, the binding site on importin beta for the unknown membrane fusion factor or factors is not contained within this region (aa 45-462).

Perhaps the most surprising difference between tagged and untagged importin beta sensitivity to Ran is the differing effect on annulate lamellae (AL) pore formation versus nuclear pore formation. Importin beta blocks AL formation, but this block is
reversed by RanGTP, whether tagged or untagged importin beta is used ([10] and data not shown), which is clearly not the case for nuclear pore assembly. One explanation could be that AL formation may not be as stringent as nuclear pore assembly, as the pore complexes in AL do not necessarily need to function, whereas nuclear pore complexes must be functional. An alternative explanation could be that the tagged importin beta blocks an assembly step that is unique to nuclear pore assembly and not found in AL assembly. Whatever the tag-sensitive block to nuclear pore assembly is, it must occur after nuclear vesicle-vesicle fusion, as the importin beta block to pore assembly is observed using membrane-enclosed BAPTA intermediates as a starting point (Figures 3 and 4) [9].

The placement of the 6-Histidine tag at either the N- or C-terminus of importin beta appears not to matter. The human importin beta used in most *Xenopus in vitro* studies [9, 10, 26, 29, 30, 35, 63, 65, 87] has a His tag at its C-terminus, while the tagged *Xenopus* importin beta constructed in this study has the tag at the N-terminus. We have not tested other types of tags on importin beta for their effect on pore assembly. Clearly, in the future functional studies using importin beta should take care to use an untagged version of importin beta or, alternatively, may specifically want to use a tagged version in order to study the mechanism of arrested nuclear pore assembly more closely.

**Conclusions**

By using species-specific importin beta for nuclear assembly studies we have now demonstrated that importin beta, human or *Xenopus*, is indeed an authentic
negative regulator of nuclear assembly and, presumably, spindle assembly. In previous studies, the action of human importin beta could easily have been due to a dominant negative mutant effect, which would have required a different model of regulation. By performing the experiments here we now provide the evidence that importin beta must truly be a negative regulator in its wild type form.

**Methods**

**Cloning and Sequencing of *Xenopus* importin beta.**

To obtain a sequence of *Xenopus* importin beta, overlapping *Xenopus* EST sequences showing homology to human importin beta were compiled from fragments present in the NIH *Xenopus* EST database. Full-length *Xenopus* importin beta was then cloned from *Xenopus* total RNA by reverse transcription and polymerase chain reaction (PCR) amplification using the forward primer 5’-CCCGGATCCATGGAGCTCGTCACCATCCTC-3’ (with BamHI site underlined) and reverse primer 5’-CCCCGCGGCGCTCAGCTAGCTTGGTTTTCAG-3’ (with NotI site underlined). The full-length *Xenopus* importin beta cDNA was cloned into the N-terminal His tag vector pET28a (Invitrogen, Carlsbad, CA) (pET28a-Xbfl). GST-*Xenopus* importin beta (pGEX6P-Xbfl) was cloned by restriction digestion of pET28a-Xbfl with BamHI and NotI, and ligation of the insert into the pGEX6P-3 vector (Amersham Biosciences, Sweden) 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’ GCTGCACTGCAAACCTGG 3’) and a reverse primer, the T7 terminator primer. Human and *Xenopus* importin beta were aligned using the Clustal-W program and highlighted using BoxShade, both available through the Workbench program of the San Diego Super Computer Center [88].

**Protein Expression and Purification.**

His-tagged proteins (*Xenopus* importin beta, human importin beta, and RanQ69L), were expressed and purified as previously described [9]. RanQ69L was loaded with GTP as described previously [9].

To purify untagged human and *Xenopus* importin beta, pGEX6P-hbfl and pGEX6P-Xbfl were transformed into Rosetta DE3 competent cells (EMD Biosciences, Germany), expanded, and induced with 0.1 mM isopropyl-beta-D-thiogalactopyranoside (IPTG) overnight at 17°C. Glutathione-Sepharose 4B beads (Amersham Biosciences, Sweden) were used to purify the GST-tagged protein as per manufacturer’s instructions. To remove the GST tag, purified proteins were cleaved on the column in the presence of 80 units of Precision Protease (Amersham Biosciences, Sweden) for 4 hours at 4°C. Untagged protein was eluted from the column and dialyzed into 5% glycerol/PBS and stored at -80°C.

**Nuclear reconstitution and immunofluorescence.**

Nuclear reconstitution and 1,2-bis (2-aminophenoxy) ethane-N,N,N,N-tetraacetic acid (BAPTA) (Calbiochem, La Jolla, CA) nuclear reconstitution reactions
were performed in the *Xenopus* egg extract system as described previously [9]. FG nucleoporins were localized using an Alexa-488 directly labelled monoclonal antibody mAb414 (Covance, Berkeley, CA). *Xenopus* egg cytosol and membranes were prepared as previously described [56], except for the use of 500 mM KCl in the membrane wash buffer. After fixation in 3% formaldehyde, membranes were visualized by the lipophilic dye 3,3-dihexyloxycarbocyanine iodide (DHCC) (Eastman Kodak, Rochester, NY). DNA was stained with 4’,6-diamidino-2-phenylindole (DAPI). Nuclei were visualized with an Axioskop 2 microscope (63X objective; Carl Zeiss, Thornwood, NY).

**Authors' contributions**

VAD carried out the comparison between human and *Xenopus* importin beta and tagged vs. untagged importin beta. VAD drafted the manuscript.

RCC conceived of the original project, drafted parts of the manuscript, directed the cloning and sequencing of *Xenopus* importin beta, and performed preliminary characterization of His-tagged *Xenopus* importin beta.

DJF significantly contributed to the intellectual content and manuscript.

All authors read and approved the final manuscript.
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**Figure 3.1 - Xenopus importin beta shows close homology to human importin beta.**

The protein sequence of *Xenopus* importin beta shows very close homology to human importin beta with 94% identities (828/876, black boxes) and 97% positives (857/876 gray and black boxes). The amino acid composition, along with the length of the protein, is well conserved between *Xenopus* and human importin beta. Three of the conservative amino acid differences between the *Xenopus* and human importin beta sequence are at residues involved in FG-domain binding (F217Y [82-84], I265V [84], and L505V [84]).
Figure 3.2 - *Xenopus* importin beta is an authentic negative regulator of the fusion events in nuclear membrane formation.

Addition of His-tagged *Xenopus* importin beta to a nuclear assembly reaction (+X-β) blocked nuclear membrane fusion, as shown by the lack of a solid nuclear rim stain by the green fluorescent membrane dye DHCC. The block to membrane fusion could be rescued by the addition of RanQ69L-GTP (+X-β +Ran). Where indicated, the added concentrations were 30µM *Xenopus* importin beta and/or 40µM RanQ69L-GTP. DNA was stained with DAPI. These observations are in accordance with experiments done with recombinant human importin beta in nuclear assembly reactions [9]. To better view the membranes, a section of the membrane stain (white dashed box) is enlarged by 3X (right panels). The bar represents 10 microns.
Figure 3.3 - *Xenopus* importin beta is an authentic negative regulator of nuclear pore assembly and is reversed by RanGTP.

Pore-free BAPTA nuclear intermediates, which have fused nuclear membranes but contain no nuclear pores (left panel), when diluted into fresh cytosol (+ buffer), incorporate nuclear pores. The addition of His-tagged human importin beta (+h-β-Tag) or *Xenopus* untagged importin beta (+X-β) prevented nuclear pore assembly. Addition of RanQ69L-GTP with His-tagged human importin beta (+h-β-Tag +Ran) could not reverse the beta block to pore assembly, as previously observed [9]. However, addition of RanQ69L-GTP with untagged *Xenopus* importin beta (+X-β +Ran) did reverse the beta block to pore assembly. Nuclear pores were detected by the monoclonal antibody mAb414, which recognizes FG nucleoporins (FG Nups). Where indicated, importin beta was added at 20µM and RanQ69L-GTP at 30µM. The bar represents 10 microns. Black squares on the drawings at the right indicate FG-staining nuclear pores.
Figure 3.4 - Altering importin beta by addition of a His-tag renders importin beta insensitive to RanGTP specifically in its block to nuclear pore assembly.

A. Pore-free BAPTA intermediates rescued in the presence of cytosol plus His-tagged *Xenopus* importin beta were not able to assemble nuclear pores (+Tag-X-β). When RanQ69L-GTP was added along with His-tagged *Xenopus* importin beta, the block to pore assembly could not be reversed (+Tag-X-β +Ran). Where indicated, importin beta was added at 10μM and RanQ69L-GTP at 50μM. The bar represents 10 microns.

B. Pore-free BAPTA nuclear intermediates rescued in the presence of cytosol and untagged human or *Xenopus* importin beta were not able to assemble nuclear pores (+X-β or +h-β). The inhibitory concentration of 10μM used here was determined to be the approximate minimum concentration for pore assembly inhibition in a separate experiment (data not shown). When RanQ69L-GTP was added along with untagged human importin beta, the block to pore assembly was partially reversed (+h-β +Ran). The *Xenopus* importin beta block was fully reversed (+X-β +Ran). To better visualize the FG-nucleoporin stain, a section of the images (white dashed box) was enlarged by 3X (right most panel). Where indicated, importin beta was added at 10μM and RanQ69L-GTP at 50μM. The bar represents 10 microns.
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CHAPTER 4

Transportin and importin beta regulate both the initial and downstream steps of nuclear pore assembly

Corine K. Lau, Valerie A. Delmar, Rene C. Chan, Douglass J. Forbes

Abstract

Post-mitotic nuclear assembly requires the precise formation of nuclear membranes and the step-wise assembly of the nuclear pore complexes. However, the mechanism, the order of assembly, and the factors involved in these highly regulated processes are still under debate. We previously showed that importin beta negatively regulates both nuclear membrane fusion and nuclear pore assembly in vitro using Xenopus laevis egg extracts. Here, we demonstrate that the transport factor, transportin, also negatively regulates nuclear envelope fusion and nuclear pore assembly. In a cell, pores are found in the nuclear envelope, but can also be found in a specialized region of the endoplasmic reticulum (ER) of rapidly growing cells as annulate lamellae (AL). We show, like importin beta, that excess transportin blocks the formation of AL-pores. By GST-pull down from Xenopus egg cytosol, we reveal that the putative soluble nucleoporin regulatory targets of importin beta and transportin for nuclear pore assembly are largely the same: the Nup107/160 complex, ELYS, the phenylalanine-glycine (FG)-containing nucleoporins, and Nup53.
Importantly, we show that both importin beta and transportin initiate their regulation as early as the first known step of nuclear pore assembly: they negatively regulate the recruitment of the pore targeting nucleoporin, ELYS to chromatin. The steps that are regulated by importin beta and transportin are counteracted by the small GTPase Ran. The interplay of these two negative regulators, along with the positive regulator Ran, allows precise choreography of nuclear assembly.

**Introduction**

The eukaryotic genome is protected by an encircling nuclear envelope. This consists of double nuclear membranes perforated by macromolecular structures called nuclear pore complexes. The nuclear pores serve as the major portals for exchange of molecules between the nucleus and the cytoplasm. Proteins larger than ~40kD, ribonucleoproteins (RNPs), and RNAs require active transport through the nuclear pores by transport receptors termed karyopherins (reviewed in [1, 2]).

Karyopherins consist of a large family of importins (import receptors) and exportins (export receptors) (reviewed in [3, 4]). The most studied import receptor of the family is importin beta. It mediates import of proteins through the use of adaptors, such as importin alpha and snurportin (reviewed in [5]). Importin alpha recognizes cargoes containing a classical nuclear localization signal (NLS) composed largely of basic amino acids. Importin beta can also bind to certain cargos directly without the use of an adaptor. A second import receptor, transportin, recognizes and imports cargoes that contain either a hydrophobic proline-tyrosine PY-NLS or a basic PY-NLS
The first protein discovered to be a substrate of transportin was hnRNP A1, which contains a 38-amino acid NLS called M9 [7].

The directionality of transport is governed by a gradient of RanGTP, the GTP-bound form of the small GTPase Ran, where the concentration of RanGTP is high near the chromatin in the nucleus, and low in the cytoplasm (reviewed in [8]). Both importin beta and transportin release their cargoes once they encounter RanGTP in the nucleus.

The basic architecture of the vertebrate nuclear pore consists of a large central framework, eight cytoplasmic filaments, and a nuclear basket. Each nuclear pore contains ~30 different proteins in multiples copies and possesses eightfold symmetry ([9-13] and references therein).

In vertebrates, the nuclear envelope breaks down and reforms during each cell cycle to allow chromosome segregation to take place. The assembly of the nucleus at the end of mitosis requires all the components including nuclear membranes and nuclear pores to be re-assembled in a step-wise manner. Vertebrate nuclear pore assembly also occurs during S-phase where nuclear pores must assemble in intact double nuclear membranes (Reviewed in [14]). Various model systems have been utilized to study the regulated process of nuclear pore assembly. Of interest, in vitro formation of nuclei using *Xenopus* egg extracts has provided a powerful way to observe and manipulate nuclear assembly [15-27]. For this, fractionated *Xenopus* egg cytosol is combined with *Xenopus* membranes, sperm chromatin, and an energy regenerating system. Nuclei competent for nuclear import and DNA replication are
formed within one hour of incubation at room temperature. Notably, the fractionated *Xenopus* cytosol contains the soluble nucleoporins in ~14 subcomplexes (Rasala et al., submitted) and these are poised for assembly into nuclear pores.

Although some nuclear assembly intermediates have been identified in the *Xenopus* system using chemical and protein inhibitors [17, 22, 23, 28-30] (reviewed in [31]), the individual steps in nuclear assembly have not been clearly defined. In addition, the precise order and regulation of nuclear assembly remain controversial. Nonetheless, one of the widely accepted models for nuclear assembly *in vitro* involves membrane vesicles first binding to sperm chromatin, followed by vesicle-vesicle fusion to become patches of membranes, from which a layer of double membranes can form over the chromatin. Nuclear pore assembly involves the recruitment of both pore membrane proteins and soluble nucleoporins to the chromatin in an orchestrated manner to the nuclear membrane [32, 33].

Amongst the soluble nucleoporins, the Nup107-160 complex is the largest, containing 9-10 members making it crucial for nuclear pore assembly [17, 19]. Vertebrate ELYS was recently discovered to be a nucleoporin and was identified as a binding partner of the critical Nup107-160 complex [34, 35]. Interestingly, ELYS has been shown to initiate nuclear pore assembly; ELYS binds to chromatin and then recruits the Nup107-160 complex [34-36]. In the absence of ELYS, pore complexes cannot assemble at the chromatin periphery, but instead form in the ER to form pores within cytoplasm, i.e. membrane stacks of which are termed annulate lamellae (AL) [34, 35]. The recruitment ELYS and the Nup107-160 complex to chromatin is a
prerequisite for other soluble nucleoporins, including the FG-repeat containing nucleoporins, to be recruited to form the mature pore. However, the mechanism of regulation of this important first step of nuclear pore assembly has not been elucidated.

Two key regulators of nuclear envelope assembly have been found: Ran is a positive regulator, while importin beta is a negative regulator [17, 19]. Nuclei formed in vitro in the presence of excess importin beta fail to undergo the vesicle-vesicle fusion necessary to form the nuclear membranes. This inhibition can be counteracted by RanGTP [15, 17, 19]. GTP bound to RanQ69L is unable to be hydrolyzed, thus this Ran mutant is constitutively found in the GTP form [37]. Excess importin beta also blocks nuclear pore assembly in nuclei that contain pre-formed nuclear membranes and in annulate lamellae [15, 17, 19], and RanGTP also reverses this inhibition [15]. These studies show a precise balance between the two regulators is required for proper nuclear assembly. Nevertheless, the molecular steps regulated in pore assembly remain an open question.

In this study, we show that transportin is a regulator of nuclear envelope assembly. Like importin beta, transportin regulates both nuclear membrane formation and nuclear pore assembly in the Xenopus nuclear assembly system and does so in a Ran-sensitive manner. A search for the regulatory targets for both importin beta and transportin of nuclear pore assembly reveals the same soluble nucleoporin targets. Furthermore, both receptors appear to differentially regulate one of the first steps of nuclear pore assembly -- ELYS binding to chromatin in a Ran-dependent manner.
Results

Transportin blocks nuclear membrane formation in a Ran-reversible manner.

Importin beta has previously been shown to act as a negative regulator of both nuclear membrane and nuclear pore assembly in the *Xenopus laevis* in vitro nuclear assembly system [15, 17, 19]. While investigating the mechanism of importin beta regulation of nuclear assembly, we also tested another nuclear import receptor, transportin, for its ability to regulate nuclear envelope assembly in the *Xenopus* system. In the control reaction, when chromatin is mixed with *Xenopus* egg cytosol and membranes, completely fused nuclear membranes were observed to encircle the chromatin by 60 minutes, as indicated by the smooth membrane stain surrounding each nucleus (Figure 1, Control). The addition of GTP$_\gamma$S, a known inhibitor of vesicle-vesicle fusion, inhibited nuclear membrane formation as evidenced by the discontinuous membrane profile indicative of unfused membrane vesicles ([23, 38]; Figure 1, GTP$_\gamma$S). Importin beta gave, as expected, a similarly discontinuous membrane profile around the chromatin (data not shown; [15, 17]). Strikingly, when excess transportin was added, unfused membranes were also observed (Figure 1, Trn). Importantly, this membrane fusion defect was counteracted by the positive regulator Ran, in the form of RanQ69L-GTP (a form of Ran that cannot hydrolyze GTP) (Figure 1, compare Trn+Ran with Trn). Therefore, transportin, like importin beta, negatively regulates nuclear membrane fusion in a Ran-reversible manner.
Transportin negatively regulates nuclear pore assembly.

To determine whether transportin separately blocks nuclear pore assembly, we first assembled nuclear intermediates containing complete nuclear membranes, but which lacked nuclear pores. This was done by performing nuclear assembly in the presence of BAPTA. Such BAPTA nuclear intermediates contain a fused nuclear envelope, but no nuclear pores as shown by electron microscopy and a lack of detectable FG nucleoporins by immunofluorescence microscopy ([15, 17, 23]; Figure 2A, left panel). Normally, it has been shown that when such BAPTA nuclei are diluted 1:10 into fresh cytosol lacking BAPTA, nuclear pore assembly quickly ensues [15, 17, 23]. This rescue is apparent from the presence of FG-nucleoporin staining (Figure 2A, +Buffer). If the Xenopus cytosol contained BAPTA, rescue was not observed (Figure 2A, +BAPTA). Importantly, when BAPTA nuclei were diluted in cytosol containing excess transportin, the incorporation of FG-nucleoporins was blocked (Figure 2A, +Trn). As in the case of importin beta ([15]; data not shown), the ability of transportin to block pore assembly was partially reversed by RanQ69L-GTP (Figure 2A, +Trn+Ran). RanQ69L-GTP alone did not have any effect on nuclear pore assembly (Figure 2A, +Ran). To quantitate these effects, seventy-five nuclei for each condition in Figure 2A were counted for the presence or absence of FG nucleoporin staining (Figure 2B). The quantitation clearly confirms that transportin blocks nuclear pore assembly.
Annulate lamellae assembly is negatively regulated by transportin.

Annulate lamellae (AL) are stacked membranes that contain cytoplasmic mimics of intact nuclear pore complexes [39-41]. In most ways these AL-pores are identical to nuclear pores, but they are assembled differently in that they do not require chromatin or the pore targeting protein, ELYS [39, 40, 42] (Rasala et al., submitted). AL is thought to serve as storehouses of nuclear pore proteins in cases when nuclear pore assembly must occur rapidly [41, 43]. A previous study found that importin beta blocks AL-pore assembly at concentrations as low as 5μM, as indicated by the absence of FG nucleoporin Nup358, Nup214, Nup153, and Nup62 incorporation into AL membranes [19]. On the other hand, transportin did not affect AL assembly when added at 10μM in the same study [19]. Since we found 20μM transportin to block nuclear pore assembly, we asked if this concentration blocked AL-pore assembly. To address this question, we compared the incorporation of several key nucleoporins into AL. We formed AL in vitro by mixing Xenopus cytosol with Xenopus membranes in the absence of a chromatin source for 90 minutes. The AL were then isolated by centrifugation and subjected to immunoblot analysis. In AL formed under normal “control” conditions, individual nucleoporins such as Nup133, Nup43, Nup93, and Nup53 were detected, as expected (Figure 3, lane 1), indicating the formation of AL-pores. As expected, when we added importin beta to an AL assembly reaction at 20μM, it blocked the incorporation of these soluble nucleoporins (Figure 3, lane 2). Strikingly, we found that 20μM transportin was indeed also able to block AL assembly (Figure 3, lane 4). The block to AL formation by importin beta or
transportin could be reversed by the addition of RanQ69L-GTP (Figure 3, lanes 3 and 5), although not completely. Importin beta and transportin also inhibited the incorporation of all tested nucleoporins, including Nup214, Nup155, Nup153, Nup98, and Nup62 into AL-pores (data not shown). Thus, these results demonstrate that transportin negatively regulates AL pore assembly.

**A subset of nucleoporins binds to both importin beta and transportin.**

Next, we wished to determine the targets of importin beta and transportin regulation of pore assembly. During import, both bind to FG-containing nucleoporins that are essential for nucleocytoplasmic transport [8, 9, 44]. One hypothesis would be that importin beta and transportin regulate pore assembly by sequestering these essential FG-nucleoporins except in the region of the chromosomes, where RanGTP is produced. The broader hypothesis would involve all of the nucleoporin subunits as targets of regulation. In addition, it is conceivable that importin beta and transportin can have certain non-overlapping targets that can be regulated differentially.

Thus, to seek a comprehensive knowledge of the nucleoporin targets for these transport receptors in the *Xenopus* system, we performed GST pull-downs from *Xenopus* cytosol using GST-importin beta or GST-transportin as bait. We then tested for the presence of 12 of the 14 known soluble nuclear pore subunits by immunoblotting. Both importin beta and transportin interacted with the FG-nucleoporins Nup358, Nup214, Nup153, Nup98, Nup62, and Nup50 (Figure 4A, lane 3), as expected [45 - 51]. The major subcomplex of the nuclear pore, the Nup107-160
complex, known to interact with importin beta, also bound to transportin as evidenced by its binding of Nup160, Nup133, and Nup85 (Figure 4A, lane 4). Interestingly, the two newly discovered Nup107-160 complex-associated proteins, ELYS and centrin [34-36, 52], bound to both importin beta and transportin (Figure 4A). Nup53 also associated with importin beta and transportin (Figure 4A). However, among the tested nucleoporins, members of the key Nup93/188/205 subcomplex and Nup155 did not show interaction with importin beta or transportin (Figure 4A).

We next tested the Ran sensitivity of nucleoporin binding to transportin. The interaction between transportin and the FG-nucleoporins Nup214, Nup98, and with the Nup107-160 complex (Nup160, Nup133, and Nup43) was abolished in the presence of RanQ69L-GTP (Figure 4B, lane 5). The interaction with Nup62 was significantly decreased. In contrast, the interaction between transportin and the FG-nucleoporins Nup358, Nup153, and Nup50 remained the same or slightly increased in the presence of RanQ69L-GTP (Figure 4B, lane 5). Interestingly, these latter three nucleoporins are unique in that each has been found to bind RanGTP on their own [51, 53-56].

Together, the above data demonstrates that both importin beta and transportin interact with the same specific subset of nucleoporins. This suggests importin beta and transportin may regulate nuclear pore assembly by a similar mechanism. It is interesting that neither interact with the Nup93/188/205 subcomplex or Nup155. The recruitment of these specific nucleoporins into the forming nuclear pore is thus not likely to be regulated by importin beta or transportin.
Importin beta and transportin regulate chromatin binding of ELYS in the first step of nuclear pore assembly.

The first known step of nuclear pore complex assembly involves recruitment of the pore-targeting nucleoporin ELYS to the chromatin [34, 36](Rasala et al., submitted). This then attracts the Nup107-160 complex and initiates pore assembly. Since both importin beta and transportin interact with ELYS and the Nup107-160 complex (Figure 4A), it is feasible that these transport receptors regulate the initial steps in nuclear pore assembly. This hypothesis was tested using a chromatin-binding assay. *Xenopus* cytosol was incubated with sperm chromatin with or without recombinant proteins, and subsequently processed for immunofluorescence. In the control, when only cytosol was added to chromatin, the chromatin binding protein Orc2 and ELYS both bound to the chromatin (Figure 5A, +Buffer). In contrast, ELYS no longer associated with chromatin in the presence of excess importin beta (Figure 5A, +beta) and was greatly reduced in the presence of transportin (Figure 5A, +Trn), while binding of Orc2 to chromatin was unaffected by either of these treatments. The inhibition was largely reversed if recombinant RanQ69L was also added (Figure 5A, +beta+Ran and +Trn+Ran). Quantitation of the ELYS binding results are shown in Figure 5B. Thus, we conclude that importin beta and transportin negatively regulate the binding of ELYS to chromatin.

The binding of ELYS to chromatin was also tested in an anchored chromatin assay. Decondensed sperm chromatin bound to poly-L-lysine coverslips was incubated with *Xenopus* cytosol, washed, and tested for the presence of ELYS and the
Nup107-160 complex by immunoblotting. In the absence of added recombinant proteins, ELYS and members of the Nup107-160 complex bound to chromatin (Figure 5C, lane 4, ELYS, Nup160, Nup133). Association of ELYS and the Nup107-160 complex with the chromatin was greatly reduced in the presence of importin beta (Figure 5C, lane 5) or transportin (Figure 5C, lane 8), while the Orc2 staining remained unchanged (Figure 5C, compare lanes 5 and 8 to lane 4). This block of ELYS and Nup107-160 complex binding to chromatin could be, in part, reversed by the addition of RanQ69L to the reaction (Figure 5C lanes 6 and 9). The data further suggest that importin beta and transportin negatively regulate recruitment of the pore targeting protein, ELYS to chromatin in a Ran-reversible manner.

If importin beta and RanGTP are dueling regulators, then it would be expected that importin beta would shield interactions between nuclear pore subunits until they are in the vicinity of RanGTP near the chromatin. When near chromatin, the nuclear pore subunits are available to interact with one another to form a nuclear pore. Indeed, such an example of RanGTP positively regulating the interaction between two nuclear pore subunits was previously shown. In the presence of excess RanQ69L-GTP, an interaction between Nup153 and the Nup107-160 complex in *Xenopus* egg extract could be induced [19]. Other data also demonstrated that Nup153 could bind to chromatin in the presence of RanGTP, presumably via the Nup107-160 complex [19] (Rasala, et al., submitted). We asked whether importin beta and transportin could prevent such interaction of Nup153 with chromatin. As expected, when RanQ69L was added to an anchored chromatin assay, we observed an induced Nup153
association with the chromatin (Figure 5C, lane 7). In contrast, Nup153 did not bind to chromatin with the addition of importin beta (Figure 5C, lane 6) or transportin (Figure 5C, lane 9) in the presence of excess RanQ69L. Therefore, both importin beta and transportin negatively regulate the Ran-induced interaction between Nup153 and chromatin.

**Discussion**

Post-mitotic nuclear assembly is a complex step-wise process that requires regulation at multiple points. Previously, excess importin beta has been shown to inhibit two steps during nuclear assembly: vesicle-vesicle fusion to form the nuclear membranes and nuclear pore assembly. In this paper, using *Xenopus* egg extract for *in vitro* nuclear assembly, we show that transportin is a novel negative regulator of these two processes. Importantly, we show that both transportin and importin beta can act as negative regulators of the earliest known step in the initiation of pore assembly by preventing ELYS and Nup107-160 complex from binding to chromatin. RanGTP plays a positive role in each of these three steps (Figure 6).

It is interesting that importin beta and transportin can have diverse functions during different parts of the cell cycle, namely as import receptors during interphase, and negative regulators of nuclear assembly. In addition, importin beta has been shown to regulate spindle activating factors by binding and sequestering them spatially and temporally until they are needed for mitotic spindle formation (Reviewed in: [57, 58]). It is not known whether transportin plays a similar role during mitosis. On the other hand, Ran in the GTP form, acts in opposite direction as a positive regulator for
nuclear assembly and mitotic spindle formation by triggering dissociation of importin beta from its binding partners (Reviewed in [57]).

Although the negative regulatory role for importin beta in nuclear assembly and in mitotic processes identified so far can be counteracted by the positive regulator Ran, excess RanQ69L-GTP was unable to fully rescue the block of nuclear pore assembly by transportin. In a study by Delmar et al., 2008, the block to pore assembly by excess human importin beta was weakly reversed by RanQ69L-GTP, whereas the block by Xenopus importin beta was readily reversed. Notably, in this present study human transportin is used [15]. One possibility for the incomplete rescue of the transportin block to pore assembly is that human transportin is less sensitive to RanGTP than its Xenopus homologue for nuclear pore assembly. Another reason could be that transportin affects the function of an unknown factor that has a more global effect on nuclear formation.

Here, we identified nucleoporin targets for the regulation of nuclear pore assembly by importin beta and transportin. Both GST-importin beta and GST-transportin associate specifically with the members of the Nup107-160 complex, ELYS, centrin, the FG-nucleoporins, and Nup53. It is possible that the binding to ELYS and centrin is mediated through the Nup107-160 complex. Importin beta and transportin do not bind members of the Nup93/188/205 complex or Nup155. These data imply that the recruitment of the Nup93/188/205 subcomplex and Nup155 are not subject to regulation by importin beta or transportin.
The binding of GST-importin beta and -transportin to ELYS and the Nup107-160 complex is significant because we demonstrate that importin beta and transportin regulate their role in the first step of nuclear pore complex assembly. However, it is not yet known at precisely which point during nuclear pore assembly do the other importin beta- and transportin-binding nucleoporins, the FG-nucleoporins and Nup53, assemble to form a nuclear pore. It would be interesting for future studies to test, for example, if importin beta and transportin regulate the interaction between Nup53 and its known interacting partner, the trans-membrane nucleoporin Ndc1[59].

The Nup107-160 complex, and FG-nucleoporins Nup214, Nup98, and a fraction of Nup62 no longer bind GST-transportin in the presence of RanQ69L-GTP. This is consistent with the idea that RanGTP promotes pore assembly by releasing these nucleoporins that importin beta and transportin have sequestered. However, Nup358, Nup153, and Nup50 remain bound to GST-transportin even in the presence of RanQ69L-GTP. It is known that these three nucleoporins can interact with RanGTP [51, 53-56], thus it is possible that they remain bound to the transportin via a different mechanism.

While nucleoporins are the most likely targets of regulation by importin beta and transportin during nuclear pore assembly, the effectors of importin beta and transportin during membrane fusion have yet to be identified.

Why is more than one nuclear import receptor used to regulate nuclear assembly? One explanation could be that although importin beta and transportin bind to the same nucleoporins, they have different affinities for binding (thus regulating)
different nucleoporins. This will be consistent with our data that a higher concentration of transportin (20µM) was needed to sequester its nucleoporin targets to block both pore assembly in AL membranes and on chromatin, while previous study showed that 5µM of importin beta is sufficient to block AL pore assembly [19]. In addition, transportin at 10µM did not affect the Ran-induced AL pore assembly [19]. Indeed, there are differences in binding affinities between these two import factors and individual nucleoporins. Furthermore we do not exclude the possibility that other transport receptors can regulate nuclear assembly in a similar way providing a mechanism to fine-tune this regulation.

In this study we discovered a newly regulated step in nuclear pore assembly by importin beta and transportin – the binding of ELYS to chromatin. This initial step in pore assembly is critical to the targeting of pore complexes to the chromatin periphery. In the absence of ELYS, pore complexes no longer form as pores in the nuclear envelope, but instead as annulate lamellae pores in the cytoplasm. Therefore the targeting of nuclear pore assembly to the chromatin must be tightly regulated. Consequently, this could lead to defects in nuclear assembly (i.e. pore spacing and lamina formation) and an excess of nuclear pores would inevitably affect other functions of the nuclear pore complex such as transcriptional activation [60].

Another important point to consider is the reason for importin beta and transportin to act at multiple steps during nuclear pore assembly. There is a need for regulation during the initiation of pore assembly around chromatin, but it would also be advantageous to regulate later steps. For example, ectopic subcomplex-
subcomplex (nup-nup) interactions between soluble nucleoporins in the cytosol, or between membrane-bound nucleoporins in the membranes need to be prevented until the correct time during nuclear pore assembly. Importin beta and transportin have the ability to bind multiple nucleoporins and regulate multiple events.

In conclusion, we demonstrate that transportin is a negative regulator of membrane fusion and nuclear pore assembly in the *Xenopus* in vitro system. Importantly, both importin beta and transportin can negatively regulate ELYS binding to chromatin, the initial step in pore assembly. Ran, on the other hand, plays an opposite role than that of importin beta and transportin to balance the effects of these negative regulators. Thus, these two import receptors, together with Ran, are choreographed precisely to regulate multiple steps of nuclear assembly.

**Methods**

**Recombinant proteins cloning, expression, and purification.**

GST-human transportin (pGEX6P-Trn) was cloned by ligating a BamHI and XhoI fragment containing full-length transportin from pET28a-Trn (Shah and Forbes, unpublished) into pGEX6P-3 vector (GE Healthcare, Piscataway, NJ). Cloning of GST-*Xenopus* importin beta was described in [15]. GST, GST-tagged *Xenopus* importin beta, and GST-tagged human transportin proteins were expressed in BL21 competent cells (EMD Chemicals, Inc., Gibbstown, NJ) by inducing with 0.1mM IPTG and grown overnight at 17°C. Glutathione-Sepharose 4B beads (GE Healthcare) were used to purify the GST-tagged protein according to manufacturer’s instructions.
GST tagged protein was cleaved using GST-Precision Protease (GE Healthcare) incubated at 4°C for 4 hours. The cleaved protein was eluted and dialyzed into 5% glycerol in PBS (8g/L NaCl, 2g/L KCl, 1.44g/L Na$_2$HPO$_4$, 0.24g/L KH$_2$PO$_4$, pH 7.4) and stored at -80°C. All the *Xenopus* importin beta and human transportin recombinant proteins added to the reactions are untagged, except in the case of pull-down experiments where GST-tagged importin beta and transportin were used. We note that 6xhis-tagged transportin behaves indistinguishably with untagged transportin.

6xHis-tagged RanQ69L was expressed, purified, and loaded with GTP as previously described [17].

**Membrane fusion and nuclear pore assembly assays.**

*Xenopus* cytosol, membrane, sperm chromatin were prepared as in [17]. Membrane fusion and nuclear pore assembly assays were performed as described previously [17], except that membrane fusion experiments comparing transportin addition to reconstituted nuclei were visualized with an Axiovert 200M microscope (Carl Zeiss, Thornwood, NY) at a magnification of 63x using an oil objective (Carl Zeiss). Images were recorded using a Coolsnap HQ (Photometerics, Tucson, AZ) camera and Metavue software (Molecular Devices Corporation, Downingtown, PA). Images were processed using ImageJ (available at http://rsb.info.nih.gov/ij/). Nuclear membrane was stained with 3,3-dihexyloxocarbocyanine iodide (DHCC) (Eastman
Kodak, Rochester, NY), and 1,2-bis(o-aminophenoxy)ethane-$N,N,N',N'$-tetraacetic acid (BAPTA) (EMD Chemicals, Inc., Gibbstown, NJ) was added at 8 mM.

**Annulate Lamellae assembly.**

*Xenopus* cytosol, membranes, glycogen, and recombinant proteins were incubated together for 1.5 hours at room temperature. Ten microliters of the reaction was diluted in 190µL ELB (10mM HEPES, pH 7.6, 50mM KCl, 2.5mM MgCl$_2$) and overlaid on to a 0.5M Sucrose cushion in ELB. These samples were spun at 25,000 rpm for 20 minutes at 2°C in a Beckman tabletop ultracentrifuge. The membrane pellet was rinsed with ELB and resuspended in 100µL 1x SDS PAGE sample loading buffer.

**GST pull-downs.**

Recombinant GST, GST-*Xenopus* importin beta, and GST-human transportin were incubated with Glutathione-sepharose beads (GE Healthcare) without cross-linking. After blocking with 20mg/mL Bovine Serum Albumin (BSA) in PBS for 30 minutes, *Xenopus* egg cytosol spun for an additional 150,000 rpm for 10 minutes at 4°C to remove residual membrane contamination, was then added to the beads and incubated for 2 hours at 4°C (25µL cytosol in 500µL PBS). After washing the beads with PBS, the bound proteins were eluted with 0.1M Glycine, pH 2.5, and then neutralized with 1M Tris pH 8 before the samples were processed for immunoblotting. One fifth of each reaction was loaded for SDS-PAGE.
Anchored chromatin assay for immunoblotting.

*Xenopus* cytosol was heat inactivated at 100°C for 3 minutes and then spun at 14,000 rpm for 20 minutes to collect the supernatant. Twenty-five µL heat inactivated cytosol was used to decondense 500,000 *Xenopus* sperm chromatin at room temperature for ~15 minutes, and the state of chromatin decondensation was monitored by DAPI staining and fluorescence microscopy. The decondensed sperm chromatin were diluted in 300µL ELB (10mM Hepes, pH 7.6, 50mM KCl, 2.5mM MgCl$_2$) and allowed to bind to poly-L-lysine coated coverslips by gravity for 2 hours at room temperature. The chromatin covered coverslips were blocked with 4% BSA in ELB. The blocked chromatin coated coverslips were then incubated with cytosol or cytosol in the presence of 20µM *Xenopus* importin beta, 20µM human transportin, or 30µM RanQ69L recombinant protein for 20 minutes at room temperature. After three washes in ELBK (10mM Hepes, pH 7.6, 100mM KCl, 2.5mM MgCl$_2$) the chromatin was lysed in 30µL 1x sample loading buffer.

Chromatin binding for immunofluorescence microscopy.

*Xenopus* cytosol, sperm chromatin, and recombinant proteins were incubated together for 20 minutes at room temperature. The reactions were diluted in 800µL ELB, overlaid on a 300µL 75% sucrose cushion in ELB, and centrifuged at 750 rpm (100x g) for 15 minutes onto poly-L-lysine treated coverslips. Coverslips were then fixed in 4% formaldehyde in PBS for 10 minutes at room temperature and processed for immunofluorescence microscopy. IgG purified anti-Orc2 (gift from John Newport)
was used at 1:100, affinity purified anti-*Xenopus* ELYS (Rasala et al., submitted) was used at 1:200, FITC-goat anti-mouse was used at 1:200 and Alexa-568 goat anti-rabbit (Invitrogen, Carlsbad, CA) was used at 1:500.
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Figure 4.1 - Excess transportin blocks nuclear membrane fusion in a Ran-reversible manner.

Nuclear formation from *Xenopus* egg extract was performed at room temperature for one hour with the addition of PBS buffer (control), 2mM GTPγS (GTPγS), 37.5μM RanQ69L (Ran), 25μM transportin (Trn), or 25μM transportin and 37.5μM RanQ69L (Trn+Ran). Membrane fusion was observed without fixation using confocal microscopy and by staining nuclei with the membrane dye DHCC (green). DNA was stained with DAPI (blue). Sections of membrane stain were magnified three times (3X) and represented to the right of the merged images. Contour images of the respective DHCC staining are shown (trace), and discontinuities in the traces indicate regions with little or no membrane fusion. Representative images are shown. Bar: 10μm.
Figure 4.2 - Excess transportin blocks nuclear pore assembly in BAPTA arrested nuclei.

A. Pore-free BAPTA nuclei intermediates were assembled by adding 8µM BAPTA in the nuclear formation reaction at t=0. At t=60’, BAPTA-arrested nuclei were diluted 1:10 in fresh cytosol in the presence or absence of recombinant proteins. To assess nuclear pore formation, Alexa-488 directly labeled antibody against FG nucleoporins was added to the nuclei at t=90’ for an additional 20 minutes before visualization by fluorescence microscopy. As expected, no staining was observed in BAPTA nuclei (left panels), while nuclear pores were formed in control nuclei with cytosol and buffer (+buffer) or with 30µM RanQ69L (+Ran). In BAPTA nuclei recovered in fresh cytosol, either adding 8 µM BAPTA (+BAPTA) or 20µM transportin (+Trn) blocked nuclear pore formation. However, weak FG staining was observed when nuclei containing 20µM transportin were added in conjunction with 30µM Ran (+Trn+Ran). DNA was stained with DAPI (blue). Representative images are shown. Bar: 10µm.

B. Quantitation of data in A. Seventy-five nuclei per experiment were counted under each condition, and the percentage of nuclei that contained FG-nucleoporin staining was plotted. Error bars represent standard deviation calculated over three independent experiments.
Figure 4.3 - Annulate lamellae pore assembly is regulated by importin beta and transportin in a Ran-mediated manner.

Decondensed sperm chromatin was incubated with *Xenopus* cytosol in the absence or presence of recombinant proteins, and the nucleoporins that bound to chromatin were detected by immunoblotting. When AL is formed, all tested nucleoporins are present (lane 1). However, when excess importin beta or transportin are added, nucleoporins no longer accumulate on AL membranes (lanes 2 and 4). This importin beta and transportin block to AL assembly can be reversed by addition of excess RanQ69L-GTP (lanes 3 and 5). RanQ69L-GTP does not affect AL pore assembly (lane 6). AL was not formed when only cytosol (lane 7) or membrane (lane 8) was added to sperm chromatin. Equal amounts of membranes were collected under each condition, as indicated by the pore membrane protein gp210 (top row).
**Figure 4.4 - Nucleoporin targets of importin beta and transportin.**

A. GST, GST-importin beta, or GST-transportin was added to *Xenopus* cytosol and incubated for 2 hours at 4°C. The bound proteins were analyzed by immunoblotting. Immunoblotting controls were shown in the left lane (Cytosol). FG-nucleoporins interact with both importin beta and transportin as expected (Nup358, Nup214, Nup153, Nup98, Nup62, and Nup50). Members of the Nup107-160 complex, representing the entire Nup107-160 complex (Nup160, Nup133, and Nup85), the Nup107-160 complex-associated proteins ELYS and centrin, and Nup53 also bind both importin beta and transportin. Nup205 binds to transportin inconsistently, but not to importin beta. Nup155 and Nup93 do not associate with importin beta or transportin.

B. GST or GST-transportin pull-downs were performed and bound proteins were analyzed as in Figure 4A, except that 10μM RanQ69L was added in the reaction (GST-Trn+Ran).
**Figure 4.5 - Importin beta and transportin regulate the initial step in nuclear pore assembly.**

A. When *Xenopus* cytosol is incubated with a chromatin source in the absence of membranes, ELYS binds to chromatin (+Buffer). However, when 20µM importin beta or 20µM transportin are added to the reaction, ELYS can no longer bind to chromatin (+beta, and +Trn). Both blocks of chromatin binding of ELYS can be reversed by the addition of 30µM RanQ69L-GTP (+beta+Ran, and +Trn+Ran). The binding of chromatin binding protein, Orc2 only moderately changes upon addition of excess recombinant protein. Increased FG-nucleoporins bind to chromatin in the presence of 30µM RanQ69L-GTP (+Ran). Representative images are shown. Bar: 10µm.

B. Quantitation of the results in Figure 5A. In each experiment, 5 nuclei per condition were analyzed. Ten 10x10 pixel sections of each nucleus were measured for pixel brightness using ImageJ software. These values were averaged per condition and normalized to the average pixel brightness value obtained for the control. The standard deviations are calculated over 4 experiments.

C. Chromatin was decondensed and allowed to settle onto poly-L-Lysine coated coverslips. The anchored chromatin was incubated with *Xenopus* egg cytosol in the presence and absence of added recombinant protein for 20 minutes at room temperature. Proteins bound to chromatin were analyzed by immunoblotting. Immunoblotting control is shown in lane 1. Decondensed chromatin incubated with recombinant importin beta or transportin but without cytosol serve as negative controls (lanes 2 and 3). The Nup107-160 complex (represented by Nup160 and Nup133) and ELYS bind to chromatin (lane 4), whereas Nup153 and Nup205 do not bind to chromatin. Orc2, a chromatin binding protein, was included as a positive control for chromatin binding. When 20µM importin beta or transportin is added, the binding of ELYS and the Nup107-160 complex to chromatin is abolished (lanes 5 and 8). This block can be reversed by addition of 30µM RanQ69L-GTP (lanes 6 and 9). RanQ69L-GTP alone does not affect the binding of ELYS and the Nup107-160 complex to the chromatin, but induces the binding of Nup153 to chromatin (lane 7).
Figure 4.6 - Model of transportin and importin beta in regulation of multiple steps during nuclear assembly.

The initial step of post-mitotic nuclear pore assembly in *Xenopus laevis in vitro* nuclear formation involves the binding of ELYS (red ovals) and the Nup107-160 complex (yellow Y-shapes) to the chromatin (blue half circles) [34-36]. Here, we demonstrate that importin beta and transportin (yellow hexagons) both negatively regulate this binding (Figure 5). The block to ELYS and Nup107-160 complex binding to chromatin is reversible by RanGTP (red circle). Membrane vesicles competent for pore formation are recruited to the chromatin (green circles) and fuse to form a double nuclear envelope (curved green lines). The fusion of the membrane vesicles to form a nuclear envelope is negatively regulated by importin beta [15, 17] and transportin in a Ran sensitive manner. After vesicle-vesicle fusion at the chromatin, remaining nucleoporins are recruited to form mature nuclear pores (red nuclear pore). This process is negatively regulated by importin beta [15, 17, 19] and transportin. Excess RanGTP counteracts the importin beta and transportin block to the recruitment of the FG-nucleoporins to the forming nuclear pore complex.
References


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Future Directions

This dissertation describes work that has furthered our understanding of the regulation of nuclear pore complex assembly. In this section I highlight several key questions that follow from this work. These questions include: What is the mechanism of the importin beta and transportin negative regulation of ELYS binding to chromatin, and what other steps of pore assembly do importin beta and transportin regulate.

What is the mechanism of the importin beta and transportin negative regulation of ELYS binding to chromatin?

The first known step for nuclear pore assembly is the binding of the pore targeting protein, ELYS, to chromatin [1, 2, 3] (Rasala et al., submitted). Thus, a simple way to downregulate NPC assembly, when needed, would be for importin beta or transportin to inhibit ELYS-chromatin binding. Indeed, in Chapter 4, I demonstrate that importin beta and transportin do negatively regulate ELYS binding to chromatin (Chapter 4, Figure 5). To molecularly define this regulated interaction, we can ask whether importin beta binds directly to ELYS or to the chromatin.

It has been shown that ELYS binding to chromatin was mediated in part by an AT-hook located near the C-terminus of ELYS and an additional site located within the last 49 amino acids of ELYS (Rasala et al., submitted). During the course of this study several useful GST-tagged ELYS constructs were made available: AT-hook+ (the last 127 amino acids containing the AT-hook and secondary binding site), ΔAT-
hook (the last 49 amino acids containing only the secondary binding site), and AT-hook-short (a region containing the AT-hook, but not the secondary binding site) (Rasala et al., submitted; Rasala, in prep.). These constructs may serve as useful tools to determine the molecular mechanism of the importin beta and transportin regulation of ELYS binding to chromatin.

First, we can assess importin beta binding to ELYS by testing for a direct interaction between untagged importin beta and the recombinant chromatin-binding GST-tagged ELYS fragments. Two of the ELYS fragments, GST-AT-hook+ and GST-ΔAT-hook, have been found to interact with importin beta by GST-pulldown from *Xenopus* cytosol (Rasala et al., in prep.). Recent work, done under my mentorship by Quang Phung, an undergraduate researcher, showed that importin beta also binds to the GST-AT-hook-short fragment. Also of note, we showed by GST-pulldown that transportin also binds to all 3 GST-ELYS fragments. However, from these pull-down experiments, it is unclear if the interactions between importin beta (or transportin) and the ELYS fragments are direct ones. Thus, the next step is to perform GST-pulldowns using the three GST-ELYS fragments plus untagged recombinant *Xenopus* importin beta or untagged recombinant human transportin in the absence of cytosol. Preliminary results indicate that GST-ELYS AT-hook+ and ΔAT-hook bind directly to importin beta (Phung, in prep.). If these results are confirmed, it will be important to test if these interactions are reversed by addition of RanQ69L-GTP. If a direct interaction between importin beta and the ELYS fragments is found, this will
provide data in support of the model that ELYS binding to chromatin is directly negatively regulated by importin beta.

Do importin beta and transportin regulate additional steps in nuclear assembly?

Although importin beta may regulate the initial step in pore assembly, it does not preclude it from regulating subsequent steps. Each step of pore assembly is in potential need for precise regulation. If one step were to go unchecked, aberrant assembly of pores with respect to place and time could occurs. We found that in Xenopus cytosol importin beta is bound to all the FG-nucleoporin subcomplexes, to the Nup107-160 complex, and to Nup53, arguing that regulation could occur at the level of assembly of any of these complexes into forming nuclear pores (Chapter 4).

Do importin beta and transportin regulate Pom121 and Ndc1 membrane vesicle recruitment?

Pom121+/Ndc1+ vesicle recruitment to the chromatin occurs soon after ELYS and Nup107-160 recruitment (Rasala et al., submitted). Indeed, we also find that a fragment of Pom121, when bound to beads, can pull down the Nup107-160 subcomplex from Xenopus egg cytosol (Rasala et al., submitted). We could next test the hypothesis that importin beta or transportin negatively regulates the interaction between the Nup107-160 complex on the surface of chromatin and Pom121+/Ndc1+ membrane vesicles.
To test this, a modified chromatin-binding assay could be performed. Chromatin can be decondensed in full *Xenopus* egg cytosol (not heat-treated) in the absence of membranes and allowed to settle onto poly-L-Lysine coated coverslips. This will allow for ELYS and the Nup107-160 complex from the cytosol to bind to the chromatin ([6]; Chapter 4; Rasala et al., submitted). After washing, the chromatin-coated coverslips could be incubated with *Xenopus* egg membranes and buffer, in the presence or absence of excess recombinant importin beta or transportin. In the absence of importin beta or transportin, Pom121\(^*/\)Ndc1\(^+\) membrane vesicles should bind to the chromatin substrate, presumably via ELYS and the Nup107-160 subcomplex, and be detectable by our fluorescently labeled anti-xPom121 antibody. If, however, added importin beta or transportin prevented this interaction, we would find no Pom121, either by IF or immunoblotting, on the harvested chromatin intermediates. This approach will allow us to determine whether importin beta and/or transportin can negatively regulate the interaction between Pom121\(^*/\)Ndc1\(^+\) vesicles and chromatin. Our data indicate that importin beta does indeed interact with Pom121 fragments in solution (Rasala et al., submitted) (Transportin was not assayed in this study). This is thus a promising starting point.

**Do importin beta and transportin regulate inner and outer nuclear membrane fusion?**

Importin beta and transportin negatively regulate the vesicle-vesicle fusion required for formation of the double nuclear membranes ([7]; Delmar et al., *in press*;
Chapter 3; Chapter 4). However, whether importin beta and transportin regulate the fusion between the inner and the outer membrane of the nuclear envelope to form the nuclear pore remains unknown. A former post-doc in the Forbes lab, Dr. Corinne Ramos, identified a cold-arrested nuclear intermediate where fusion between the inner and outer nuclear membranes had not yet occurred (Ramos et al., in prep.). To address whether importin beta or transportin regulates the important inner/outer membrane fusion step, we could form cold-arrested nuclei, add an excess of importin beta/transportin or a control protein, and allow the intermediates to then assemble for a further time. Untreated nuclei, upon continued incubation, should readily form pores that are competent for diffusion of an Alexa-labeled 3-kDa fluorescent dextran into the nucleus and thus would show brightly labeled nuclei after anti-Alexa antibody quenching. If importin beta/transportin prevents the formation of the fusion event and pore assembly, however, we would expect the 3-kDa fluorescent dextran to be excluded from such nuclear intermediates and the nuclei to thus appear dark. To control for nuclei that have simply broken, we could also include an Alexa-568 fluorescently labeled anti-DNA antibody. This antibody will only access the DNA if the nuclei are broken, as the antibody is too large to fit through a nuclear pore. This study would be one of the first characterizations of potential regulation of fusion between the inner and outer nuclear membranes. The experiments described above are directed at attacking the molecular mechanism of regulation of nuclear pore assembly by importin beta and transportin. The prevailing theory is that importin beta and transportin interact with nucleoporin subcomplexes and prevent them from interacting
with one another. When for example, an importin beta-inhibited nucleoporin subcomplex comes into contact with RanGTP in high concentration around chromatin, it is thought that importin beta releases its hold on the nucleoporin, permitting nucleoporin-nucleoporin interaction [8, 9]. This mechanism could be applicable to any nuclear assembly step before the formation of an enclosed nuclear envelope that involves the recruitment of an importin beta- or transportin-binding protein.

**Does transportin regulate mitotic spindle assembly?**

Importin beta plays a key role in regulation of mitotic spindle assembly by binding to spindle activation factors (SAFs), preventing them from performing their function. In some cases, the interaction of importin beta with the SAFs is mediated by importin alpha. However, in others the interaction of the SAFs with importin beta is direct [10-14]. Therefore, it is interesting to speculate that transportin may also have an effect on spindle assembly by sequestering particular transportin-binding SAFs.

A simple way to address this question would be to add recombinant untagged transportin to mitotic *Xenopus* cytosol in the presence of chromatin and check for the presence of properly formed spindles by addition of rhodamine-labeled tubulin. Addition of excess importin beta would act as an important control for spindle assembly inhibition. If transportin does in fact regulate spindle assembly, one could identify potential regulatory targets by investigating the amino acid sequences of known spindle assembly factors for the M9 or A1 type NLSs that are recognized by transportin in its cargo binding.
**Which member of the Nup107-160 complex does importin beta bind to?**

It is known that importin beta interacts with the key Nup107-160 complex ([9] Chapter 4). However, it is not known which of the 9 nucleoporins of the Nup107-160 complex with which importin beta interacts. The answer is not known because most of the 9 nucleoporins are insoluble when recombinantly expressed. Therefore, performing the obvious direct binding assay is not practical. However, if we were able to narrow down our options (i.e., have 2 vs. 9 potential candidates) this would make determining the answer feasible.

One way to narrow down the candidates would be to try a blot overlay approach. In this approach, one would immunoprecipitate the Nup107-160 complex with anti-Nup133 (or an antibody to another complex member). The immunoprecipitate would be run out on a gel and transferred to PVDF membrane, as if treating for western blot. The membrane could be incubated with recombinant importin beta to allow importin beta the opportunity to bind to specific Nup107-160 complex members, separated by molecular weight, and immobilized on the membranes. An antibody to importin beta (or to its purification tag if applicable) could be used to identify where on the blot importin beta bound. If, for example, anti-importin beta recognized a band at 43kDa it would suggest that importin beta could directly bind to Nup43 of the Nup107-160 complex. Recombinant direct binding assays can be used to confirm this interaction.
The answers to the questions in this Future Directions section would provide substantial mechanistic insight into the roles of importin beta and transportin in the regulation of nuclear pore complex assembly.
References


