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Nuclear dynamics: from importin beta regulation of nuclear pore assembly to identification of novel vertebrate proteins involved in mRNA export.

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

Master of Science

in

Biology

by

Quang Phung

Committee in charge:

      Professor Douglass Forbes, Chair
      Professor Maho Niwa
      Professor James Wilhelm

2009

The Thesis of Quang Phung is approved, and it is acceptable in quality and form for

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ABSTRACT OF THE THESIS

Nuclear dynamics: from importin beta regulation of nuclear pore assembly to identification of novel vertebrate proteins involved in mRNA export.

by

Quang Phung

Master of Science in Biology

University of California, San Diego, 2009

Professor Douglass Forbes, Chair

The cell nucleus contains the genetic materials of an organism and allows selective nucleocytoplasmic transport via the nuclear pores embedded in the nuclear membranes. In interphase, importin beta is a well-studied nuclear import receptor mediating the classical nuclear import pathway. In mitosis, importin beta negatively regulates key mitotic events of mitotic spindle formation and nuclear pore assembly. Here, we showed that importin beta directly interacts with ELYS, the pore-targeting protein, and negatively interferes with the initiating step of nuclear pore assembly. We also showed that both Xenopus and human importin beta directly interacts with human centrin2, a small Ca-binding protein involved in centrosomes dynamics as well as protein and mRNA export. While nuclear import of transcritption factors into the nucleus is required to initiate gene transcription, mRNA transcripts need to be exported out to the cytoplasm for translation into new proteins. In this study, we showed that both over-expression of human Shd1 and Eny2 causes nuclear accumulation of poly(A)+ RNA, suggesting potential roles in an unknown vertebrate TREX2 in mRNA export. Furthermore, Eny2 was shown to biochemically associate with multiple nuclear transport

factors, notably TAP, the major mRNA export receptor. In conclusion, these data provide

supporting evidence for a potential vertebrate TREX2 involved in mRNA export.

THESIS INTRODUCTION

The cell nucleus

As the biological level of complexity increased from prokaryotes to eukaryotes, it became necessary for the eukaryotic cell to evolve regulatory strategies to coordinate cellular processes in a spatially and temporally organized fashion. The nucleus takes center stage in the eukaryotic cell providing a protective environment for the genome containing the "information of life". The nuclear envelope provides spatial separation between the nucleoplasm, the site of DNA replication and transcription, and the cytoplasm, the site of protein synthesis and many other cellular processes (Anderson et al., 2008). The nuclear dynamics are also temporally regulated. In interphase, the nucleus encloses the highly ordered chromatin structure of the genome that is contained within the nuclear interior. However, when it is time for a cell to enter mitosis, the metazoan nucleus undergoes a dynamic structural re-organization by disassembling at the start of prophase to allow the faithful separation of the doubled chromosomes and reforming at the end of telophase (Bodoor et al., 1999; Kutay et al., 2008).

A full understanding of the molecular components of the nucleus is of intense interest to understand how the nucleus achieves the regulatory functions discussed above. Structurally, the nucleus is enclosed by the two double nuclear membranes, the outer and inner nuclear membranes separated by the perinuclear space (Burke et al., 2002). The nuclear lamins lie immediately underneath the inner nuclear membrane and are thought to provide structural support for the membrane and to function as a docking framework to connect the chromosomes to the inner nuclear membrane (Gerace et al., 1994). Information exchange between the nucleus and cytoplasm is controlled by nuclear pores

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and it is these that regulate the entry and exit of molecular messages and signals.

Nuclear Pore Complexes

Nuclear pores are embedded in the nuclear envelope and function as gateways for exchange of molecular information between the nucleus and cytoplasm in response to extra- and intra-cellular changes. Nuclear pore complexes (NPCs) are composed of ~30 proteins termed nucleoporins (or Nups for pore-associated proteins). Each protein is present in multiple copies. Many are present in different structural subunits of the entire pore. A single nuclear pore is a macromolecular complex that is approximately 124 MDa and is ~30 times the size of a ribosome (Rout et al., 2000; Lim et al., 2008). Structurally, an individual nuclear pore consists of eight symmetrically-organized cytoplasmic filaments, a central transporter region, and a nuclear basket ring facing the nucleoplasm (Antonin et al., 2008). Each structural component of the pore provides a functional contribution to the overall role of the pore, which is to mediate coordinated transport of molecular complexes into and out of the nucleus. The cytoplasmic filaments have been shown to contain Phenylalanine-Glycine (or FG-) repeats that interact with transport receptors and serve as initial docking sites for cargo-receptor complexes to be imported into the nucleus (Hetzer et al., 2005). After initial docking, the transport complex passes through the central transporter region where it is hypothesized to interact with the FG-repeats lining the center core of the pore. These successive interactions help to translocate the import complex into the nucleus (Gorlich et al., 1999; Beck et al., 2008)

Nucleocytoplasmic transport

Nucleocytoplasmic transport is mediated by a large number of transport receptors belonging to the karyopherin-beta superfamily (Kutay et al., 1999; Cook et al., 2007).

Together, importin beta and importin alpha form a bipartite receptor complex to mediate classical nuclear import pathway (Gorlich et al., 1995; Macara et al., 2001).

Mechanistically, importin alpha first recognizes a NLS-containing protein cargo on one side and binds to importin beta on the other side. This heterotrimeric complex of cargo, adaptor, and receptor formed in the cytoplasm, migrates to the nuclear pores. Importin beta then binds to the FG cytoplasmic filaments to initially dock the import complex onto the pores. Subsequent interactions with the FG filaments lining the pore mediate the translocation of the import complex into the nucleus (Pemberton et al., 2005). Once the entire import complex is in the nucleus, RanGTP binds to importin beta, mediates the dissociation of the cargo and the adaptor importin alpha and receptor importin beta to complete import (Kutay et al., 1999; Ben et al., 2001; Beck et al., 2008).

In addition to importin alpha and importin beta, a second receptor, transportin, was next identified. Transportin is the nuclear import receptor for M9 sequence-containing protein cargoes (Pollard et al; 1996). However, unlike importin beta, transportin recognizes and directly binds to its cargoes without requirement for any adaptor protein (Gorlich et al., 1999). One well-characterized import cargo for transportin is the heterogeneous nuclear ribonucleoprotein A1 (hnRNP A1) with basic sequence containing a proline(P) and tyrosine(Y), which is defined as PY-NLS (Chook et al., 2006). Taken together, importin alpha/importin beta and transportin provide two major import pathways into the nucleus, although other import receptors also exist.

Crm-1 as the major nuclear protein export receptor

CRM1 (or chromosome region maintenance) was originally identified as a temperature-sensitive mutant of a 115-kD nuclear protein which caused abnormal

morphology of nuclear chromosome in *Schizosaccharomyces pombe* at the restrictive temperature (Adachi, et al., 1989). The role of Crm1 in nuclear transport was first suggested by various studies showing that the nuclear export of proteins bearing nuclear-export signals (NES) was inhibited in Crm1-mutant strains of budding and fission yeast (Fukuda, et al., 1997; Stade et al., 1997). Additional studies in mammalian systems showed that leptomycin B, a chemical inhibitor of Crm1 (Nishi, et al., 1994), was able to inhibit nuclear export of leucine-rich NES-containing proteins (Wolff, et al., 1997; Ossareh-Nazari et al., 1997). Interestingly, Crm1 shared structurally and functional similarities with members of the importin beta super-family in that it had an N-terminal region containing a RanGTP-binding domain and the ability to interact with nucleoporins (Fornerod, et al., 1997). These data demonstrated the involvement of Crm1 as an export receptor for nuclear proteins containing leucine-rich NES. In the nucleus, Crm1 cooperatively binds to NES-cargoes and RanGTP, the latter of which helps stabilize the formation of a trimetric export complex and promote nuclear protein export (Petosa et al., 2004). Translocation of the Crm1-mediated export complex was shown to be mediated by direct interaction with Nup214 and Nup358 (Hutten and Kehlenbach, et al., 2006). Once in the cytoplasm side of the NPC, two cytoplasmic proteins, RanGTP-binding protein RanBP1 and RanGAP work in concert to mediate GTP hydrolysis to dissociate of the export complex and release the export cargo into the cytoplasm (Gorlich et al., 1999).

Role of Ran gradient in directionality of nuclear transport

Even though the major nuclear transport factors were identified, it was unclear what control the directionality of nuclear transport and how the import and export processes are coordinated. It was later shown by various studies that Ran, a small GTPase

protein that is present either in its GDP-bound or GTP-bound form, is involved in nuclear transport (Gorlich et al., 1999; Arnaotov et al., 2005). It was shown that a Ran-GEF (for Guanine-Exchange factor, also named RCC1) was found to be bound to chromatin in the nucleus and its function was to induce RanGDP conversion into RanGTP. Meanwhile, on the cytoplasmic side of NPCs, a RanGAP (for GTPase-Activating protein) acted to induce GTP hydrolysis of RanGTP into a GDP-bound form (Kutay and Gorlich, et al., 1999). The presence of these two Ran cofactor proteins helps to establish and maintain a "Ran gradient" with high RanGTP in the nucleus and high RanGDP in the cytoplasm. This Ran gradient imposes directionality on nuclear transport by binding to import receptors in the nucleus, leading to the release of the imported cargoes, while promoting the formation of export complexes in nuclear export pathways (Dasso, et al., 2001).

Nuclear mRNA export

Eukaryotic gene expression occurs in a well-coordinated process where gene transcription in the nucleus produces messenger RNAs (mRNAs), which then must be exported through NPCs into the cytoplasm to be translated into proteins. Nuclear export of mRNA transcripts is an intricate step that is required for the production of functional proteins involved in many important processes of the cell and ultimately the life of the organism. Defects in mRNA export have been implicated in important human diseases (Hurt et al., 2004). For example, a human bone disorder called osteogenesis imperfecta type I was characterized by a genetic mutation in a collagen-encoding gene which causes improper splicing and nuclear retention of the mRNA transcripts and ultimately results in decreased production of collagen proteins important for normal bone formation (Johnson et al., 2000). A second type of mRNA-associated human disease was found in a disorder

called lethal congenital contracture syndrome, characterized by a mutation in an mRNA export factor that results in reduced export of mRNAs involved in motor neuron and skeletal muscle development (Nousiainen et al., 2008). Given the importance of proper mRNA export to normal functions and survival of the organism, further studies will undoubtedly advance our understanding of the basic molecular and cellular aspects of nuclear mRNA export and its implications in treatment of human diseases.

Like protein import and export, several specific transport factors exist to mediate nuclear mRNA export. Early studies of mRNA export using genetic approaches in yeast identified a number of key factors implicated in the nuclear export of mRNAs. For example, in situ hybridization experiments in yeast strains showed that a conditional temperature-sensitive mutant in a yeast protein named Mex67p caused a temperature-induced retention of mRNAs in the nucleus at the restrictive temperature, indicating Mex67p as a nuclear mRNA export factor (Segref, et al., 1997). Further studies subsequently identified functional homologue of Mex67p in metazoan known as TAP (Pasquinellie, et al., 1997). Additional experiments in Xenopus oocytes and human cell culture showed that over-expression of TAP increased export of mRNA transcript and rescued mRNA accumulation phenotypes, validating function of TAP in mRNA export (Herold, et al., 2000; Braun et al., 2001). Identification of yeast Mex67p and mammalian homologue TAP led to further studies looking into the translocation mechanism of mRNA export. Both Mex67p and TAP were shown to shuttle between the nucleus and cytoplasm, cross-link to mRNA in vivo, and interact with FG-nucleoporins as other known nuclear transport receptors would. However, one striking feature of Mex67p and TAP-dependent export of mRNAs was that Ran binding was shown to be dispensable for

their export function (Zenklusen, et al., 2001; Cullen et al., 2003). Thus mRNA export presents an exception to the rule of Ran-dependent nuclear transport. It is of interest to note that Crm1, previously identified as a major protein export receptor, also had an unexpected role in nuclear mRNA export of unspliced viral mRNA (Wolff, et al., 1997) and some cellular mRNAs involved in some specialized cellular processes of differentiation (as reviewed in Kehlenbach et al., 2007). Thus, there are two major pathways of nuclear mRNA export: Mex67p or TAP-dependent and Ran-independent export of majority of cellular mRNA and Crm1/RanGTP-mediated export of virally unspliced mRNA and some cellular mRNAs.

With respect to the TAP-dependent mRNA export pathway, additional studies have recently showed that the process of nuclear export of mRNAs is tightly linked to the upstream steps of gene transcription, pre-mRNA processing, formation of large messenger-ribonucleoprotein particles (mRNPs), and their targeting to the nuclear pores for export (Erkmann et al., 2004; Vinciguerra et al., 2004). For example, genetic and biochemical studies in yeast have revealed the existence of a TREX (Transcription and Export) complex consisting of transcriptional elongation factors as well as splicing factors involved in mRNA processing and formation of export competent mRNPs (Strasser et al., 2002). Subsequent studies have identified another complex named TREX-2, which is involved in targeting the mRNPs to the nuclear pores and recruiting of additional export factors (Kohler et al., 2007).

In summary, in this introductory chapter of my thesis, I have presented a general overview of the structural aspects of the cell's nucleus and nuclear pores as well as an overview of their functional involvement in the nucleocytoplasmic transport of proteins

and mRNA. In the next chapter, I will focus on two major aspects of my study. The first topic will include importin beta regulation of nuclear pore assembly and a direct interaction of importin beta with centrin. The second topic will be a discussion of my work on identification and characterization of a potential vertebrate homologue of the yeast TREX2 complex with involvement in mRNA export.

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Chapter 1: Transportin regulates major mitotic assembly events:

from spindle to nuclear pore assembly

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Running head: Transportin and mitotic assembly events

Abbreviations: AL, annulate lamellae; FG, phenylalanine-glycine; GST, glutathione-S-transferase; NLS, nuclear localization signal; PY, proline-tyrosine; SAF, spindle assembly factors; Trn, transportin

ABSTRACT

Mitosis in higher eukaryotes is marked by the sequential assembly of two massive structures, the mitotic spindle and nucleus. Nuclear assembly itself requires the precise formation of both nuclear membranes and nuclear pore complexes. Previously, importin alpha/beta and RanGTP were shown to act as dueling regulators to ensure that these assembly processes occur only in the vicinity of the mitotic chromosomes. We now find that the distantly related karyopherin, transportin, negatively regulates nuclear envelope fusion and nuclear pore assembly in *Xenopus* egg extracts. We show that transportin -- and importin beta -- initiate their regulation as early as the first known step of nuclear pore assembly, recruitment of the critical pore-targeting nucleoporin ELYS/MEL-28 to chromatin. Indeed, each karyopherin can interact directly with ELYS. We further define the nucleoporin subunit targets for transportin and importin beta and find them to be largely the same: ELYS, the Nup107/160 complex, Nup53, and the FG nucleoporins. Equally importantly, we show that transportin negatively regulates mitotic spindle assembly. These negative regulatory events are counteracted by RanGTP. We conclude that the interplay of the two negative regulators, transportin and importin beta, along with the positive regulator RanGTP, allows precise choreography of multiple cell cycle assembly events.

INTRODUCTION

During mitosis, the intracellular architecture of the higher eukaryotic cell undergoes constant and dramatic change. The chromosomes condense, the nuclear envelope breaks down, a mitotic spindle forms and separates the chromosomes, and finally a nuclear envelope is reassembled around each set of daughter chromosomes. The movement of the cell cycle from one stage of mitosis to the next involves the coordinated action of multiple kinases and phosphatases. Recently, however, new regulators have been identified that contribute not to the timing but to the *spatial* control of assembly of both the mitotic spindle and the nucleus: karyopherins and RanGTP.

Karyopherins are most well known for their function in interphase as the transport receptors for nuclear import and export [reviewed in (Tran and Wente, 2006; Stewart, 2007)]. Nuclear proteins larger than ~20-40kD are actively imported, while snRNAs, tRNAs, and other cargoes are exported through the nuclear pores by karyopherins. Karyopherins consist of a large family of importins (import receptors) and exportins (export receptors) (reviewed in Weis, 2003; Pemberton and Paschal, 2005). The first and most studied import receptor of the karyopherin family was importin beta (Adam and Adam, 1994; Chi *et al.*, 1995; Gorlich *et al.*, 1995; Imamoto *et al.*, 1995; Radu *et al.*, 1995; Floer *et al.*, 1997). Importin beta mediates the nuclear import of many proteins through the use of a specific adaptor, such as importin alpha (reviewed in Cook *et al.*, 2007). Importin alpha recognizes cargoes containing classical or bipartite nuclear localization signals (NLSs), which are composed largely of basic amino acids (reviewed in Stewart, 2007). Importin beta can also bind to certain cargoes directly without the use of an adaptor. The second major import receptor discovered was transportin (or

karyopherin beta-2), which is distantly related to importin beta (24% identical) (Nakielny *et al.*, 1996; Pollard *et al.*, 1996; Bonifaci *et al.*, 1997; Fridell *et al.*, 1997; Siomi *et al.*, 1997; Chook and Blobel, 1999). Transportin imports proteins without an adaptor. Transportin most commonly recognizes cargoes that contain a different consensus NLS, one with a terminal proline-tyrosine (PY) dipeptide preceded either by a hydrophobic or basic region, depending on the protein (Lee *et al.*, 2006; Cansizoglu *et al.*, 2007; Suel *et al.*, 2008).

The directionality of transport is governed by a gradient of RanGTP, the GTP-bound form of the small GTPase Ran. The concentration of RanGTP is high in the nucleus and low in the cytoplasm (reviewed in Madrid and Weis, 2006), a result of the exclusive localization of the Ran-GEF to chromatin. Both importin beta and transportin release their cargoes upon encountering RanGTP in the nucleus.

From yeast to vertebrates, the basic architecture of the 60-120 megadaltons nuclear pore consists of a large central scaffold, eight cytoplasmic filaments, and a nuclear pore basket. Each nuclear pore contains ~30 different proteins or nucleoporins in multiples copies and possesses eightfold symmetry (Rout *et al.*, 2000; Cronshaw *et al.*, 2002; Stoffler *et al.*, 2003; Beck *et al.*, 2007; Beck and Medalia, 2008; Brohawn *et al.*, 2008; Debler *et al.*, 2008; Jovanovic-Talisman *et al.*, 2008; Lim *et al.*, 2008). During transport, the karyopherins interact with a specific subset of nuclear pore proteins which have in common phenylalanine-glycine or FG repeats (Gorlich and Kutay, 1999; Chook and Blobel, 2001; Macara, 2001; Denning *et al.*, 2003; Fahrenkrog *et al.*, 2004; Strawn *et al.*, 2004; Peters, 2005; Madrid and Weis, 2006; Tran and Wente, 2006; Cook *et al.*, 2007; Stewart, 2007; Lim *et al.*, 2008). In organisms from *Drosophila* to

vertebrates, the nuclear envelope disassembles and reforms during each cell cycle. Disassembly involves the complete disassembly of the nuclear pores into subunits and the simultaneous retraction of the nuclear membranes into the ER. Assembly of the nucleus at the end of mitosis requires all the components including nuclear membranes and nuclear pores to reassemble in a step-wise manner (reviewed in Burke and Ellenberg, 2002; Antonin *et al*., 2008; Kutay and Hetzer, 2008). Progress continues to be made on the order of assembly of the nucleoporins and/or nucleoporin subcomplexes into the pore, although this order remains approximate (Bodoor *et al*., 1999; Belgareh *et al*., 2001; Daigle *et al*., 2001; Walther *et al*., 2003a; Dultz *et al*., 2008; Rasala *et al*., 2008).

One valuable model system that has been used to study the regulated processes of mitotic spindle assembly and nuclear assembly, including nuclear pore assembly, is derived from extracts of *Xenopus* eggs. Mitotic *Xenopus* egg extracts can successfully recapitulate spindle assembly (see below). Similarly, interphase egg extracts have provided a powerful way to observe and manipulate nuclear assembly (Lohka and Masui, 1984; Newport, 1987; Smythe and Newport, 1991; Powers *et al*., 1995; Ullman and Forbes, 1995; Macaulay and Forbes, 1996; Goldberg *et al*., 1997; Hetzer *et al*., 2001; Harel *et al*., 2003a; Harel *et al*., 2003b; Walther *et al*., 2003a; Walther *et al*., 2003b; Chan and Forbes, 2006; Clarke and Zhang, 2008; Delmar *et al*., 2008). For nuclear assembly, fractionated *Xenopus* egg cytosol is combined with *Xenopus* egg membranes, sperm chromatin, and an energy-regenerating system. Notably, the fractionated *Xenopus* cytosol contains the soluble nucleoporins in ~14 subcomplexes (Rasala *et al*., 2008) and these, poised for assembly, quickly assemble into nuclear pores. The vesiculated ER derived from lysing of the eggs serves as a source of membrane for nuclear membrane

formation *in vitro*. [*In vivo*, the reticular ER itself serves as the source of nuclear

membrane; see (Yang *et al*., 1997; Anderson and Hetzer, 2008) and refs therein]. Within

~one hour of incubation at room temperature nuclei competent for nuclear import and

DNA replication are formed *in vitro*.

Intermediates in nuclear assembly have been identified in the *Xenopus* system

using chemical and protein inhibitors (Newmeyer and Forbes, 1988; Finlay and Forbes,

1990; Macaulay and Forbes, 1996; Goldberg *et al*., 1997; Shumaker *et al*., 1998; Harel *et

al*., 2003a; Hetzer *et al*., 2005) or by visualization using electron microscopy (Goldberg

et al., 1997; Wiese *et al*., 1997). When chromatin, vesicles and cytosol are mixed without

any preincubation, electron microscopy reveals membrane vesicle binding to chromatin,

followed by vesicle-vesicle fusion into patches of double membranes, nuclear pore

assembly within the double membrane patches, and finally closure of any gaps in the

nuclear envelope by additional fusion (Macaulay and Forbes, 1996; Burke and Ellenberg,

2002; Harel and Forbes, 2004; Baur *et al*., 2007; Rasala *et al*., 2008). However, the

precise order and regulation of components in nuclear assembly remain an area of intense

study (Antonin *et al*., 2008; D'Angelo and Hetzer, 2008; Rasala *et al*., 2008).

Recently, the formation of nuclear pores was found to be initiated by the

recruitment of a new pore-targeting protein, ELYS/MEL-28 to chromatin. ELYS recruits

the Nup107-160 complex, the largest pore subcomplex, which contains 9-10 members (Li

et al., 1995; Goldstein *et al*., 1996; Siniossoglou *et al*., 1996; Siniossoglou *et al*., 2000;

Belgareh *et al*., 2001; Lutzmann *et al*., 2002; Harel *et al*., 2003b; Walther *et al*., 2003a;

Loiodice *et al*., 2004; Zuccolo *et al*., 2007; Boehmer *et al*., 2008; Brohawn *et al*., 2008).

Indeed, vertebrate ELYS was identified biochemically as a binding partner of the critical

vertebrate Nup107-160 complex (Rasala *et al*., 2006; Franz *et al*., 2007; Gillespie *et al*., 2007). Strikingly, in the absence of ELYS, pore complexes do not assemble at the chromatin periphery, but instead form pore mimics in the ER; these cytoplasmic membrane stacks of pores are termed cytoplasmic annulate lamellae (AL) (Rasala *et al*., 2006; Franz *et al*., 2007; Rasala *et al*., 2008). Furthermore, the recruitment of ELYS and the Nup107-160 complex to chromatin was found to be a prerequisite for the recruitment of the integral membrane pore proteins POM121 and NDC1, followed by the bulk of the soluble nucleoporins, including the FG repeat nucleoporins (Rasala *et al*., 2008), to form the final mature nuclear pore perforating the double nuclear membranes. Interestingly, a fraction of ELYS moves with the Nup107-160 complex to kinetochores at mitosis, and mutation or RNAi of either causes cell cycle defects in yeast, *C. elegans*, zebrafish, and humans (Bai *et al*., 2004; Fernandez and Piano, 2006; Rasala *et al*., 2006; Davuluri *et al*., 2008; de Jong-Curtain *et al*., 2008).

Clearly, the formation of large cellular structures such as the mitotic spindle, nuclear membranes, and nuclear pores would be predicted to be the subject of careful and coordinated regulation. Two key regulators of all these assembly events have been identified, as stated above: RanGTP acts as a positive regulator, while importin beta acts as a negative regulator for spindle assembly (Kalab *et al*., 2002; Quimby and Dasso, 2003; Clarke and Zhang, 2008; Kalab and Heald, 2008) and for nuclear membrane and pore assembly (Ryan and Wente, 2002; Harel *et al*., 2003a; Ryan *et al*., 2003; Walther *et al*., 2003b; Clarke and Zhang, 2004; Harel and Forbes, 2004; Hetzer *et al*., 2005; D'Angelo *et al*., 2006; Ryan *et al*., 2007; Antonin *et al*., 2008). In the case of spindle assembly, importin beta, often in conjunction with importin alpha, binds to spindle

assembly factors (SAFs) such as NuMA and TPX2 and sequesters them in an inactive

form, preventing them from promoting microtubule assembly during mitosis --- except in

the area of the mitotic chromosomes (Gruss *et al*., 2001; Nachury *et al*., 2001; Wiese *et al*., 2001; Du *et al*., 2002; Schatz *et al*., 2003; Trieselmann *et al*., 2003; Ciciarello *et al*., 2004; Ems-McClung *et al*., 2004; Blower *et al*., 2005; Maresca *et al*., 2005; Albee *et al*., 2006; Ribbeck *et al*., 2006; Sillje *et al*., 2006; Tahara *et al*., 2008). RanGTP, produced

only in the vicinity of the chromosomes, releases importin alpha and/or beta from nearby

spindle assembly factors, allowing a spindle to form specifically around the mitotic

chromosomes. Thus, importin beta and RanGTP act as *spatial* regulators or mitotic

spindle assembly. For nuclear assembly, when excess importin beta is added, membrane

vesicles are recruited to the chromatin, but fail to undergo the vesicle-vesicle fusion

necessary to form the double nuclear membranes. This inhibition of fusion can be

counteracted by the inclusion of increased RanGTP (Harel *et al*., 2003a; Walther *et al*., 2003b; Delmar *et al*., 2008). If excess importin beta is added to membrane-enclosed

nuclear intermediates, it separately blocks nuclear pore assembly (Harel *et al*., 2003a; Walther *et al*., 2003b; Delmar *et al*., 2008). RanGTP reverses the inhibition of nuclear

pore assembly, but only when untagged importin beta is used as the negative regulator

(Delmar *et al*., 2008). Excess Ran alone causes excessive pore formation, both as nuclear

annulate lamellae and as cytoplasmic annulate lamellae (Harel *et al*., 2003a; Walther *et al*., 2003b). These studies demonstrated a precise balance between the two regulators

required for proper nuclear envelope assembly. For nuclear assembly, we believe that

this regulatory system would be utilized *in vivo* to carefully regulate nuclear membrane

fusion to occur where RanGTP is high, as on the surface of mitotic chromosomes, in

order to prevent spatially or temporally undesirable fusion. Undesirable fusion would include the formation of intranuclear or cytoplasmic annulate lamellae, or the formation of disproportionate ratios of inner to outer nuclear membrane (Harel *et al.*, 2003a; Walther *et al.*, 2003b; Delmar *et al.*, 2008). With respect to pore assembly, the importin beta negative regulatory system would prevent nuclear pores from forming in excess amounts on the nucleus or in inappropriate places as annulate lamellae elsewhere in the cell.

Analysis of the above studies poses a number of major questions: (1) Is the critical first step of nuclear pore assembly, the recruitment of ELYS to chromatin, regulated by importin beta? (2) Which downstream nucleoporin subunits are targets for importin beta regulation? (3) And perhaps most interestingly, do other transport receptors also regulate the steps of nuclear assembly -- or spindle assembly?

In this study, we have found that transportin is indeed a regulator of cell cycle assembly events. Transportin negatively regulates nuclear membrane formation, nuclear pore assembly, and spindle assembly in the *Xenopus* nuclear *in vitro* system and does so in a Ran-sensitive manner. Focusing on the events of nuclear pore assembly, a search for the regulatory targets of transportin and importin beta in nuclear pore assembly was done and revealed largely the same soluble nucleoporin targets. Furthermore, both transportin and importin beta affect the critical first step of nuclear pore assembly, ELYS/MEL-28 binding to chromatin to initiate pore assembly.

MATERIALS AND METHODS

Recombinant protein cloning, expression, purification, and antibodies

GST-human transportin (pGEX6P-Trn) was cloned by ligating a BamHI and XhoI fragment containing full-length transportin (Transportin 1; AAH40340) from pET28a-Trn (S. Shah and D. Forbes, unpublished data) into pGEX6P-3 vector (GE Healthcare, Piscataway, NJ). Cloning of GST-*Xenopus* importin beta was described in (Delmar *et al.*, 2008). 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. To obtain untagged importin beta and transportin, the 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_2HPO_4, 0.24g/L KH_2PO_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 GST-tagged and 6xHis-tagged transportin behaves indistinguishably from untagged transportin.

6xHis- and GST-tagged RanQ69L-GTP were expressed, purified, and loaded with GTP as previously described (Harel *et al.*, 2003a). GST-ELYS fragments were expressed and purified as previously described (Rasala *et al.*, 2008).

Antibodies used in this study included rabbit anti-gp210 (Harel *et al.*, 2003a), anti-hNup133 and anti-xNup160 (Harel *et al.*, 2003b), anti-xNup43 (Orjalo *et al.*, 2006), anti-Nup93 (Miller and Forbes, 2000), anti-Nup53 and anti-xELYS (Rasala *et al.*, 2008), anti-Orc2 (generous gift from Dr. John Newport), anti-*Xenopus* importin beta (Rasala *et*

al., 2006), and mouse anti-human transportin and anti-Nup62 (BD Biosciences, San Jose, CA).

Membrane fusion and nuclear pore assembly assays

Xenopus high speed cytosol and membranes, both from interphase *Xenopus* eggs, and *Xenopus* sperm chromatin were prepared as described in (Harel *et al.*, 2003a). Membrane fusion and nuclear pore assembly assays were performed as described previously (Harel *et al.*, 2003a). Nuclear membrane assembly was analyzed (Figure 1A) using the fluorescent membrane dye 3,3-dihexyloxacarbocyanine iodide (DHCC) (Eastman Kodak, Rochester, NY) and visualized with an Axiovert 200M confocal microscope (Carl Zeiss) and a 63X objective. Images from the confocal microscope were recorded using a Coolsnap HQ (Photometrics, Tucson, AZ) camera and Metavue software (Molecular Devices Corporation, Downingtown, PA). Images were processed using ImageJ (available at http://rsb.info.nih.gov/ij/) and Adobe Photoshop. For dextran diffusion analysis of membrane integrity (Figure 1B), *in vitro* nuclear reconstitution reactions were carried out as above, except that at the beginning of the reaction additions were made as follows: GST, as a control (25 µM); GTPγS (2 mM); BAPTA (7.5 mM); RanQ69L-GTP (37.5 µM); GST-Trn (25 µM); or RanQ69L-GTP (37.5 µM) plus GST-Trn (25 µM). Assembly was allowed to proceed for 60 min, then WGA (100 µM; EY Laboratories, San Mateo, CA) was added for 10 min to further ensure a tight seal of any nuclear pores present (although nuclear pores are already expected to exclude 70 kD dextran). Reactions were stopped on ice, then rhodamine-labeled dextran (70 kD; 2.5 µg; Molecular Probes, Eugene, OR) was added to a 25 µl reaction, followed by 15 min

incubation on ice. Reactions were diluted 1:1 and fixed with egg lysis buffer containing 7.4% paraformaldehyde. Dextran exclusion was visualized with an Axioskop2 microscope (Carl Zeiss, Thornwood, NY) at a magnification of 100X using an oil objective (Carl Zeiss). Nuclear pore assembly (Figure 2) was visualized with the same microscope and a 63X objective as determined by staining for FG nucleoporins with mAb414 antibody (Covance Inc., Berkeley, CA) which was directly labeled with Alexa 488 (Invitrogen, Eugene, OR) per manufacturer protocol. BAPTA [1,2-bis(2-aminophenoxy)ethane-*N,N,N,N*-tetraacetic acid] (EMD Chemicals, Inc., Gibbstown, NJ) was added at 8 μM.

Annulate lamellae assembly

Xenopus cytosol (40 μL), membranes (5 μL), glycogen (5 μL), and the indicated amount of recombinant proteins were incubated together for 90 minutes at room temperature. Ten microliters of the reaction were diluted in 190 μL ELB (10mM HEPES, pH 7.6, 50 mM KCl, 2.5 mM $MgCl_2$) and overlaid onto a 300 μL sucrose cushion (0.5 M) in ELB. The samples were spun at 25,000 rpm for 20 minutes at 2°C in a TL-100 tabletop ultracentrifuge (Beckman Coulter Inc., Fullerton, CA). The membrane pellet was collected and rinsed with ELB, and resuspended in 100 μL 1x SDS-PAGE sample loading buffer. One tenth of the volume was loaded for SDS-PAGE and processed for immunoblotting.

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 20 mg/mL Bovine Serum Albumin (BSA) in PBS for 30 minutes,

Xenopus egg cytosol was spun for an additional 150,000 rpm for 10 minutes at 4°C to

remove residual membrane contamination, then added to the beads and incubated for 2

hours at 4°C (25 μL cytosol in 500 μL PBS). After washing the beads with PBS, the

bound proteins were eluted with 0.1 M Glycine, pH 2.5, and neutralized with 1 M Tris,

pH 8.0. One fifth of each reaction was loaded for SDS-PAGE and processed for

immunoblotting.

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 800 μL ELB,

overlaid on a 300 μL 25% 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

immunofluoresence microscopy. Nuclei were visualized with an Axioskop 2 microscope

(63X objective; Carl Zeiss, Thornwood, NY).

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 microliters of heat-

inactivated cytosol, which contains primarily the protein nucleoplasmin, 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 300 μL ELB (10 mM HEPES, pH 7.6, 50 mM KCl, 2.5 mM 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 20 μM *Xenopus* importin beta, 20 μM human transportin, or 30 μM RanQ69L-GTP recombinant protein for 20 minutes at room temperature. After three washes in ELBK (10 mM HEPES, pH 7.6, 100 mM KCl, 2.5 mM MgCl$_2$) the chromatin was lysed in 30 μL 1x SDS-PAGE sample loading buffer, and the bound proteins were subjected to SDS-PAGE and immunoblotting.

ELYS direct binding assay

To assess direct binding, 1.0 μM untagged *Xenopus* importin beta or human transportin was incubated with 1 μM GST, GST-RanQ69L, GST-ELYS AT-hook+, GST-ELYS ΔAT-hook, or GST-ELYS short AT-hook+ in 1x binding buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 0.2 mM EDTA, 1 mM DTT, 0.2% BSA, 1 μg/ml μg/ml pepstatin, 1 μg/ml leupeptin, 1 μg/ml protease inhibitor TPCK, 1 mM benzamidine, 1 mM PMSF) with or without 5 μM RanQ69L-GTP on ice for 1 hour. Meanwhile, glutathione-sepharose beads (GE Healthcare, Piscataway, NJ) were washed and blocked with 20 mg/mL BSA (Sigma-Aldrich, St. Louis, MO) for 30 minutes at room temperature in 1x binding buffer. Ten microliters of the glutathione beads were added to the recombinant

proteins in each reaction and incubated for additional 30 minutes at 4°C in a total volume

of 100 μL, before washing 3X with 1X binding buffer (without BSA) and 2X with PBS.

The bound proteins were eluted with 27 μL 0.1 M glycine, pH 2.5, followed by

neutralization with 3 μl 1 M Tris, pH 8.0, and addition to 10 μl 4X SDS-PAGE sample

loading buffer. Samples were boiled and loaded onto 12% SDS-PAGE gels, which were

silver stained, stained with Coomassie blue, or transferred to PVDF for immunoblotting.

Spindle assembly experiments

Frogs for interphase extract preparation were induced to ovulate with injection of

500 U of human chorionic gonadotropin (Sigma-Aldrich, St-Louis, MO), and put into

individual buckets containing 100 mM NaCl for 18 hr. The frogs laid eggs overnight at

18°C and the eggs were collected the next morning. After removing most of the NaCl

solution from the best batches, eggs were dejellied with a 2% cysteine solution, pH 7.7.

Activated and dead eggs were constantly removed using a transfer pipette (Fisher

Scientific, Pittsburg, PA). When dejellied, eggs were washed extensively 3 times with

XB buffer (100 mM KCl, 0.1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, 50 mM sucrose,

pH 7.7). Using a cut transfer pipette, eggs were transferred in 2 ml polypropylene tubes

(Beckman) and crushed by centrifugation using a TL55S rotor in a Beckman TL100

ultracentrifuge for 15 minutes at 15,000 rpm. The cytoplasmic layer was removed with a

18-guage needle on a 3ml syringe, gently drawn out and supplemented with cytochalasin

B (50μg/ml) and a protease inhibitor cocktail (10 μg/ml aprotinin, 10 μg/ml leupeptin).

The cytoplasmic extract was then centrifuged a second time for 15 minutes at 15,000

rpm. The light layer was recovered and used as the interphase extract.

The mitotic extract (cytostatic factor or CSF extract) was made exactly as the interphase extract, except for the use of the buffer CSF-XB (100 mM KCl, 0.1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, 50 mM Sucrose, 7 mM EGTA, pH 7.7) instead of XB.

For spindle assembly, ~6.5 x 10^4 sperm chromatin DNA (3000 SpC/μl) were added to 20 μl of interphase extract, as well as ATP energy mix (10 mM phosphocreatine, 80 μg/ml creatine kinase, 1 mM ATP, 2 mM $MgCl_2$, 100 μM EGTA). At t=90' after the DNA addition, the extract now containing assembled nuclei was supplemented with rhodamine-tubulin (final concentration 20 μg/ml) and 20 μl of mitotic extract to induce entry into mitosis. Where indicated, GST-transportin was added to a final concentration of 20 μM and RanQ69L-GTP to a final concentration of 5 μM. At t=15' or 60' after mitotic extract addition, aliquots were withdrawn and fixed with 1 μl of fixation buffer (48% glycerol, 11% formaldehyde, 10 mM HEPES, pH=7.5) supplemented with 5μg/ml Hoechst DNA dye. Spindle formation was monitored using a Zeiss Axioskop 2 microscope using a 63X objective.

RESULTS

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

Importin beta has been shown to act as a negative regulator of both nuclear membrane and nuclear pore assembly (Harel *et al*., 2003a; Walther *et al*., 2003b; Delmar *et al*., 2008). We asked whether another nuclear import receptor, transportin, could

regulate nuclear envelope assembly in the *Xenopus laevis in vitro* nuclear assembly system. In a control reaction, when chromatin was mixed with *Xenopus* egg cytosol and membranes, completely fused nuclear membranes were observed to encircle the chromatin by 60 minutes, as indicated by a smooth membrane stain surrounding each nucleus (Figure 1A, Control). The addition of GTPγS, a known inhibitor of vesicle-vesicle fusion, inhibited nuclear membrane formation as evidenced by the discontinuous membrane profile, indicative of unfused membrane vesicles (Figure 1A, GTPγS; (Newport and Dunphy, 1992; Macaulay and Forbes, 1996). Importin beta gave, as expected, a similarly discontinuous membrane profile around the chromatin (data not shown; (Harel *et al*., 2003a; Delmar *et al*., 2008). When excess transportin was added, unfused membranes were also observed (Figure 1A, 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 (Hughes *et al*., 1998)] (Figure 1A, compare Trn+Ran with Trn), as previously seen with importin beta (Harel *et al*., 2003a; Delmar *et al*., 2008). We also measured membrane fusion (or lack thereof) by determining whether the structures formed around chromatin after 60 minutes of reconstitution were able to exclude 70 kD rhodamine-labeled dextran. 70 kD dextran is known to be unable to diffuse through nuclear pores in completely formed nuclear membranes, but can diffuse into nuclei if there are gaps in the nuclear membranes or if nuclear membrane assembly never proceeds beyond the vesicle binding stage. We found that when either transportin or GTPγS was added, then exclusion of rhodamine 70 kD dextran was not observed (Figure 1B, red). Observation of a discontinuous nuclear rim by differential interference contrast (DIC) microscopy confirmed this lack of fusion when

transportin or GTPγS were added (Figure 1B, gray). When Ran was added with

transportin, dextran exclusion and a continuous rim were now observed. Taken together,

these results showing a discontinuous rim by DIC microscopy (Figure 1B) and by DHCC

membrane labeling (Figure 1A), as well as an absence of dextran exclusion (Figure 1B,

red) all indicated that an intact nuclear membrane does not form in the presence of excess

transportin. We conclude that transportin negatively regulates nuclear membrane fusion

and does so in a Ran-reversible manner.

Transportin negatively regulates nuclear pore assembly.

To determine whether transportin separately blocks mature nuclear pore

assembly, we formed nuclear intermediates that contained complete nuclear membranes,

but which lacked nuclear pores. This was done by performing nuclear assembly in the

presence of the calcium chelator BAPTA (Macaulay and Forbes, 1996; Harel *et al.*,

2003a; Delmar *et al.*, 2008). BAPTA nuclear intermediates contain a fused nuclear

envelope, but no mature nuclear pores, as previously shown by electron microscopy and a

lack of detectable FG nucleoporins by immunofluoresence with mAb414 (Macaulay and

Forbes, 1996; Harel *et al.*, 2003a; Delmar *et al.*, 2008). When such BAPTA nuclear

intermediates are diluted 1:10 into fresh cytosol lacking BAPTA, nuclear pore assembly

quickly ensues (Macaulay and Forbes, 1996; Harel *et al.*, 2003a; Delmar *et al.*, 2008).

This rescue was typically apparent from the acquisition of FG-nucleoporin staining by the

rescued nuclei as shown in Figure 2A (+Buffer). If the added *Xenopus* cytosol instead

contained a second addition of BAPTA, rescue was not observed (Figure 2A, +BAPTA).

However, if BAPTA nuclei were diluted into cytosol containing excess transportin at

either 10 or 20 μM concentrations, the incorporation of FG-nucleoporins was substantially reduced by 10 μM transportin (Supplemental Figure 1) and was completely blocked by 20 μM transportin (Figure 2A, +h-Trn; Supplemental Figure 1). We found that the ability of transportin to block nuclear pore assembly was partially reversed by RanQ69L-GTP: FG-staining pores were now observed, albeit fewer in number (Figure 2A, +h-Trn+Ran; see also high magnification insert) than in the control (+buffer). This partial rescue of pore assembly by Ran-Q69L was also observed when importin β was the inhibitory agent (Figure 2A, +x-beta +Ran). When RanQ69L-GTP was added alone, it did not have any negative effect on nuclear pore assembly (Figure 2A, +Ran). To quantitate these effects, seventy-five nuclei for each condition in each of three different experiments were counted for a strong FG nucleoporin rim, a partial FG rim, or the absence of FG staining. The quantitation, as shown in Figure 2B, clearly confirms that transportin blocks nuclear pore assembly and that this block is partially reversed by Ran-GTP.

Annulate lamellae assembly is negatively regulated by transportin.

Annulate lamellae (AL) are stacked membranes within the ER membrane network that contain cytoplasmic mimics of intact nuclear pore complexes (Merisko, 1989; Dabauvalle *et al*., 1991; Meier *et al*., 1995). In most ways these AL pores are identical to nuclear pores, but they are assembled differently in that they do not require the pore-targeting protein ELYS or chromatin in order to form (Kessel, 1989; Merisko, 1989; Dabauvalle *et al*., 1991; Meier *et al*., 1995; Miller and Forbes, 2000; Rasala *et al*., 2008). A previous study found that 10 μM transportin did not block AL pore assembly (Walther

et al., 2003b). However, in that electron microscopic experiment, transportin was only tested in the presence of 5 μM RanQ69L-GTP, and thus was likely lower in effective concentration. We observed in our experiments above that 20 μM transportin was required to completely block *nuclear* pore assembly. [We note that our assembly extracts are ~twice as concentrated as those used in (Walther *et al*., 2003b).] We next set out to ask whether transportin (20 μM, with no added RanQ69L-GTP) could block in vitro *AL* pore assembly, as determined by immunoblotting of membrane pellets for nucleoporins. To form AL, *Xenopus* egg cytosol was mixed with *Xenopus* membranes in the absence of chromatin and incubated for 90 minutes. The AL were isolated by centrifugation and subjected to immunoblot analysis, and the incorporation of multiple key nucleoporins into AL was assessed. When AL were formed under control conditions, all the individual nucleoporins tested including Nup133, Nup43, Nup93, and Nup53 were detected as present, indicating the formation of AL pores (Figure 3C, lane 1). When we added 20 μM importin beta to an AL assembly reaction, as expected from previous work, it blocked the incorporation of these soluble nucleoporins into membranes (Figure 3C, lane 2). Addition of 20 μM transportin also clearly blocked AL assembly (Figure 3C, lane 4). Indeed, importin beta and transportin inhibited the incorporation of all tested nucleoporins, which in addition to the ones shown in Figure 3C, included Nup214, Nup155, Nup153, Nup98, and Nup62 (data not shown). The block to AL formation by importin beta and transportin was significantly reversed by the addition of RanQ69L-GTP (Figure 3C, lanes 3 and 5). These results demonstrate that transportin is mirroring its affect on nuclear pore assembly and negatively regulating AL pore assembly.

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

We next wished to determine the nucleoporin subunit targets of transportin and importin beta regulation of pore assembly. During import in intact cells, both karyopherins are known to bind to FG-containing nucleoporins that are essential for nucleocytoplasmic transport (Madrid and Weis, 2006; Terry *et al.*, 2007; Lim *et al.*, 2008). One hypothesis would be that transportin and importin beta regulate pore assembly by sequestering the essential FG-nucleoporins, except in the region of the chromosomes where RanGTP is high. A broader hypothesis might predict that *all* nucleoporin subunits are targets of regulation. Lastly, it is conceivable that transportin and importin beta might have overlapping and non-overlapping nucleoporin targets that can be regulated differentially.

To address the potential nucleoporin subunit targets of receptor inhibition in the *Xenopus* system in a comprehensive manner, we performed GST pull-downs from *Xenopus* cytosol using GST, GST-importin beta, or GST-transportin as bait. We probed for the different pore subunits by immunoblotting using antibodies to 12 of the 14 soluble nuclear pore complex subunits with the exception of Aladin and Tpr (Rasala *et al.*, 2008). We found that both importin beta and transportin clearly interacted with the FG-nucleoporins Nup358, Nup214, Nup153, Nup98, Nup62, and Nup50 (Figure 3A, lanes 3 and 4; Figure 3B, lane 4), as expected (Moroianu *et al.*, 1995; Shah and Forbes, 1998; Shah *et al.*, 1998; Yaseen and Blobel, 1999; Fontoura *et al.*, 2000; Ben-Efraim and Gerace, 2001; Lindsay *et al.*, 2002). Nup53, which contains a low number of FGs (Marelli *et al.*, 1998; Devos *et al.*, 2006) also associated with importin beta and transportin (Figure 3A, lanes 3 and 4).

The major subunit of the nuclear pore, the Nup107-160 complex, exists in two complexes, one containing 9 core nucleoporins (Nup107, Nup160, Nup133, Nup96, Nup85, Nup43, Nup37, Sec13, and Seh1) and a second complex that also contains the pore-targeting nucleoporin ELYS/MEL-28. A previous study has shown that an ELYS/Nup107-160 complex immunoprecipitated by anti-ELYS antibody from human cell lysates does not contain importin beta (Rasala *et al*., 2006). In contrast, immunoprecipitates of ELYS/Nup107-160 complex from *Xenopus* egg extracts with anti-ELYS antibodies contain importin beta [(Franz *et al*., 2007) and our unpublished observations]. When GST-importin beta was used here to perform a pull-down assay from *Xenopus* egg cytosol, all of the tested members of the Nup107-160 complex were observed (Figure 3A, lane 3). The complex was also observed in GST-transportin pull-downs (Figure 3A, lane 4; Figure 3B, lane 4). Strikingly, the two newly discovered Nup107-160 complex-associated proteins, ELYS and centrin (Rasala *et al*., 2006; Franz *et al*., 2007; Gillespie *et al*., 2007; Resendes *et al*., 2008), were also found to bind to both GST-importin beta and GST-transportin pulldowns (Figure 3A, lanes 3 and 4).

Not all nucleoporin subunits were found to bind to importin beta and transportin: members of the key Nup93/188/205 scaffold subunit (Grandi *et al*., 1997; Hawryluk-Gara *et al*., 2005), as well as another subunit of the pore scaffold, Nup155 (Aitchison *et al*., 1995; Franz *et al*., 2005), did not show interaction with importin beta or transportin (Figure 3A, lanes 3 and 4).

The above results show that exogenously added importin beta and transportin can bind to a specific and similar subset of nucleoporin complexes in *Xenopus* egg cytosol. To ask whether importin beta or transportin is found naturally in endogenous nucleoporin

subcomplexes, we performed immunoprecipitation with several different anti-Nup antibodies and probed for the presence of importin beta and transportin. We found that endogenous importin beta and transportin were each present in immunoprecipitates with anti-Nup62, anti-Nup50, and anti-Nup98 antibodies (Supplemental Figure 2A-B). Significantly, these nucleoporins are all known to be present in *Xenopus* extracts in separate Nup subcomplexes (see, for example, Rasala *et al.*, 2008). We conclude from this sampling of subcomplexes that importin beta and transportin are clearly present in endogenous Nup subcomplexes *in vivo*.

We looked further at the effect of Ran-GTP on the nucleoporin targets of transportin. The interaction between GST-transportin and the large Nup107-160 complex (Nup160, Nup133, and Nup43) was abolished by the presence of RanQ69L-GTP (Figure 3B, lane 5). Similarly, the binding of transportin to the FG-nucleoporins Nup214 and Nup98 was also prevented by the presence of RanQ69L-GTP (Figure 3B, lane 5), and 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 increased in the presence of RanQ69L-GTP (Figure 3B, lane 5). Interestingly, these latter three proteins are unique among all nucleoporins in that they have the ability to bind RanGTP on their own (Wu *et al.*, 1995; Yokoyama *et al.*, 1995; Saitoh *et al.*, 1996; Nakielny *et al.*, 1999; Lindsay *et al.*, 2002).

Together, the above data demonstrate that importin beta and transportin interact with a near identical and specific subset of nucleoporins. This suggests a model where importin beta and transportin regulate nuclear pore assembly by a similar mechanism. It is interesting that neither importin beta nor transportin interact with the Nup93/188/205

subcomplex or with Nup155. We conclude that the recruitment of these latter

nucleoporins into the forming nuclear pore is not likely to be regulated by importin beta

or transportin. Thus, it appears that transportin and importin beta regulate not all, but a

subset, of nucleoporins and that this subset is substantially similar between the two

karyopherins.

Transportin and importin beta 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/MEL-28 to AT-rich regions of chromatin.

Importantly, this recruitment can occur either in the absence or presence of the Nup107-

160 complex in *Xenopus* egg extracts (Rasala *et al*., 2006; Franz *et al*., 2007; Gillespie *et

al*., 2007; Rasala *et al*., 2008). However, the Nup107/160 complex cannot bind to

chromatin in the absence of ELYS (Franz *et al*., 2007). ELYS thus initiates nuclear pore

assembly. Since we have found that both transportin and importin beta interact with

ELYS and the Nup107-160 complex (Figure 3A), it is feasible that these transport

receptors *regulate* the initial step of nuclear pore assembly. We tested this hypothesis

using a chromatin-binding assay. For this assay, *Xenopus* cytosol and sperm chromatin

were mixed in solution with or without the recombinant receptor proteins and incubated

for 20 minutes at room temperature. The chromatin templates were centrifuged onto

polylysine-coated coverslips and processed for immunofluorescence. In the control,

where cytosol was added to chromatin without any added importin beta or transportin, the

chromatin binding proteins ELYS and Orc2 both bound to the chromatin (Figure 4A,

+Buffer). In contrast, ELYS no longer associated with chromatin if excess importin beta was present in the reaction (Figure 4A, +beta). ELYS was also greatly reduced on chromatin in the presence of transportin (Figure 4A, +Trn). The binding of Orc2 to chromatin was unaffected by either of these karyopherins (Figure 4A, + beta; +Trn). The inhibition of ELYS binding to chromatin by transportin was substantially prevented if recombinant RanQ69L-GTP was included (Figure 4A, +Trn+Ran). A similar effect was observed with the simultaneous addition of importin beta and Ran-GTP (Figure 4A, +beta+Ran). These effects on ELYS binding were quantitated in four different experiments and are shown in Figure 4B. We conclude that importin beta and transportin negatively regulate the binding of ELYS to chromatin, as judged by this immunofluorescence assay.

The binding of ELYS to chromatin was also tested biochemically, using instead an anchored chromatin assay. In this assay, decondensed sperm chromatin was bound to poly-L-lysine coverslips, then incubated with *Xenopus* cytosol. The unbound cytosolic proteins were washed away, while the chromatin-bound proteins were collected in SDS sample buffer, subjected to SDS-PAGE, and tested for the presence of ELYS and the Nup107-160 complex by immunoblotting. In the absence of any added recombinant receptor proteins, ELYS and members of the Nup107-160 complex bound to chromatin, as expected (Figure 5, lane 5; ELYS, Nup160, Nup133, Nup43). We have previously shown that AT-rich DNA sequences capture ELYS (Rasala *et al*., 2006; Franz *et al*., 2007; Gillespie *et al*., 2007; Rasala *et al*., 2008). ELYS then recruits the Nup107-160 complex (Rasala *et al*., 2006; Franz *et al*., 2007; Gillespie *et al*., 2007; Rasala *et al*., 2008). We found that association of the Nup107-160 complex with chromatin was greatly

reduced in the presence of importin beta (Figure 5, lane 6) or transportin (Figure 5, lane 9). The Orc2 signal present on the chromatin remained unchanged whether or not importin beta or transportin were added (Figure 5, compare lanes 6 and 9 to lane 5). The block of ELYS and Nup107-160 complex binding to chromatin could, in large part, be prevented by the addition of RanQ69L-GTP (Figure 5, lanes 7 and 10). Taken together, the data indicate that importin beta and transportin can negatively regulate the recruitment of the pore targeting protein ELYS to chromatin and do so in a Ran-regulated manner.

Transportin and importin beta bind to ELYS directly

As shown above, transportin and importin beta negatively regulate nuclear pore assembly, prevent ELYS from binding to chromatin, and interact with ELYS in pull-down assays from *Xenopus* egg cytosol. To determine whether importin beta or transportin can bind to ELYS directly, we performed a direct binding assay using different regions of the C-terminus of ELYS which are known to associate with chromatin (Rasala *et al*., 2008). The ELYS fragments used were GST-tagged versions of ELYS AT-hook+ (aa 2281-2408), ELYS ΔAT-hook (aa 2359-2408), and ELYS-short AT-hook+ (aa 2281-2359) (Figure 6A). All three fragments bind to chromatin, although GST-ELYS AT-hook+ binds somewhat more strongly, as it possesses two chromatin binding domains, [(Rasala *et al*., 2008); and data not shown)]. The GST-tagged versions of each of these ELYS fragments were incubated with untagged importin beta or untagged transportin in the absence of any cytosolic proteins. The bound fraction was analyzed by silver stain to detect importin beta (Figure 6B). GST-RanQ69L-GTP, a

positive control, clearly bound importin beta (Figure 6B, lane 1). GST alone, a negative

control, did not bind importin beta (Figure 6B, lane 2). We found that both ELYS AT-

hook+ and ELYS ΔAT-hook directly bound importin beta (Figure 6B, lanes 3 and 4,

respectively). However, ELYS-short AT-hook+ did not bind to importin beta (Figure 6B,

lane 5).

We further found that both ELYS AT-hook+ and ELYS ΔAT-hook bound to

transportin directly (Figure 6C, lanes 3 and 4), while GST-ELYS-short AT-hook+ did not

bind transportin (Figure 6C, lane 5), as determined by immunoblotting. RanGTP

substantially prevented the binding of transportin to ELYS AT-hook+ and ELYS ΔAT-

hook (Figure 6E, lanes 8 and 6, respectively), a significant reversal also observed with

importin beta and Ran (Figure 6D, lanes 4 and 6). These data indicate that transportin

and importin beta can bind directly to the extreme C-terminus of ELYS in a region that

does not include the AT-hook.

Transportin blocks spindle assembly in a Ran-sensitive manner.

Importin beta has been shown in multiple studies using mitotic *Xenopus* egg

extracts to regulate spindle assembly. Mitotic spindles readily assemble *in vitro* around

chromatin added to such an extract (see Kaleb and Heald, 2008; Clarke and Zhang, 2008

for review). It has been proposed that the location of spindle assembly specifically in the

area of mitotic chromosomes in cells is orchestrated by importin beta and its adaptor

protein importin alpha, which act to inhibit spindle assembly factors elsewhere (Clarke

and Zhang, 2008; Kalab and Heald, 2008). RanGTP, produced by its chromatin-bound

RanGEF, releases spindle assembly factors from inhibition in the vicinity of

chromosomes; thus Ran acts as a positive regulator of spindle assembly and importin alpha and/or beta act as negative regulators. When excess exogenous Ran is added to *Xenopus* mitotic cytosol, abundant asters then are induced to form throughout the cytosol, even in the complete absence of chromatin (Carazo-Salas *et al.*, 1999; Kalab *et al.*, 1999; Ohba *et al.*, 1999; Wilde and Zheng, 1999).

Here we asked whether the import receptor *transportin* would show a similar ability to regulate spindle assembly. We reasoned that this could very well be the case since, like importin beta, transportin negatively inhibits both nuclear membrane assembly and nuclear pore assembly (Figures 1-5).

To assess effects of transportin on mitotic spindle assembly, nuclei were assembled by adding sperm chromatin to a *Xenopus* interphase egg extract (20 μl) and allowing them to assemble into nuclei for 90 minutes (Figure 7A, top left panel, 0'). During this time, it has been demonstrated that the DNA present faithfully replicates (Newport, 1987; Tutter and Walter, 2006). Mitotic egg extract (20 μl) was then added to convert the reaction to a mitotic state. Rhodamine-labeled tubulin was simultaneously added to allow the monitoring of mitotic spindle (or aster) assembly. Transportin (20 μM) was added with or without RanQ69L-GTP (5 μM) in parallel reactions to test for effects on spindle assembly. At 15 minutes after the addition of mitotic cytosol, essentially all of nuclei in the reactions had been converted to condensed chromatin figures which had an attached rhodamine-labeled half spindle, as is typical of this time point in spindle assembly assays in *Xenopus* mitotic extracts (Mitchison *et al.*, 2004; Maresca and Heald, 2006; Orjalo *et al.*, 2006) (Figure 7A, 15'). We noted that when transportin was present the half spindles appeared somewhat smaller. At 30 minutes,

small bipolar spindles were beginning to form in both the control and transportin

reactions (data not shown). By 60 minutes, the control showed abundant robust bipolar

spindles organized around condensed chromatin (66%) (Figure 7A, 60'). However, the

chromatin in reactions with added transportin were for the most part now devoid of

associated microtubules (89%) (Figure 7A, 60'). In contrast, when RanQ69L-GTP was

included with the transportin, robust (40%) and weak (22%) bipolar spindles formed

(Figure 7A, 60'). Addition of RanGTP alone caused large numbers of asters to form

independent of chromatin (52%) as well as the formation of multipolar spindles in the

vicinity of chromatin (38%).   The data from three such experiments has been quantitated

in Figure 7B. The 60' Control results are shown in blue, transportin results are in red, and

transportin plus RanGTP results shown in yellow. We conclude that transportin, like

importin beta, is an inhibitor of mitotic spindle assembly *in vitro* and this inhibition can

be counteracted by RanGTP.

DISCUSSION

Mitosis is a complex step-wise process that requires regulation at multiple points. Here, we show that transportin is a negative regulator of spindle assembly, nuclear membrane formation, and nuclear pore assembly (Figures 1, 2, and 7). Moreover, transportin also negatively regulates assembly of the cytoplasmic mimic of nuclear pores, annulate lamellae (Figure 3C). The nucleoporin targets of transportin and importin beta involve many, but not all nucleoporins. The targets include the FG-nucleoporins and the large critical Nup107-160 complex, but do not include the Nup93-188-205 or Nup155 pore scaffold subunits (Figure 3, A and B). These data imply that the recruitment of certain nucleoporins is regulated by transportin and importin beta, but that the recruitment of other Nups is not. When tested, RanGTP reversed the binding of transportin to key Nup subunits (Figure 3 A and B; Figure 6), consistent with the model that RanGTP promotes pore assembly by releasing nucleoporins from karyopherin sequestration (Harel *et al.*, 2003a; Hetzer *et al.*, 2005; Antonin *et al.*, 2008; Delmar *et al.*, 2008; Lim *et al.*, 2008).

We provide evidence for the first time that both transportin and importin beta can negatively regulate the earliest known step in the initiation of pore assembly, the recruitment to chromatin of the pore-targeting protein ELYS/MEL-28 (Figures 4 and 5). We further find that transportin and importin beta can bind directly to the C-terminus of ELYS in a chromatin-binding region that is downstream from the ELYS AT-hook (Figure 6A, CBD2). Prevention of ELYS from binding to chromatin has been previously shown to block the recruitment of the Nup107-160 complex to chromatin and to prevent all further pore assembly (Rasala *et al.*, 2006; Franz *et al.*, 2007; Gillespie *et al.*, 2007;

Rasala *et al.*, 2008). Thus, negative regulation of ELYS/chromatin binding downregulates nuclear pore assembly (Figure 8). We hypothesize that transportin and importin beta act in a multiplex manner by also binding to nucleoporin targets other than ELYS, i.e., the FG-Nups and the Nup107-160 complex (Figure 3, A and B), in order to ensure that neither pore assembly nor assembly of abortive nucleoporin aggregates occur in inappropriate regions of the cell. These inappropriate regions would include intranuclear annulate lamellae, cytoplasmic annulate lamellae, and smaller soluble aggregates of Nups in the cytoplasm. RanGTP appears to play a counteracting role to the karyopherin regulators in both the initial and downstream steps of pore assembly.

Dueling regulators

Ran reversal of importin beta inhibition is, for the most part, a hallmark of the studies of importin beta regulation to date. When tagged human importin beta was shown to block mitotic spindle assembly and nuclear membrane assembly in *Xenopus* extracts, both processes were reversed by RanGTP (Nachury *et al.*, 2001; Wiese *et al.*, 2001; Harel *et al.*, 2003a; Ems-McClung *et al.*, 2004; Blower *et al.*, 2005; Albee *et al.*, 2006; Ribbeck *et al.*, 2006; Tahara *et al.*, 2008). An exception to this was the inhibition of nuclear pore assembly by tagged human importin beta, where RanGTP did not reverse the inhibition (Harel *et al.*, 2003a). It was later found, however, that when an untagged version of either human or *Xenopus* importin beta was used to inhibit nuclear pore assembly, the inhibition was RanGTP-reversible (Delmar *et al.*, 2008). In the present study, we used untagged human transportin, but found that its block to pore assembly was only partially reversed by excess RanGTP. One possibility is that human transportin may

be less sensitive to RanGTP than importin beta is, specifically in the area of nuclear pore

assembly. An alternate explanation is that there may be a separate effector of transportin

in addition to the GTPase Ran involved in pore assembly. Potentially consistent with

this, we did observe that although RanGTP was able to release transportin from certain

nucleoporins (the FG nucleoporins Nup98 and Nup214 and the Nup107-160 complex),

RanGTP left intact transportin's binding to the FG nucleoporins Nup358, Nup153, and

Nup50 (Figure 3B), perhaps arguing for the existence of a second positive regulator for

transportin. It is known that these latter proteins are unique among nucleoporins in that

they contain known RanGTP-binding domains (Wu *et al*., 1995; Yokoyama *et al*., 1995;

Saitoh *et al*., 1996; Nakielny *et al*., 1999; Lindsay *et al*., 2002). It is possible that their

regulation by transportin is reversed via a different mechanism.

Multiple points of regulation in pore assembly

In mammalian cells, the order of assembly of the Nups has been very broadly

categorized using GFP-tagged nucleoporins into early, intermediate, and late-assembling

nucleoporins (Bodoor *et al*., 1999; Dultz *et al*., 2008). Assuming this order is followed in

Xenopus cells as seems likely (Walther *et al*., 2001; Harel *et al*., 2003b; Walther *et al*.,

2003b; Rasala *et al*., 2006; Rasala *et al*., 2008), we conclude that transportin and importin

beta bind to nucleoporin subunit targets in each of the temporal stages of pore assembly.

Transportin and importin beta bind to ELYS and the Nup107-160 complex which

assemble early, Nup98 which has an intermediate assembly time (Dultz *et al*., 2008), and

Nup50 and Nup214 which assemble late, as well as Nup153 which has both early and late

assembling populations (Bodoor *et al*., 1999; Dultz *et al*., 2008). Thus, it appears that

there could be sequential points of regulation by transportin and importin beta during nuclear pore assembly.

Multiple regulators

Why is more than one nuclear import receptor used to regulate nuclear assembly? One explanation would be that, although transportin and importin beta bind to a similar subset of nucleoporins, they may bind with different affinities. The nucleoporins could then be differentially regulated in a more controlled manner. Another possibility is that the availability or concentration of importin beta accessible for regulation *in vivo* might be quite different than that of transportin, providing another mechanism for fine tuning the regulation of steps in nuclear assembly. Lastly, we do not exclude the possibility that additional import receptors may regulate nuclear assembly in a similar way.

Regulation of the initial step in nuclear pore assembly

The binding of ELYS to chromatin is the initial step in pore assembly. We show this to be regulated by both transportin and importin beta (Figures 4 and 5). In the absence of the recruitment of ELYS to chromatin, pore complexes no longer form in the nuclear envelope, but instead form as annulate lamellae pores in the cytoplasm (Rasala *et al.*, 2006; Franz *et al.*, 2007; Rasala *et al.*, 2008). We would predict that if an excess of ELYS were allowed to bind to chromatin, then excessive nuclear pores would form which would lead to defects in nuclear assembly including, for example, aberrant assembly of intranuclear annulate lamellae as previously observed in the presence of

excess Ran (Harel *et al.*, 2003a), and defects in pore spacing or nuclear lamina formation. A disproportion of nuclear pore numbers could also adversely affect the novel functions recently recognized to be mediated by the nuclear pore complex, such as transcriptional activation of certain genes (Casolari *et al.*, 2004; Cabal *et al.*, 2006; Taddei *et al.*, 2006; Brown *et al.*, 2008) and DNA break repair (Nagai *et al.*, 2008). In consequence, we believe it is likely that pore number would be tightly regulated. Interestingly, most vertebrate cultured cell lines have a very similar number of nuclear pores (~2000-5000), while inactive B cells have many fewer nuclear pores (~400) (Maul, 1977). In reality, little is known of the mechanism of pore number regulation. Regulation of the targeting of ELYS to chromatin by transportin and importin beta could be one such mode of regulation.

Karyopherin binding sites on the C-terminus of ELYS

The C-terminal 50 aa of *Xenopus* ELYS binds both importin beta and transportin directly (aa 2359-2408, Figure 6A, yellow/red). Sequence analysis reveals that the last 18 amino acids contain 10 positively charged residues in two clusters (RRTRRRIIAKPVTRRKMR$_{COOH}$) (Figure 6A, red). Importin beta is well known to bind to positively charged regions in proteins ranging from its adaptor, importin alpha, to cargoes that importin beta binds directly, such as HIV Rev and ribosomal proteins L23a and S7 (Gorlich and Kutay, 1999). Thus, each basic cluster may bind importin beta directly. The most recently reported transportin NLS consensus sequences contain a proline-tyrosine (PY) dipeptide preceded by various alternate upstream amino acid sequences, one of which contains 5-8 basic amino acids (basic-enriched$_{5-8}$-X$_{8-10}$-PY) (Lee

et al., 2006). Although this abundant class of transportin NLSs was pre-selected by computer analysis on the basis of containing a PY element, the protein HuR, a known transportin cargo, contains a PG in place of PY in its NLS (Lee *et al.*, 2006). Also, of two known cargoes for the *S. cerevisiae* transportin homologue kap104, Hrp1 contains a PY, while Nab2 has a PY-type NLS where a PL replaces the PY (aa 200-250). Each is essential for kap104 binding of these cargoes (Siomi *et al.*, 1998; Truant *et al.*, 1998; Lee and Aitchison, 1999; Marfatia *et al.*, 2003; Lange *et al.*, 2008). Here we found that the C-terminus of *Xenopus* ELYS directly binds transportin, even though it does not contain a PY; it does, however, contain a PV dipeptide which maps between the two basic clusters at the ELYS C-terminus (Figure 6A, red). It is possible that this PV serves in place of the PY. Indeed, the ELYS sequence RRTRRRIIAK**PV**TRRKMR$_{COOH}$ resembles somewhat the predicted transportin NLS of the human Metal Response Element-Binding Transcription Factor 2 (KKGKKKSVGRPPG**PY**TRKM) (Lee *et al.*, 2006).

For vertebrate ELYS, transportin and importin beta have both been shown to bind to the last 50 amino acids (Figure 6), although they may also bind elsewhere on ELYS. If it is found that they bind to the identical sequence or to adjacent sequences within the positively charged ELYS C-terminus, it may be that they contribute to the negative regulation of ELYS binding to chromatin in an interchangeable manner. Alternatively, one of the karyopherins may be present in greater abundance *in vivo* or be higher in its binding affinity, and thus act as the dominant regulator of that particular site. It is also highly possible that importin beta and transportin bind to additional regions of ELYS and that multiple binding events are required for controlling ELYS. In any case, their goal presumably would be to achieve negative regulation of ELYS *except* in areas of high

RanGTP, such as at the surface of chromatin.

Multiplex regulation of spindle assembly

Lastly, we demonstrated that transportin has the ability to negatively regulate an entirely different cell cycle structure, the mitotic spindle, and does so in a Ran-sensitive manner (Figure 7). Our data thus support the idea that, like importin beta, transportin has diverse functions during different parts of the cell cycle, namely as an import receptor during interphase and as a negative regulator of mitotic spindle and nuclear assembly during mitosis. Importin beta was demonstrated to regulate mitotic spindle assembly by binding and sequestering spindle assembly factors (SAFs) in areas away from the mitotic chromosomes (reviewed in Harel and Forbes, 2004; Funabiki, 2005; Kalab and Heald, 2008). RanGTP, produced on the mitotic chromosomes by the RanGEF RCC1, acts as a positive regulator for mitotic spindle formation by triggering dissociation of importin beta from its SAF binding partners (Kalab and Heald, 2008). An interesting question will be whether transportin binds to the same SAFs as importin alpha and beta or different ones. At present, at least ten SAFs have been identified that are direct targets of importin beta and/or alpha (including NuMA, TPX2, and the kinesin/MAP XCTK2) (Clarke and Zhang, 2008; Kalab and Heald, 2008). A cursory analysis of these SAFs reveals that several contain a PY dipeptide (D. Forbes, unpublished), but whether these are authentic transportin binding sites is not yet known. Significantly, the *S. cerevisiae* kap104 mutant kap104-E604K has been found to speed up mitosis *in vivo*, consistent with a negative regulatory role for transportin in mitosis (Asakawa and Toh-e, 2002). However, because yeast retain an intact nucleus during their closed mitosis, this has also been suggested to

result from a defect in the import role of kap104.

An interesting question is whether other karyopherins similarly sequester SAFs. At present it is known that the export karyopherin Crm1 acts during mitosis but in a different manner, targeting to kinetochores and aiding in the binding of a set of proteins important for kinetochore function (Arnaoutov *et al.*, 2005; Arnaoutov and Dasso, 2005; Knauer *et al.*, 2006). As export receptors are stabilized by Ran, one might expect that only import receptors could duel with RanGTP to regulate assembly of the large mitotic cellular structures around the chromatin. However, export receptors could also regulate these assembly events if they used an entirely different way to reverse target sequestration such as, for example, being dissociated by RanGAP.

Summary

In conclusion, we have demonstrated that transportin is a negative regulator of nuclear membrane formation and pore assembly in the *Xenopus in vitro* system, and that this regulatory complexity extends to spindle assembly. Focusing on nuclear pore assembly, we showed that both transportin and importin beta negatively regulate the initial step in pore assembly, ELYS binding to chromatin. RanGTP acts in an opposite manner from that of transportin to balance the effects of this new negative regulator. Both transportin and importin beta bind directly to the C-terminus of ELYS, a region critical for ELYS recruitment to chromatin. The precise choreography of regulation provided by the new regulatory karyopherin, transportin, reveals yet another level of complexity to the regulation of mitotic spindle and nuclear assembly.

ACKNOWLEDGEMENTS

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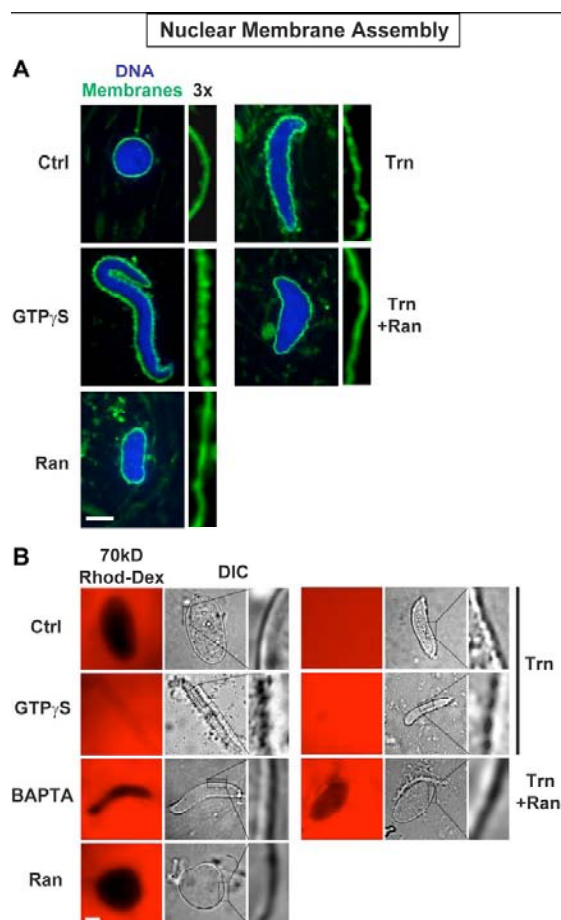
Figure 1.1: Excess transportin blocks nuclear membrane fusion in a Ran-regulated manner.

(A) Nuclear formation from *Xenopus* egg extract was performed at room temperature for one hour with the addition of PBS buffer (control), 2 mM GTPγS (GTPγS), 37.5 µM RanQ69L-GTP (Ran), 25 µM transportin (Trn), or 25 µM transportin and 37.5 µM RanQ69L-GTP (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. Discontinuities in the DHCC staining indicate regions with little or no membrane fusion. Representative images are shown. Bar: 10 µm.

(B) To determine membrane integrity, six *in vitro* nuclear reconstitution reactions were set up and supplemented with the following additions: GST as a control (25 µM); GTPγS (2mM); BAPTA (7.5 mM); RanQ69L-GTP (37.5 µM); GST-Trn (25 µM); or RanQ69L-GTP (37.5 µM) plus GST-Trn (25 µM). After 60 min of assembly, the nuclei were treated as described in the Materials and Methods. The entry or exclusion of rhodamine-labeled 70 kD dextran (70 kD Rhod-Dex) was visualized using fluorescence microscopy. The membrane integrity of the same nuclei was also assessed as visualized with differential interference contrast microscopy in the right panels (DIC). Bar: 10 µm.
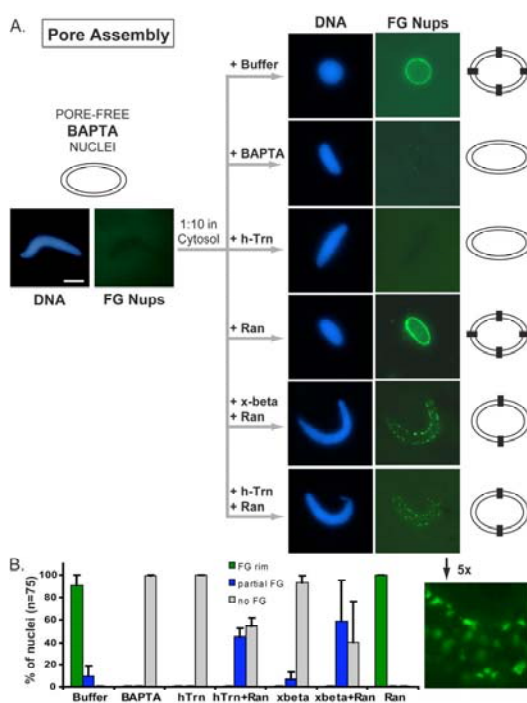
Figure 1.2: Excess transportin blocks nuclear pore assembly.
(A) Pore-free BAPTA nuclei intermediates were assembled by adding 8 μM BAPTA in
the nuclear formation reaction at t=0 minute. At t=60 minutes, BAPTA-arrested nuclei
were diluted 1:10 in fresh cytosol in the presence or absence of recombinant proteins. To
assess nuclear pore formation, Alexa-488 directly labeled antibody against FG
nucleoporins (mAb414) was added to the nuclei at t=90 minutes for an additional 20
minutes before visualization by fluorescence microscopy. As expected, no FG pore
staining was observed in the starting BAPTA nuclei (left panels). When the BAPTA
nuclei were then diluted 1:10 into fresh cytosol to which a small amount of control buffer
was added (+buffer), or to which 30 μM RanQ69L-GTP (+Ran) was added, nuclear pores
formed, as detected by anti-FG staining. If, however, BAPTA (8 μM) were included in
the fresh cytosol, pore assembly was prevented (+BAPTA). Strikingly, human
transportin (20 μM; +h-Trn) blocked nuclear pore formation, as did *Xenopus* importin
beta [20 μM; data not shown and (Delmar *et al.*, 2008)]. In contrast, significant, albeit
not full, FG staining was observed when 20 μM transportin was added in conjunction
with 30 μM RanQ69L-GTP (+h-Trn+Ran), or when 20 μM *Xenopus* importin beta was
added with 30 μM RanQ69L-GTP (+x-beta+Ran). A 5x magnification of a
representative Trn+Ran nucleus is shown, with the signal of the FG Nup staining
brightened to show detail. DNA was stained with DAPI (blue). Representative images
are shown. Bar: 10 μm. **(B)** Quantitation of data in (A). Seventy-five nuclei per
experiment were counted under each condition, and the percentage of nuclei that
contained strong, partial, or no FG-nucleoporin staining was plotted. Error bars represent
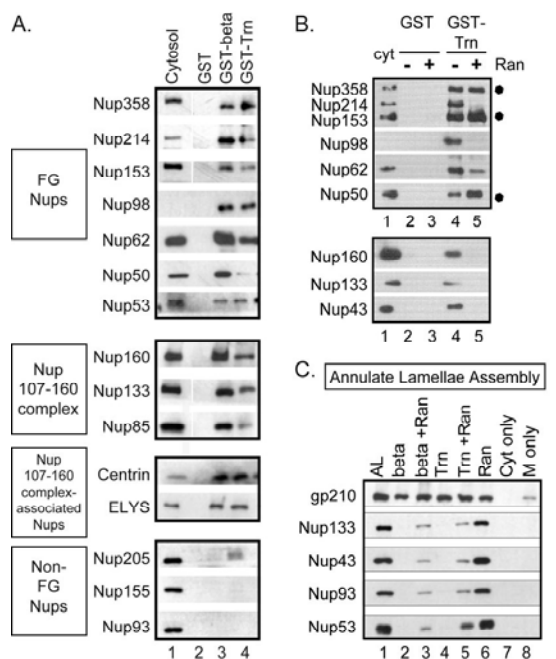standard deviation calculated over three independent experiments.

Figure 1.3: 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 lane 1 (Cytosol). GST alone did not interact
with any of the tested nucleoporins (lane 2). FG-nucleoporins (Nup358, Nup214,
Nup153, Nup98, Nup62, Nup50, and Nup53) interact with both importin beta and
transportin as expected (lanes 3 and 4). Members of the Nup107-160 complex (Nup160,
Nup133, and Nup85), the Nup107-160 complex-associated proteins ELYS and centrin
also bind both importin beta and transportin. Nup155 and Nup93 do not associate with
importin beta or transportin. In the Nup205 immunoblot, a higher non-specific band was
observed. **(B)** GST or GST-transportin pull-downs were performed and bound proteins
were analyzed as in (A), except that 10 µM RanQ69L-GTP was added in the reactions in
lanes 3 and 5 (GST+RanQ69L, and GST-Trn+RanQ69L).
**(C) Annulate lamellae pore assembly is regulated by transportin in a Ran-mediated
manner.** *Xenopus* cytosol and membranes were incubated with or without recombinant
proteins for 90 minutes. AL membranes were isolated by centrifugation and the
associated nucleoporins were detected by immunoblotting. When AL is formed, all
tested nucleoporins (gp210, Nup133, Nup43, Nup93 and Nup53) are present (lane 1).
However, when excess importin beta or transportin (20 µM) are added, these
nucleoporins no longer accumulate on AL membranes (lanes 2 and 4). The importin beta
and transportin block to AL assembly can be largely reversed by addition of excess
RanQ69L-GTP (30 µM) (lanes 3 and 5). Excess RanQ69L-GTP alone 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
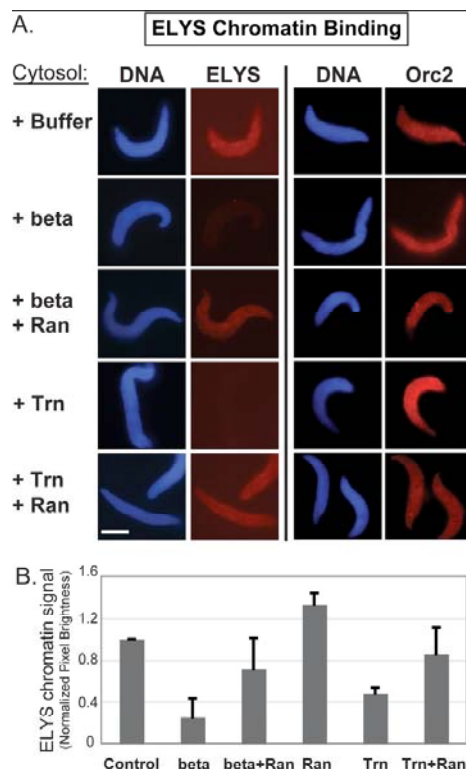under each condition, as indicated by the pore membrane protein gp210 (top row).

Figure 1.4: Transportin and importin beta regulate the initial step in nuclear pore assembly, ELYS recruitment.
(A) *Xenopus* cytosol, sperm chromatin, and recombinant proteins were incubated together for 20 minutes at room temperature, before centrifugation of the chromatin onto coverslips and processing for immunofluorescence with anti-ELYS and anti-ORC2 antibodies. Representative images are shown. When *Xenopus* cytosol was incubated with a chromatin source in the absence of membranes, ELYS bound to chromatin (+Buffer). However, when 20 μM importin beta or 20 μM transportin were added to the reaction, ELYS no longer bound to chromatin (+beta, and +Trn). Both blocks to chromatin binding of ELYS were substantially prevented by the inclusion of 30 μM RanQ69L-GTP (+beta+Ran, and +Trn+Ran). The binding to chromatin of the known chromatin-binding protein, Orc2, was not significantly changed upon addition of an excess of any of the recombinant proteins. DNA was stained with DAPI. Bar: 10 μm.
(B) Quantitation of the data from four experiments performed as in (A) are plotted. For each experiment, 50 areas per condition were quantitated. Specifically, ten sections of 10x10 pixels in each of five nuclei per condition 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 the 4 experiments.
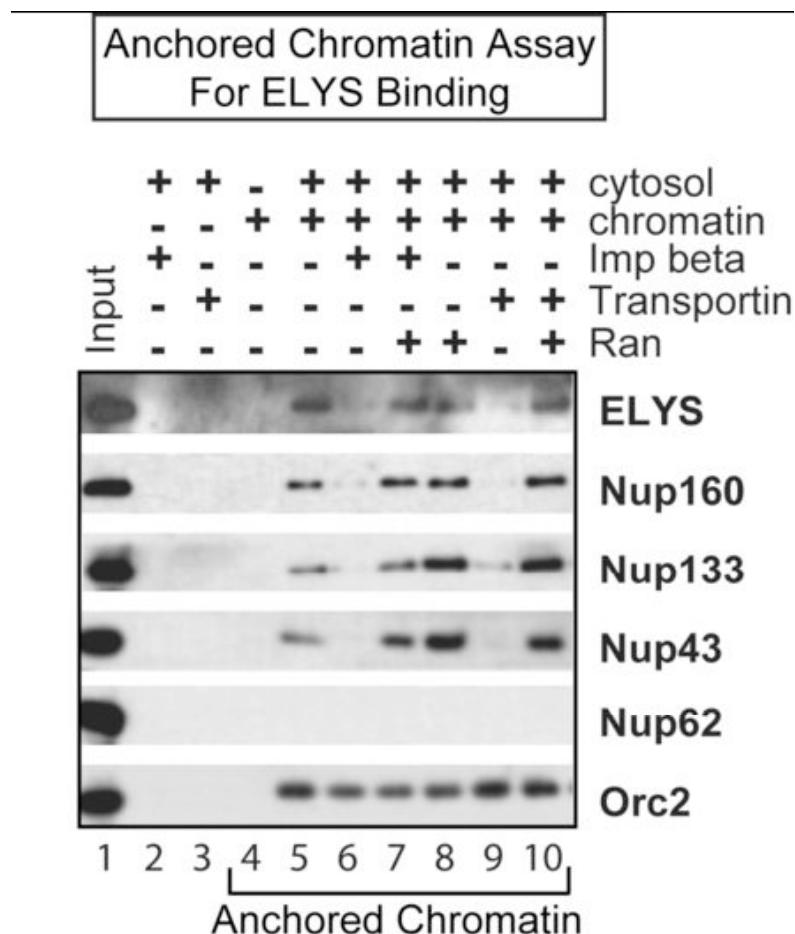
Figure 1.5: Transportin and importin beta block ELYS from binding to anchored chromatin. Chromatin was decondensed and allowed to settle onto poly-L-Lysine coated coverslips. The anchored chromatin was incubated with *Xenopus* egg cytosol in the presence and absence of added recombinant protein for 20 minutes at room temperature. Proteins bound to chromatin were isolated and analyzed by immunoblotting. An immunoblotting control of total *Xenopus* egg cytosol is shown in lane 1. Negative controls included cytosol incubated with either recombinant importin beta or transportin but without chromatin (lanes 2 and 3), or decondensed chromatin templates with no further addition (lane 4). The Nup107-160 complex (represented by Nup160 Nup133, and Nup43) and ELYS bind to chromatin (lane 5), whereas Nup62 does not bind to chromatin. Orc2, a chromatin binding protein, was included as a positive control for chromatin binding. When 20 μM importin beta or transportin was added, the binding of ELYS and the Nup107-160 complex to chromatin was largely abolished (lanes 6 and 9, respectively). This block could be significantly reversed by inclusion of 30 μM RanQ69L-GTP (lanes 7 and 10). RanQ69L-GTP alone did not adversely affect the binding of ELYS and the Nup107-160 complex to the chromatin (lane 8).
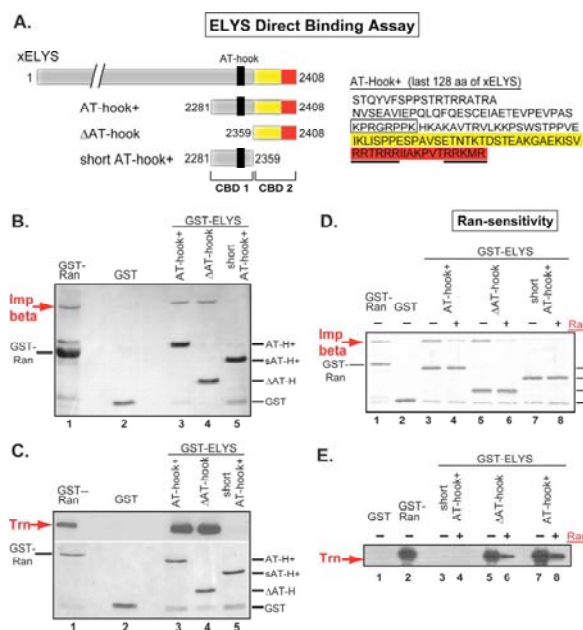
Figure 1.6: Transportin and importin beta directly bind to ELYS.

(A) Schematics of the *Xenopus* ELYS and three GST-tagged *Xenopus* ELYS C-terminal fragments used in the direct binding assay. ELYS AT-hook+ contains two chromatin binding domains (CBD 1 and CBD 2) including an AT-hook motif (black bar) in CBD 1. ELYS ΔAT-hook contains the last 50 amino acids of the C-terminus of ELYS (yellow and red) and lacks the AT-hook. ELYS short AT-hook+ (CBD 1) includes the AT-hook but lacks CBD 2. The sequence of the final 128 amino acids of *Xenopus* ELYS is shown at the right with the AT-hook boxed, the initial portion of CBD 2 in yellow, and the basic region of CBD 2 in red (see text). **(B and C)** GST-RanQ69L-GTP, GST alone, or one of three GST-ELYS fragments (GST-ELYS AT-hook+, GST-ELYS ΔAT-hook, or GST-ELYS short AT-hook+) were incubated with 1.0 μM untagged recombinant importin beta (B) or transportin (C) in the presence of BSA but the absence of any *Xenopus* nuclear or cytoplasmic proteins. The GST-tagged proteins and bound protein were isolated on glutathione-sepharose beads and subjected to gel electrophoresis and silver stain analysis in (B) or Western blotting and Coomassie staining in (C). The gel in (D) was Coomassie stained, while (E) is an immunoblot using anti-transportin antibody. GST-RanQ69L-GTP served as a positive control for importin beta and transportin direct binding (lane 1 in B-D; lane 2 in E), while GST served as a negative control (lane 2 in B-D; lane 1 in E). Importin beta and transportin were found to directly bind to GST-ELYS AT-hook+ and GST-ELYS ΔAT-hook, but not to GST-ELYS short AT-hook+ (see lanes 3-5 in B-C; lanes 3-8 in D-E), indicating that binding requires a region in the extreme C-terminus (aa 2359-2408) of ELYS (yellow + red). Comparable amounts of GST recombinant proteins were recovered and loaded on the gels as determined by silver or Coomassie staining; these are marked with hatchmarks (B-D) on the right of each panel. **(D, E)** Experiments were performed as in (B) and (C) except with the addition of RanQ69L-GTP (5 μM) in parallel reactions (lanes 4, 6, and 8).
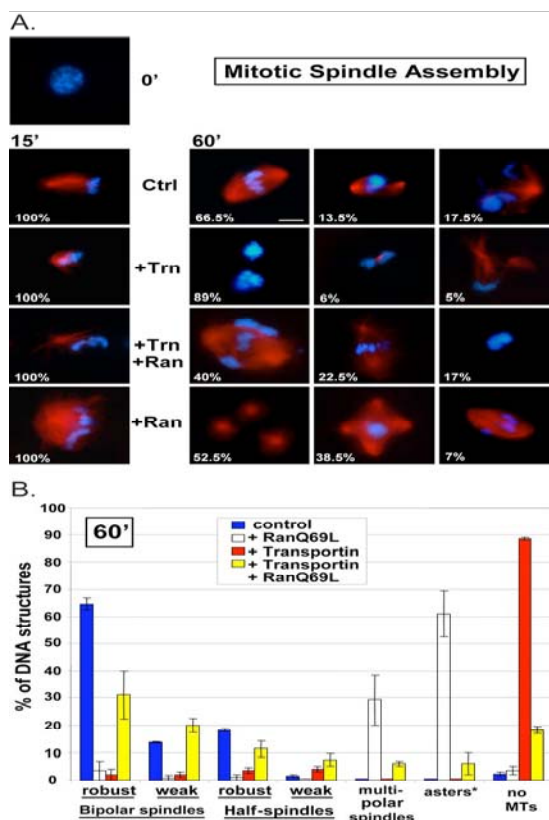
Figure 1.7: Transportin negatively regulates mitotic spindle assembly.
(A) *Xenopus* interphase egg extract was supplemented with sperm chromatin and energy mix to allow nuclear formation and DNA replication for 90 minutes (see Methods). Spindle assembly was then induced and followed by the addition of 20 μl of *Xenopus* mitotic extract (CSF extract) and rhodamine-labeled tubulin. Human GST-Transportin (20 μM; + Trn) or RanQ69L-GTP (5 μM; + Ran) were added where noted. A representative nucleus visualized at t = 0' after mitotic extract addition corresponds to 90' after adding sperm chromatin to the *Xenopus* interphase extract (upper left picture). After adding mitotic extract, aliquots were withdrawn at 15' (left panels) and 60' (right panels), prefixed with fixation buffer, and the structures formed were counted for Figure 7B. DNA was visualized with 5 μg/ml Hoechst DNA dye. Bar: 10 μm.
(B) Quantitation was done to enumerate the *in vitro* spindles or defective spindles observed after no addition, transportin addition, and/or RanGTP addition. The graph represents the quantitation of the 60 minute time points derived from three different experiments done as in Figure 7A. For each condition, approximately 60-80 structures were counted and classified into the categories shown on the x-axis: robust or weak bipolar spindles, robust or weak half spindle, multipolar spindles, asters, or DNA with no microtubules (MTs) at all. All the structures counted contained DNA except in the case of asters (*); added RanGTP caused the formation of ~60% asters (no associated DNA) and 29% multipolar spindles (contained DNA).
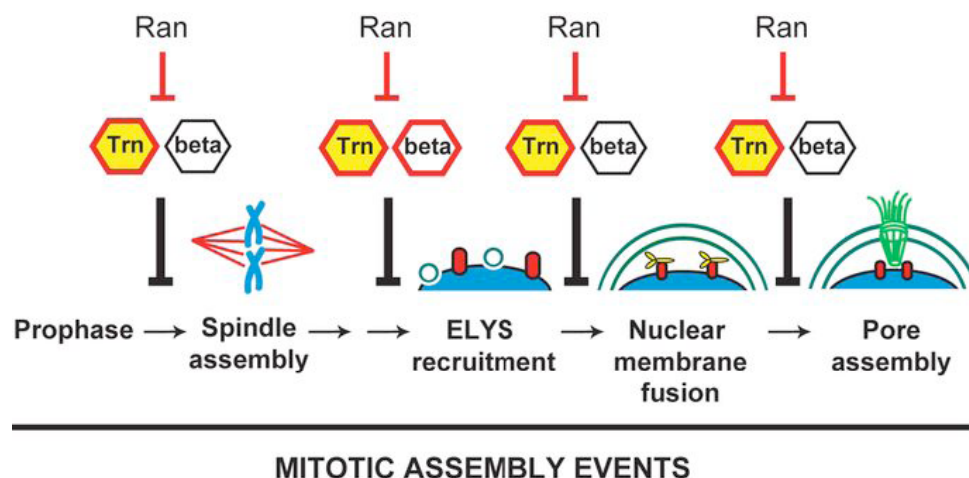
Figure 1.8: Transportin and importin beta regulate multiple mitotic assembly events: from spindle assembly to pore assembly.

Upon induction of mitosis, sequential assembly of a number of large cellular structures is required. Following prophase, a mitotic spindle must be assembled around the metaphase chromosomes. Transportin (Trn) negatively regulates this assembly event (Figure 7), as was previously shown for importin beta. The next large assembly event is that of the nucleus. In early telophase, the initial step of nuclear pore assembly involves the binding of ELYS (red ovals) to chromatin (blue half circles). Here, we demonstrate that transportin and importin beta both negatively regulate the binding of ELYS to chromatin (Figures 4 and 5). This inhibition is counteracted by RanGTP. ELYS next recruits the Nup107-160 pore subunit (yellow Y-shapes). During this period, membrane vesicles (white circles) and/or sheets are also recruited to the chromatin, then fuse to form a double nuclear membranes (curved lines). The fusion of membrane vesicles to form a nuclear envelope is negatively regulated by transportin (Figure 1) and importin beta (Harel *et al.*, 2003a; Delmar *et al.*, 2008) in a RanGTP-sensitive manner (Figure 1). After vesicle-vesicle fusion at the chromatin to form double membrane patches, the bulk of nucleoporins are recruited into the membrane to form mature nuclear pores (green nuclear pore). This latter process is negatively regulated by transportin (Figure 2) and importin beta (Harel *et al.*, 2003a; Walther *et al.*, 2003b; Delmar *et al.*, 2008) and positively regulated by RanGTP. The transportin and importin beta hexagons outlined in red represent findings presented here for the first time.
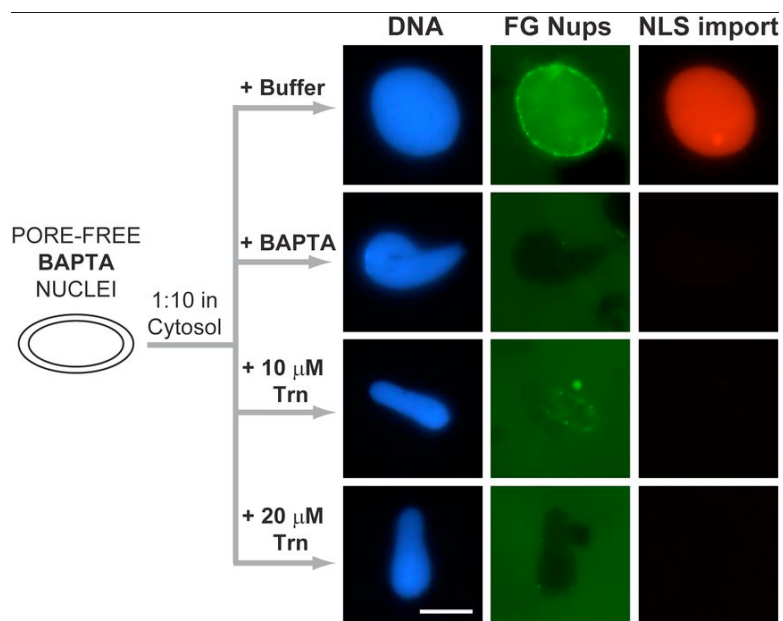
Figure 1.9: The block to nuclear pore assembly *in vitro* by transportin is dosage-dependent.

Pore-free BAPTA nuclear intermediates were assembled by adding 8 μM BAPTA to a nuclear formation reaction at t=0 minute. At t=60 minutes, aliquots of BAPTA-arrested nuclei were diluted 1:10 into fresh cytosol in the presence of: buffer, additional BAPTA (8 μM), or differing amounts of recombinant transportin. Alexa-488-directly labeled antibody against FG nucleoporins (mAb414) was used to assess nuclear pore formation, while TRITC-SV40 NLS-HSA was used to assess nuclear import. We observed that addition of BAPTA (+BAPTA) to the rescuing cytosol prevented pore formation. Some FG staining was observed when BAPTA nuclei were incubated in the presence of 10 μM transportin (+10 μM Trn) in the rescuing cytosol. However, 20 μM human transportin in the cytosol completely blocked nuclear pore formation (+20 μM Trn). Nuclear import was not observed in any of the three experimental conditions. DNA was stained with DAPI (blue). Representative images are shown. Bar: 10 μm.
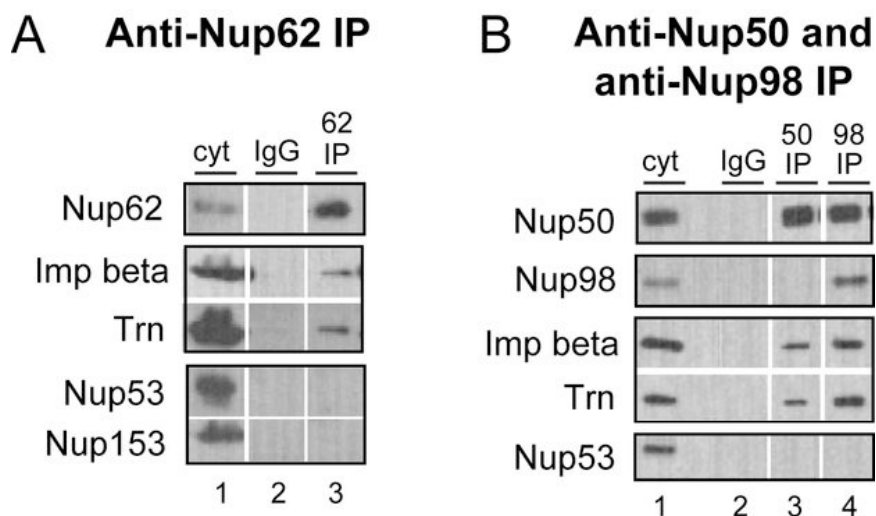
Figure 1.10: Endogenous importin beta and transportin interact with nucleoporin subcomplexes in *Xenopus* egg extract.

(A) Immunoprecipitation from high speed *Xenopus* egg cytosol was performed using control rabbit IgG or anti-*Xenopus* Nup62 immunopurified antibodies. The immunoprecipitates were then immunoblotted with anti-Nup62, anti-importin beta, anti-human transportin, anti-Nup53, and anti-hNup153 antibodies.

(B) Immunoprecipitation was performed from *Xenopus* egg cytosol using control rabbit IgG, anti-*Xenopus* Nup50, or Nup98 affinity purified antibodies. The immunoprecipitates were then immunoblotted with anti-Nup50, anti-Nup98, anti-importin beta, anti-human transportin, and anti-Nup53.

SUPPLEMENTAL MATERIALS AND METHOD

Nuclear pore assembly assay

BAPTA nuclei were assembled as in Figure 2 and diluted 1:10 into fresh cytosol with or without additions. Thirty min later, TRITC-SV40 NLS-HSA transport substrate and Alexa 488-labeled mAb414 antibody were added and incubated for 20 min. The samples were then fixed in 3% paraformaldehyde and visualized by fluorescence microscopy.

Analysis of transportin and importin beta in endogenous *Xenopus* nucleoporin subcomplexes

Immunoprecipitation from high speed *Xenopus* egg cytosol was performed using control rabbit IgG (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), anti-*Xenopus* Nup62 (E. Meier and D. Forbes, unpublished data), anti-xNup50 (R. Sekhorn and D. Forbes, unpublished data), or anti-rat Nup98 GLFG antibody (Harel *et al*., 2003b). The immunoprecipitates were probed by immunoblotting with these antibodies and with additional antibodies including anti-*Xenopus* importin beta (Rasala *et al*., 2008), anti-human transportin (BD Biosciences, San Jose, CA), anti-Nup53 (Rasala *et al*., 2008), and anti-hNup153 (Harel *et al*., 2003b).

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Chapter 2: The search for a vertebrate TREX2 complex: characterizing the roles of human Shd1 and Eny2 in mRNA and protein transport.

Introduction

Eukaryotic gene expression requires unidirectional export of mRNA transcripts from the sites of transcription within the nucleus to the ribosomes for translation in the cytoplasm. Previous studies have identified the yeast protein Mex67 and its human homologue TAP/NXF1 protein as the major mRNA export receptor. In addition, two other protein complexes that are important for mRNA export in yeast have been identified. The TREX complex (Transcription-Export complex), consisting of THO, Yra1, and Sub2, couples transcriptional elongation to mRNA processing (Strasser et al., 2002; Katahira et al., 2009). The separate TREX2 complex functions in coordinating SAGA-dependent transcriptional activation with nuclear pore targeting and subsequent mRNA export (Kohler et al., 2007; Pascual et al., 2008). TREX-2 is composed of four protein subunits Thp1, Sac3, Sus1, and a yeast centrin Cdc31 (Kohler et al., 2007). The complex was shown to localize at the nucleoplasmic side of the NPC and associate with pore proteins (Gallardo at el., 2003; Fischer et al., 2004).

The discovery of the TREX2 complex came from the identification of an interaction between Sac3 and the Thp1 protein, which was implicated in transcription elongation (Gallardo et al., 2001). Prior to the identification and purification of the entire TREX2 complex, two separate studies identified Thp1 and Sac3 as localized to the nucleoplasmic side of the yeast nuclear pores (Gallardo et al., 2001; Fischer et al., 2002). In addition, functional studies showed defects in mRNA export in both single and double

mutants of Sac3 and Thp1, suggesting a role for this complex in mRNA export in yeast (Fischer et al., 2002). It is proposed that the Sac3-Thp1 complex functions in yeast mRNA export by serving as a docking site at the nuclear pore to recruit the mRNA export receptors Mex67/Mtr2 which are coupled to the early steps of transcriptional elongation and processing mediated by the TREX complex (Fischer et al., 2002).

Thp1 was first identified in yeast as a novel protein that functions in transcription elongation. A deletion mutation in Thp1 causes transcription elongation arrest and mitotic recombination (Gallardo et al., 2001). Later studies revealed that Thp1 interacts with Sac3 to form a stable Sac3-Thp1 complex, which docks at the nuclear pore and connects transcription elongation to mRNA export (Fischer et al., 2002; Gallardo et al., 2003). In a recent study in Drosophila cells, using a genome-wide RNAi screen of mutants for defects in mRNA export, Silver and colleagues identified 72 factors required for mRNA export. The *Drosophila* PCID2 protein was one of these and is the *Drosophila* homologue of yeast Thp1. DmPCID2 was found to shuttle between the nucleus and the cytoplasm. The PCI domain of dmPCID2 protein was shown to be dispensable for its interaction with the mRNA export receptor NXF1; however, it is required for interaction with a translation initiation factor eIF3 associated with the polysomes (Farny et al., 2008). Taken together, yeast Thp1 and Drosophila PCID2 play a role in mRNA export and possibly, in the case of dmPCID2, function in connecting mRNA export to translation in the cytoplasm. Given that they reported that a putative human homologue of Thp1 exists, it is an area of interest to further characterize its potential function in vertebrate mRNA export.

Subsequent studies to identify additional mRNA export factors unexpectedly

found that a third protein, Sus1, which had been previously identified as a functional

component of the SAGA (Spt-Ada-Gcn5-Acetyltransferase) transcriptional coactivation

complex (Wu et al., 2004; Cabal et al., 2006), is also a component of the yeast TREX-2

complex, suggesting a role of Sus1 in mRNA export (Rodriguez et al., 2004). Consistent

with this, loss-of-function Sus1 mutants in yeast resulted in both transcriptional and

mRNA export defects (Rodriguez et al., 2004). Furthermore, together with Sac3, Sus1 is

shown to function in repositioning and anchoring of the SAGA-dependent GAL1 gene

locus to the nuclear pores, presumably via interaction with the Sac3 and other nucleoprins

(Kurchaskova et al., 2007; Chekanova et al., 2008).

The fourth identified member of the yeast TREX2 complex is Cdc31, a yeast

centrin protein. Centrin proteins are traditionally associated with microtubule organizing

centers. Indeed, Cdc31 was shown to function in the duplication of spindle pole body, the

yeast equivalent of the mammalian centrosome (Ivanovska et al., 2001; Paoletti et al.,

2003). Interestingly, subsequent study also found Cdc31 at the yeast nuclear pore (Rout

et al., 2000) and showed a connection of Cdc31 to mRNA export through interaction with

Sac3-Thp1-Sus1 complex (Fischer et al., 2004). Overproduction of the C-terminal

domain, termed CID for Cdc31-interacting domain, of Sac3 sequestered Cdc31 and

resulted in a block in spindle pole body (SPB) duplication. Interestingly, however, Cdc31

mutants and the deletion of Sac3 CID also domain resulted in mislocalization of Cdc31

released from the nuclear pores and strong defect in mRNA export. Together with

previous studies, it is suggested that the CID motif of Sac3 recruits Cdc31 to the Sac3-

Thp1-Sus1 complex at the nuclear pore to couple transcription to mRNA export (Fischer

et al., 2004).

Since the mitotic function of centrin in microtubule nucleation is evolutionarily conserved from the flagella of algae to the spindle pole body of yeast to the vertebrate centrosomes, the unexpected finding of yeast centrin cdc31 at the yeast nuclear pores and its function in mRNA export raised the question of whether vertebrate centrin functions in a similar manner. Indeed, human centrin2 (hCentrin2) has been found by the Forbes lab to associate biochemically with nucleoporins such as Nup107-160 complex, and Nup153 (Resendes et al., 2008), both of which are known to play a role in mRNA export in vertebrates (Ullman et al., 1999; Vasu et al., 2001). Immunofluorescence studies using the mild detergent digitonin also revealed that hCentrin2 is, in fact, at the nuclear pores of reconstituted *Xenopus* and Hela nuclei. Furthermore, functional studies showed that overexpression of either the N- or C-terminal calcium-binding domains of human centrin 2 caused a dominant-negative effect on both mRNA and protein export while leaving protein import unaffected. These results demonstrated that human centrin 2 plays a role in vertebrate mRNA and protein export (Resendes et al., 2008). Since human centrin 2 was identified to play a role in mRNA export, we asked whether the entire TREX-2 complex is present in vertebrates.

We began by looking at Eny2 protein, a human homologue of yeast Sus1. Previous studies showed that yeast Sus1 interacts with protein components of the SAGA histone acetylase complex (Rodriguez et al., 2004) as well as localizes to the yeast nuclear pore through interaction with Sac3 and Thp1 component of the TREX-2 complex which mediates subsequent mRNA export (Kohler et al., 2007). In recent studies, Kurshakova and colleagues identified Eny2 (enhancer of yellow 2), a *Drosophila* homologue of Sus1, as a subunit of the STAGA/TFTC type histone acetyl transferase

complex in *Drosophila*, which is functionally equivalent to the yeast SAGA complex.

They further showed that Eny2 concentrates at the Drosophila nuclear periphery and

biochemically interacts with X-mas2, the Drosophila homologue of yeast Sac3

(Kurshakova et al., 2007). In addition, human Eny2 has been shown to associate with the

human STAGA/TFTC complex with functions in histone acetylation and

deubiquitination to initiate transcription activation (Zhao et al., 2008), a functional

homologue of yeast and *Drosophila* SAGA-like complex. These findings prompted me to

also examine Eny2 for a potential evolutionarily conserved function in vertebrate mRNA

export. We have identified several transport related binding partners for human Eny2,

including both nucleoporins and transport receptors. In addition, using fluorescence based

assays, we have showed that Eny2 plays a potential role in vertebrate mRNA export.

Another critical member of the yeast TREX-2 complex is Sac3 protein (~150kD).

The *SAC3* gene (SAC = suppressor of actin) of *Saccharomyces cerevisiae* was originally

isolated in a genetic screen for suppressors of a temperature-sensitive mutation in the

essential actin gene (Novick et al., 1989). Immunofluorescence studies of Sac3p showed

that it is localizes to the nucleus and is required for normal mitotic progression of cell

cycle (Bauer et al., 1996). Sac3 was later demonstrated to have both genetic and

biochemical interactions with known mRNA export factors. For example, Fischer and

colleagues (2002) showed that Sac3 genetically interacts with Yra1 and Sub2, the two

protein subunits of the TREX complex, which conserved from yeast to Drosophila and

mammals (Straber et al., 2000 and 2001), (Strasser et al., 2002).In addition, subsequent

study showed that Sac3p interacts with mRNA export receptor Mex67/Mtr2 and

nucleoporins Nup1 and Nup60 (Lei et al., 2003).

Several Sac3-related proteins have been identified in higher species. In

Drosophila, Xmas-2 protein (130 kDa) shows significant homology at its C terminus to

the yeast Sac3 protein. Immunofluorescene and RNAi-knockdown studies of Xmas-2

demonstrated that X-mas2 localizes to the nuclear pores and plays a role in mRNA export

in *Drosophila* similar to the role of Sac3 in yeast (Kurshakova et al., 2007). In

vertebrates, three proteins related to yeast Sac3 have been identified. These includes

human GANP (210kD), human MCM3AP (100 kD), and human Shd1 (50kD) (Abe et al.,

2000; Kuwahara et al., 2001; Khuda et al., 2004). Both B-cell germinal center-associated

protein GANP and MCM3 associated protein (MCM3AP) are derived from alternative

splicing of a single gene. While these proteins have 23% homology with a region of yeast

Sac3 containing its centrin-interaction domain, they also contain other non-Sac3 domains

with functions in DNA replication (Abe et al., 2000). In contrast, the third vertebrate

relative of Sac3, Shd1 (Sac3 homology domain protein 1), is only one-third the size of

yeast Sac3 and lacks the yeast-centrin interaction domain. However, it has been shown to

localize to centrosomes and Shd1 RNAi induces abnormalities in centrosome duplication

and spindle formation, similar effects to knockdown of human centrin 2 (Salisbury et al.,

2002). We hypothesize that human Shd1, like human centrin 2, might have dual roles at

the centrosome and in mRNA export. Thus, we chose to study Shd1 for a potential role in

vertebrate mRNA export. Using fluorescence-based assays, we showed that Shd1 is

involved in vertebrate mRNA export, but not hormone-induced protein transport.

Results and Discussions

Eny2 biochemically interacts with multiple nuclear transport factors

To dissect the potential role of Eny2 in mRNA export, we first set out to identify

its interacting partners in order to determine whether Eny2 has any functional association with the nuclear transport machinery. First, we cloned a GST-Eny2 construct (see Materials and Methods) and purified the bacterially-expressed GST-Eny2 recombinant proteins. To identify interacting partners of Eny2, we performed GST-Eny2 pulldown from Hela cell lysates and probed for various nuclear transport factors by immunoblotting. Interestingly, we found that GST-Eny2 interacted with key factors known to be involved in mRNA export in vertebrates, specially, the mRNA export receptor TAP and the nucleoporins Nup160 and, variably, Nup153 (Figure 1, top panel). In addition, Eny2 also interacted with several factors involved in nuclear protein import, including importin alpha, importin beta, transportin and the regulatory GTPase Ran (Fig1, middle panel). In contrast, Eny2 did not interact with the protein export receptor Crm1 or the Nup62 (Fig 1, bottom panel). This biochemical data revealed that Eny2 interacts with the known mRNA export factors. Because Sus1, the yeast homologue of human Eny2, interacts with yeast centrin Cdc31 as part of the yeast TREX2 mRNA export complex, we also asked if Eny2 interacted with human centrin2, which plays a role in vertebrate mRNA and protein export (Resendes et al., 2008). Surprisingly, GST-Eny2 failed to pulldown human centrin 2 (Fig1, bottom panel).

Our data are consistent with previous studies in yeast where it was shown that yeast Sus1 genetically interacted with Mex67, the yeast mRNA export receptor (Hurt et al., 2004). Furthermore, Sus1 was shown to biochemically associate with the yeast nucleporins Nup1, which physically interacts with yeast Sac3 (Tamas et al., 2002). Strikingly, our data showed that Eny2 biochemically interacts with the human mRNA export receptor, TAP, the human homologue of yeast Mex67. Moreover, we showed that

Eny2 also pull-downs human Nup153 and Nup160, which is the human homologue of yeast Nup1, both of which are functionally involved in mRNA export (Forbes et al., 1999; Vasu et al., 2001), suggesting that Eny2 potentially interacts with this Nup107-160 complex and Nup153 during initial docking of an mRNA export complex at the nuclear pore. Taken together, this interaction data suggest that Eny2 may play a functional role in vertebrate mRNA export.

Eny2 overexpression causes nuclear accumulation of poly(A)+ RNA

Previous studies revealed that both yeast Sus1 and Drosophila E(y)2 have been found in the SAGA complex (Rodriguez et al., 2004) and TREX2 (Susana et al., 2004). Later studies connected the two functions and demonstrated that SAGA-dependent transcription activation is coupled to TREX2-mediated mRNA export (Kurshakova et al, 2007; Pau et al., 2008). Recently, human Eny2 was identified as a functional component of the STAGA/TFTC complex in mammalian cells (Zhao et al., 2008). Given that it is possible that human Eny2 also has dual roles in gene activation and mRNA export in vertebrates, a function in mRNA export was our next area of interest.

We asked whether overexpression of Eny2 would have an effect on mRNA export in human cells. Hela cells seeded on cover slips were transfected with either full length HA-tagged Eny2 or an negative control HA-tagged Lap2 (lamin protein 2) construct. After 24 hours of incubation, the transfected cells were identified by immunofluorescence using an anti-HA primary antibody, followed by Oregon green fluorescent secondary antibody. Poly(A)+ RNA localization was simultaneously monitored in the cells by hybridization with red fluorescent Cy3-oligo(dT), as previously done in Resendes et al. (2008). mRNA localization was assessed in 500 HA-transfected cells per experiment

condition and the full experiment was done in triplicate with the final quantitative percent averaged from three different experiments.

Human cells transfected with the negative control Lap2 gene exhibited blocked mRNA export in only ~6.5% of the HA-positive transfected cells (Fig. 2A). Strikingly, overexpression of Eny2 led to nuclear accumulation of poly(A)+ RNA in ~18% of the positively transfected cells. For comparison sake, overexpression of centrin 2 dominant negative fragments caused 20% to 25% nuclear accumulation in transfected Hela cells (Resendes et al., 2008). Sample images of the transfected human cells inhibited for mRNA export by Eny2 expression are shown in Fig. 2B, where red indicates poly(A)$^+$ RNA and green indicates HA transfection. Thus, these results indicated that Eny2 plays a role in mRNA export.

Our results are consistent with what has been seen for the Drosophila homologue E(y)2, where Kurshakova and colleagues showed that E(y)2 siRNA knockdown lead to nuclear accumulation of poly(A)+ RNA in Drosophila cells (Kurshakova et al., 2007). Because our current data shows that overexpression of HA-Eny2 inhibited mRNA export, we propose that this excess of Eny2 acts as a dominant negative and may be preventing the formation of complete endogenous TREX2 export complex. It will be of future interest to determine whether siRNA knockdown of human Eny2 also affects mRNA export as in Drosophila. However; at present, the lack of an available Eny2 antibody to determine efficient knockdown prevents us from performing this assay. It will also be of interest to determine the intracellular localization of Eny2, which we would predict to be at the nuclear pore in association with the other components of the mRNA export complex. Thus, from our biochemical interaction (Fig 1) and functional results (Fig 2),

we conclude that, in addition to its functional association with the STAGA/TFTC complex, human Eny2 also plays a role in vertebrate mRNA export.

Eny2 expression has no effect on either protein import or export.

Since Eny2 overexpression inhibits mRNA export, we next asked whether this is a general block of all nuclear transport or a specific block of mRNA export. To address this question, we examined the effect of Eny2 overexpression on nuclear protein transport. To study nuclear protein import and export, we used an NES/NLS-bearing protein construct termed RGG (Rev-Glucocorticoid-GFP). This chimeric protein contains: 1) the human immunodeficiency virus (HIV) protein REV fused to its NES, 2) the ligand binding domain of the glucocorticoid receptor which contains a hormone-dependent NLS, further fused to the green fluorescent protein, GFP (Hanover et al., 1998 and Resendes et al., 2008). It is important to note that the mechanism of nuclear transport of the RGG protein is hormone-dependent in that the transfected RGG protein stays in the cytoplasm until the addition of dexamethasone, which induces its import into the nucleus. Upon removal of dexamethasone, the RGG construct protein is exported through the Crm-1 export receptor via its interaction with the NES of the Rev protein (Hanover et al., 1998; Freedman and Yamamoto 2004; Resendes et al., 2008).

HA-Eny2 or HA-Lap2 construct was transfected into RGG2.2 cells, a Hela cell derivative stably transfected with the RGG construct, for 24 hours. Transfection of the constructs was determined by immunofluorescence using primary anti-HA antibody and secondary Texas-red fluorescent antibody. Overexpression of HA-Lap2, as a negative control, had no significant effect on either protein import or export as 95% of transfected cells showed import and export of RGG equivalent to control RGG2.2 cells (Fig 3A and

Fig 4A). Similarly, normal import and export of the RGG protein were observed in 92%

(for import) (Fig 3A) and 94% (for export) (Fig 4A) of Eny2-transfected cells.

Representative images of nuclear protein import are shown in Figure 3C and of nuclear

protein export in Figure 4B. The lack of an effect of Eny2 overexpression having on

export of RGG protein was consistent with our biochemical data where Eny2 failed to

interact with Crm1. Taken together, we concluded that Eny2 expression had no effect on

hormone-dependent protein import and Crm1-mediated protein export.

Shd1 overexpression causes an mRNA export defect

In the data above, we showed that the human homologue of yeast Sus1, Eny2 has

a conserved role in mRNA export. Thus, we reasoned that it was possible that other

members of the yeast TREX2 complex also have vertebrate homologues and together

they comprise a potential vertebrate TREX2 complex. Previous studies identified Shd1 as

one vertebrate homologue of yeast Sac3 and the most like Sac3 in behavior. Recent study

of Shd1 showed its localization at centrosome at interphase and at spindle poles and

mitotic spindles during M phase (Sefat et al., 2004). In addition, our recent work revealed

that human centrin 2, traditionally associated with centrosome, also localizes to the

vertebrate nuclear pore and plays a role both in mRNA export and nuclear protein export

(Resendes et al., 2008). Because Shd1 and hCentrin2 have a similar centrosomal-

associated function, we asked whether Shd1 mirrors human cenrtin2 in that it associates

with the vertebrate nuclear pores and plays a role in vertebrate mRNA export.

To ask whether overexpression of Shd1 has an effect on mRNA export, Hela cells

were seeded on coverslips and were transfected with either full length myc-tagged Shd1

construct or an negative control myc-tagged PK (pyruvate kinase). After 24 hours of

incubation, the successfully transfected cells were identified using FITC labeled myc Ab. The localization of poly(A)+ RNA was monitored by hybridization with red fluorescent Cy3-oligo(dT). mRNA localization was assessed in 500 myc-transfected cells per condition and the full experiment was done in triplicate, with the final quantitative percent averaged from these three different experiments. Human cells transfected with the negative control PK gene exhibited blocked mRNA export in only ~10% of the myc-positive transfected cells (Fig. 5A). However, overexpression of Shd1 led to the accumulation of poly(A)+ RNA in ~48% of the positively transfected cells (Fig 5A), an even percentage than seen with Eny2 or centrin 2. Sample images of the transfected human cells inhibited for mRNA export by Shd1 expression are shown in Fig. 5B, where red indicates poly(A)$^+$ RNA and green indicates myc transfection. Thus, our result indicate that Shd1 plays a role in vertebrate mRNA export. We propose that this excess of Shd1 is preventing the formation of the complete endogenous TREX2 export complex in manner similar to that of overexpresses Eny2.

Interestingly, a recent study identified Shd1 as interacting partner of STAT5, one of the major cytokine-inducible signal transducers and transcriptional activators, and demonstrated that Shd1 acts a negative feedback regulator of this cytokine signal pathway by specifically repressing STAT-dependent transcription (Nakajima et al., 2009). It is possible that Shd1 also has a dual function, similar to that of Eny2, acting in both transcriptional regulation and mRNA export. Just as Eny2 functionally associates with the STAGA/TFTC complex (Zhao et al, 2008), it is possible that Shd1 might also be linked to an unknown regulatory complex of a STAT-dependent transcriptional module to be revealed by future studies.

Shd1 overexpression has no effect hormone-dependent RGG protein transport.

Because Shd1 overexpression caused a similar defect in mRNA export to that of Eny2, we further asked whether Shd1 has a role in nuclear protein transport. We used an NES/NLS-bearing protein construct RGG (Rev-Glucocorticoid-GFP) to study nuclear protein import and export. Either myc-Shd1 or myc-PK construct was transfected into RGG2.2 stable transformant for 24 hours. Protein expression was determined by FITC-labeled anti-myc antibody. Overexpression of myc-PK, the negative control, had no significant effect on either protein import or export (Fig 6A and Fig 7A). Similarly, normal import and export of RGG protein were observed in ~90% of Shd1-transfected cells (for import) (Fig 6A) and ~92% (for export) (Fig 7A) of Shd1-myc transfected cells. Representative images of protein import are shown in Figure 6C and for protein export in Fig7B.

To summary, our data showed that Shd1 overexpression did not have a statistically significant effect on the hormone-dependent nuclear import or export of RGG protein upon the removal of hormone. It will be of future interest to determine whether Shd1 biochemically interacts with any known nuclear transport factors, as these biochemical data will further complement our functional data. However, due to the technical challenge of creating a GST-tagged Shd1 construct to date, these biochemical experiments have not yet been possible.

In conclusion, the yeast TREX2 complex localizes to the nuclear face of the nuclear pore, where it couples mRNA export to upstream steps of transcription elongation and formation of export-competent mRNPs. Human centrin 2 was identified as the human homologue of the yeast Cdc31, a member of the yeast TREX2 complex.

Our recent work revealed that human centrin 2 localizes at the nuclear pore and plays a role in both protein and mRNA export in vertebrates. In addition, vertebrate homologues of other protein members of the yeast TREX2 complex have been identified, with human Shd1 and Eny2 are the closest homologues to yeast Sac3 and Sus1, respectively. In this study, we showed that over-expression of either Shd1 or Eny2 causes nuclear accumulation of mRNA, suggesting a functional role in vertebrate mRNA export. Moreover, GST pulldown assay revealed that Eny2 biochemically interacts with nucleoporins Nup160 with known functional involvement in vertebrate mRNA export as well as human TAP, a major mRNA export receptor.

Although having strong similarity in function, can we say the two TREX2 complexes in yeast and in vertebrate are necessarily identical in structure? There is precedence for homology of function but diversity of structure. For example, the yeast Nup60 and its homologue, human Nup153, both play role in mRNA export despite their differences in structural homology. This supports how human Shd1, despite being only a third of yeast Sac3 and lacking of the centrin-interacting domain, can fulfill both mitotic and interphase roles similar to that of Sac3. In conclusion, we propose that both human Shd1 and Eny2 have functional involvement in a potential vertebrate homologue of yeast TREX2 complex as shown in Figure 8.

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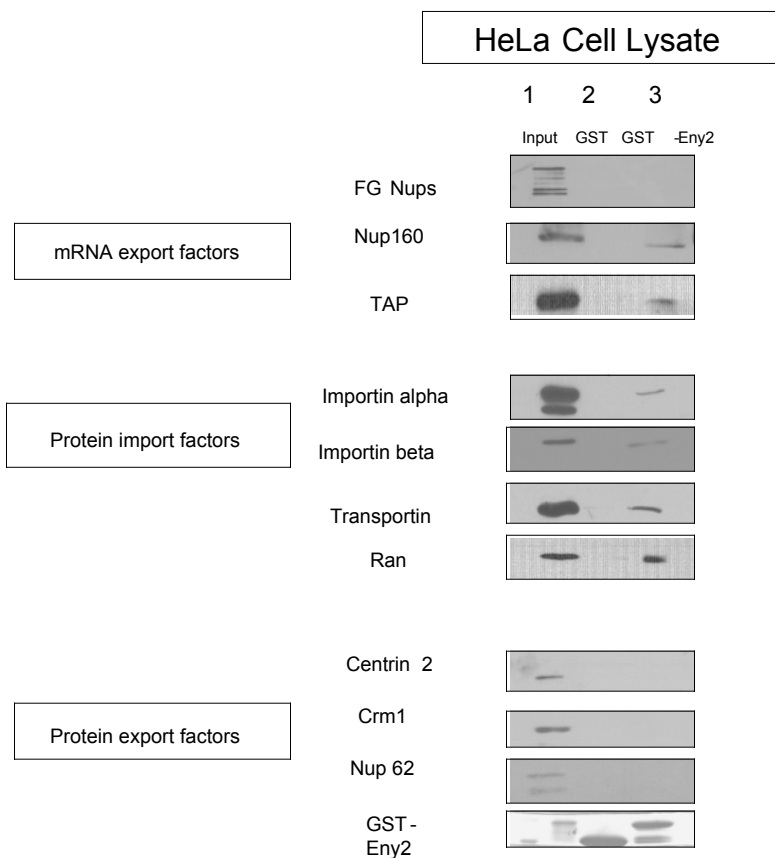
Figure 2.1 Eny2 biochemically interacts with multiple nuclear transport factors from Hela cells. GST-Eny2 (lane 3) or GST (lane 2) (as a negative control) were bound to glutathione beads and were then incubated with Hela cell lysate. Top panel: GST-Eny2 interacts with mRNA export factors**:** TAP, Nup160, Nup153 variably, but not Nup214/358 (lane 3). Middle panel: GST-Eny2 interacts with nuclear import factors including import alpha, importin beta, transportin, as well as Ran protein. Bottom panel: GST-Eny2 did not interact with hCentrin2, Nup62 (non-member of Nup107-160 complex), or Crm1. Input indicates a fraction of Hela cell lysate (lane 1). The negative control GST did not interact with any of the tested proteins.
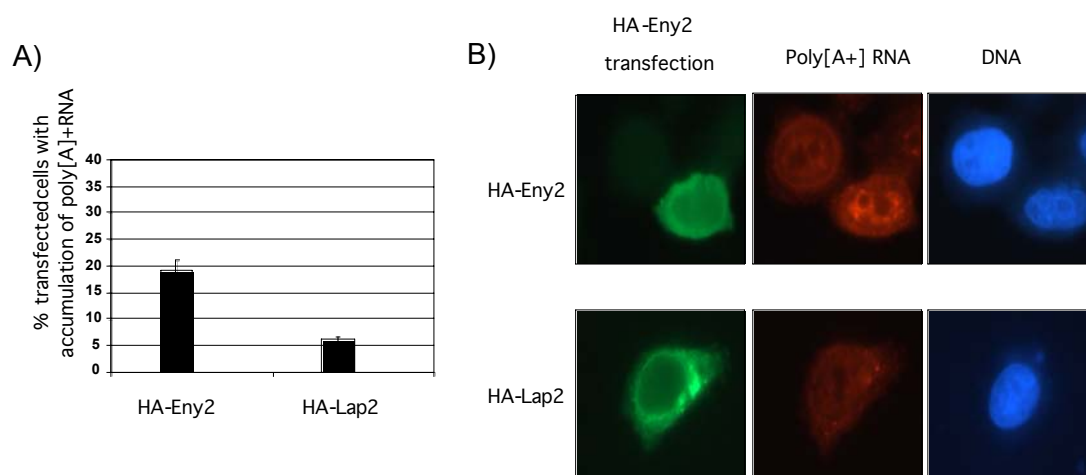
Figure 2.2 Overexpression of Eny2 causes nuclear accumulation of poly(A)+ RNA.
A) Overexpression of the human Eny2, but not negative control Lap2, causes the nuclear accumulation of poly(A)$^+$ RNA. HeLa cells were transfected with the indicated construct 24 h before the poly(A)$^+$ RNA accumulation assay was performed. Quantitation of nuclear poly(A)$^+$ RNA accumulation in cells transfected with either of human Eny2 or Lap2 gene was done with 500 cells per experiment. The percentage of transfected HeLa cells with nuclear poly(A)$^+$ RNA accumulation was calculated in three independent experiments and averaged.
(B) Typical views of HeLa cells successfully transfected with the HA-Eny2 or HA-Lap2 control gene are shown. The cells were transfected as described for panel A. The left panels show expression of the HA-tagged constructs using primary anti-HA antibody and Oregon green goat-anti-mouse secondary (green). The center panels are the same cells hybridized with Cy3-oligo(dT)$_{50}$ to show nuclear poly(A)$^+$ RNA accumulation (red). The right panels show the complete field of cells by DAPI DNA staining.
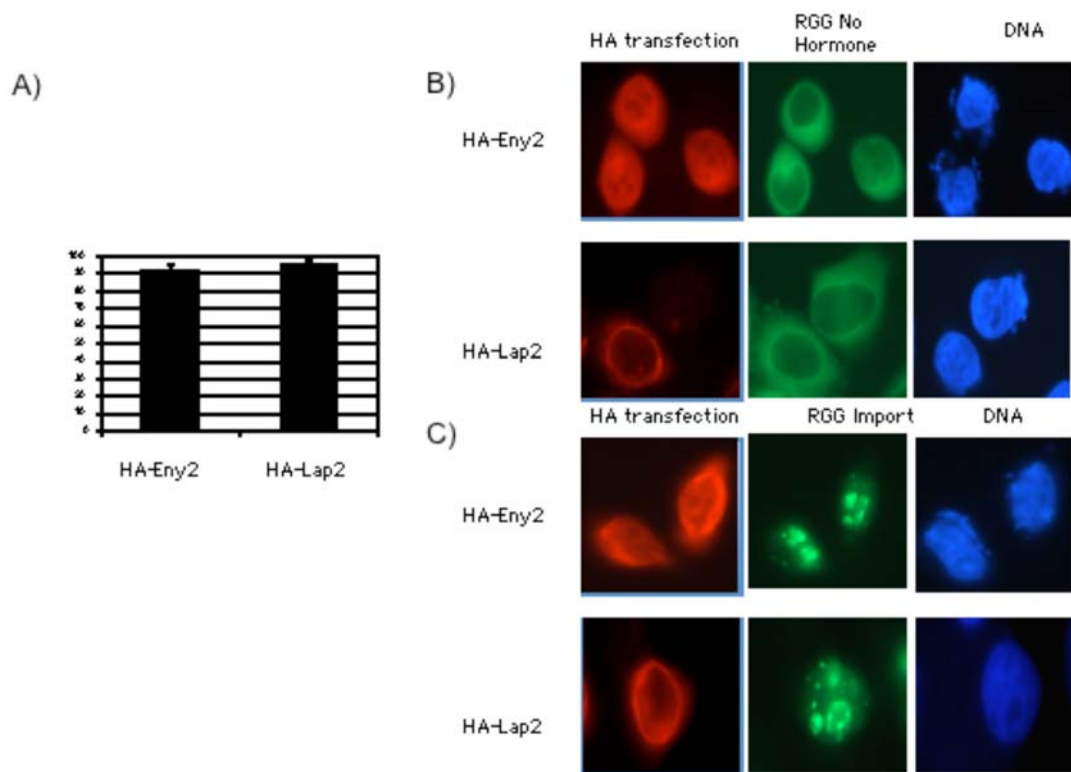
Figure 2.3 Overexpression of Eny2 does not affect hormone-dependent RGG protein import.
(A-C) Overexpression of Eny2, or Lap2, has no effect on RGG protein import. The RGG2.2 cells, which are stably transfected with the RGG construct were transiently transfected with the indicated constructs before the addition of dexamethasone to induce RGG protein import.A) Quantitation of nuclear import in cells transfected with either of human Eny2 or Lap2 gene was done with 500 cells per experiment. Overexpression of HA-Lap2, as negative control, had no significant effect on either protein import as 95% of transfected cells showed normal import. Similarly, normal import of RGG protein was observed in 92% of HA-Eny2 transfected cells. Typical views of RGG2.2 cells successfully transfected with the HA-tagged constructs before (B) or after (C) induced protein import are shown. The left panels show expression of the HA-tagged constructs using primary anti-HA antibody and secondary Texas-red secondary antibody (red). The middle panels are the same cells showing the location of the RGG protein via its GFP tag (green). Typically, the RGG protein targets the nucleoli within the nucleus as observed here. The right panels indicated DAPI staining of DNA of the same cells.
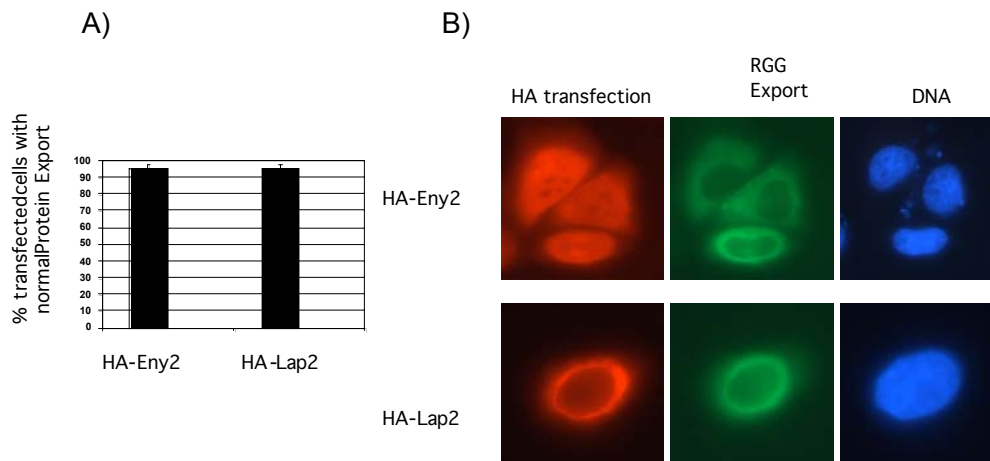
Figure 2.4 Overexpression of Eny2 does not affect Crm1-mediated RGG protein export.

(A) Typical views of RGG cells successfully transfected with the HA-tagged constructs are shown. The left panels show expression of the HA-tagged using primary anti-HA antibody and secondary Texas-red secondary antibody (red). The middle panels are the same cells showing the location of the RGG protein via its GFP tag (green), which are in the cytoplasm, indicating normal export. The right panels indicated DAPI staining of DNA of the same cells.

(B) Overexpression of human Eny2, or Lap2, had no effect on RGG protein export. Following the RGG protein import as described above, the cells were switched to fresh medium lacking dexamethasone for 2 h to induce RGG protein export. Overexpression of HA-Lap2, as negative control, had no significant effect on protein export as 95% of transfected cells showed normal export.
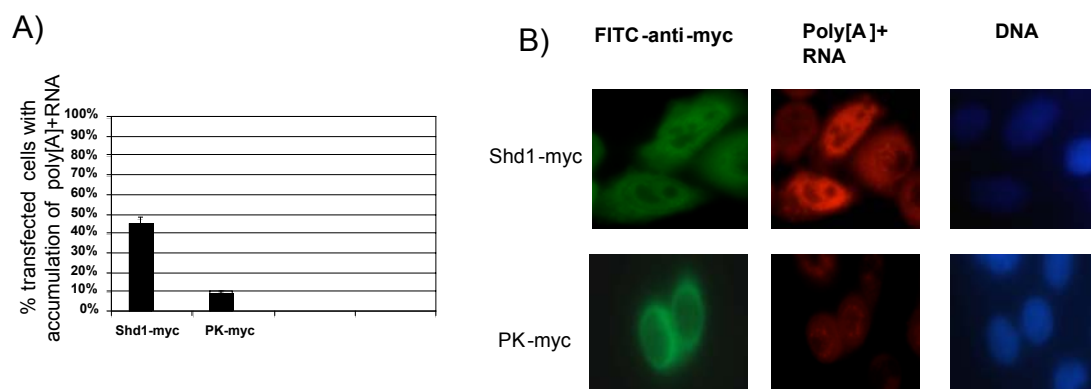
Figure 2.5 Overexpression of Shd1 causes nuclear accumulation of poly(A)+ RNA.
(A) Overexpression of the Shd1, but not PK (pyruvate kinase) used as negative control,
causes the nuclear accumulation of poly(A)$^+$ RNA. HeLa cells were transfected with the
indicated construct 24 h before the poly(A)$^+$ RNA accumulation assay was performed.
Quantitation of nuclear poly(A)$^+$ RNA accumulation in cells transfected with either of
human Shd1 or PK gene was done with 500 cells per experiment. The percentage of
transfected HeLa cells with nuclear poly(A)$^+$ RNA accumulation was calculated in three
independent experiments and averaged.
(B) Typical views of HeLa cells successfully transfected with the Shd1-myc and PK-myc
control gene are shown. The cells were transfected as described for panel A. The left
panels show expression of the myc-tagged constructs using FITC labeled anti-myc
(green). The center panels are the same cells hybridized with Cy3-oligo(dT)$_{50}$ to show
nuclear poly(A)$^+$ RNA accumulation (red). The right panels show the complete field of
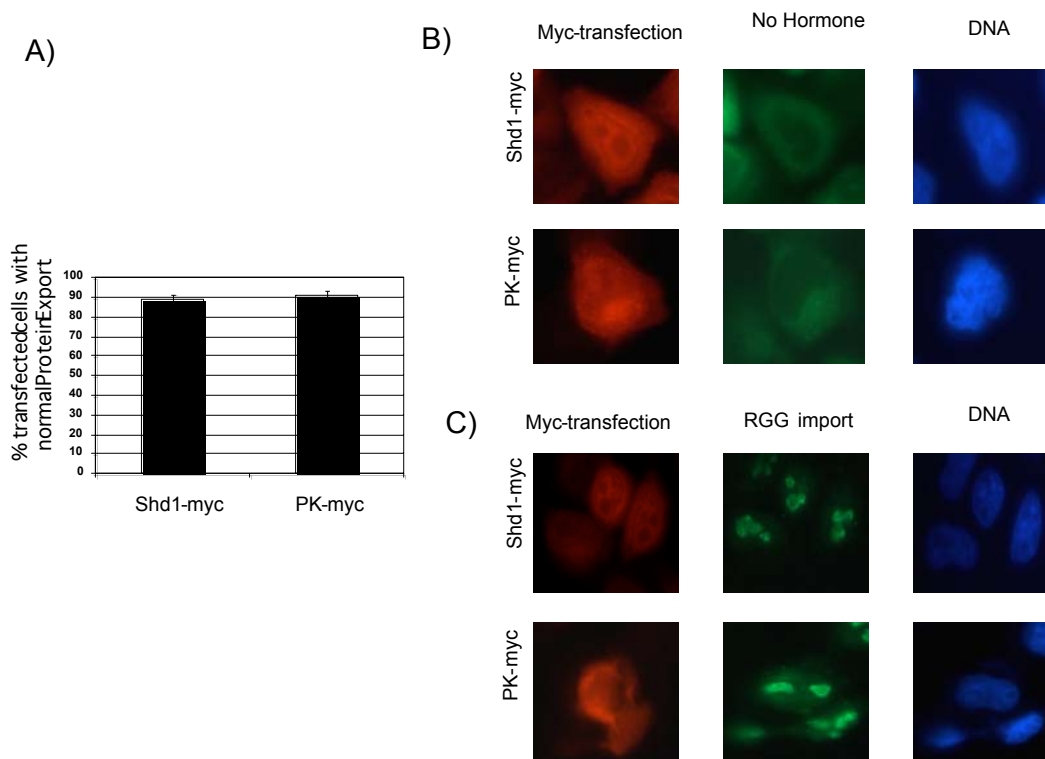cells by DAPI DNA staining.

Figure 2.6 Overexpression of Shd1 does not affect hormone-dependent RGG protein import.
(A-C). Overexpressions of Shd1, or PK have no effect on RGG protein import. RGG2.2 cell line stably transfected RGG construct were transfected with the indicated constructs before the addition of dexamethasone to induce RGG protein import. (A) Quantitation of overexpression of myc-PK, as negative control, had no significant effect on both protein import as 93% of transfected cells showed normal import. Similarly, Shd1 verexpression had no effect on protein import as normal import were observed in ~90% transfected cells. Typical views of RGG2.2 cells successfully transfected with the myc-tagged constructs before (B) or after (C) induced protein import are shown. The left panels show expression of the myc-tagged constructs TRITC labeled anti-myc (red). The middle panels are the same cells showing the location of the RGG protein via its GFP tag (green). Typically, the RGG protein targets the nucleoli within the nucleus as observed here. The right panels indicated DAPI staining of DNA of the same cells.
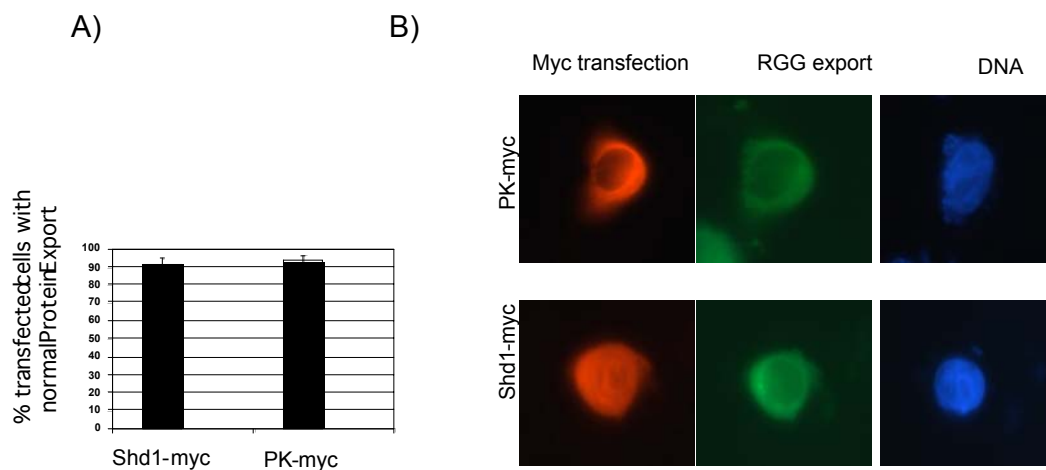
Figure 2.7 Overexpression of Shd1 does not affect Crm1-mediated RGG protein export
(A) Overexpression of human Shd1, or PK, have no effect on RGG protein export. Following the RGG protein import as described above, the cells were switched to fresh medium lacking dexamethasone for 2 h to induce RGG protein export. Quantitation of overexpression of myc-PK, as negative control, had no significant effect on protein export as 95% of transfected cells showed normal export. Similarly, Shd1 overexpression had no effect on protein export as normal export were observed in ~90% transfected cells. (B) Typical views of RGG cells successfully transfected with the myc-tagged constructs are shown. The left panels show expression of the myc-taggedTRITC labeled anti-myc. The middle panels are the same cells showing the location of the RGG protein via its GFP tag (green), which are in the cytoplasm, indicating normal export. The right panels indicated DAPI staining of DNA of the same cells.
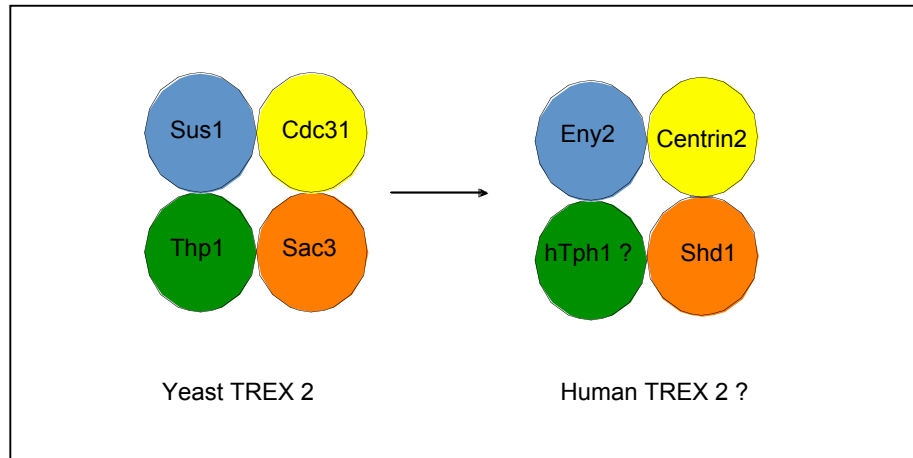
Figure 2.8 Model of a potential vertebrate TREX2 complex.
The vertebrate homologues of the yeast TREX2 complex have been identified. In our study, we showed 2 proteins, Shd1 and Eny2, play potential roles in vertebrate mRNA export, suggesting a possibility of a vertebrate TREX2 complex.

Chapter 3: Importin beta directly interacts with human centrin 2.

Implications for centrin function

Introduction

During interphase, importin beta positively regulates several processes. The best characterized interphase function of importin beta is as an import receptor working together with importin alpha to import NLS-containing cargoes into the nucleus (Gorlich et al., 1995). During interphase protein import, the small Ran-GTP binds to importin beta and causes the dissociation of the import cargo from the import receptor complexes. In addition, importin beta functions as a cytoplasmic chaperone, perventing proteins with highly basic domains from self-aggregating (Jakel et al., 2002), Interestingly, importin beta acts as microtubule motor adaptor used by viruses to target their genomes to the nucleus along the microtubule tracks (Campbell and Hope, 2003; see review in Harel and Forbes, 2003).

In contrast, during mitosis, importin beta functions as a negative regulator of multiple major mitotic events. These range from its control of nuclear membrane and nuclear pore assembly to mitotic spindle assembly and centrosome dynamics (as reviewed in Harel and Forbes, 2003). In mitosis, Ran functions as a positive regulator to counteract the inhibitory effect of importin beta. The working model of the dual regulatory functions of Ran and importin beta, with respect to mitotic spindle assembly, proposes that importin beta and at time, importin alpha, binds to mitotic factors such as the spindle assembly factors (SAFs) required for spindle assembly. It is thought to also bind to other mitotic factors required for mitotic centrosomes dynamics and kinetochore attachments. In of all these mitotic events, the role of importin beta and importin alpha is

to keep these mitotic proteins inactive until they are targeted to the close vicinity of chromosomes where the RanGEF-induced high concentration of Ran-GTP causes their dissociation from importin beta and activate their mitotic functions (Nachury et al., 2001; Harel et al., 2003; Funabiki et al., 2005).

Centrins are small calcium-binding proteins that function in structures that control microtubule nucleation in a manner conserved from algae and yeast to humans (Baum et al., 1986 and Salisbury et al., 1995). Yeast centrin, Cdc31, associates with the yeast spindle pole bodies and is required for their proper duplication (Ivanovska et al., 2001). Of particular interest to us, Cdc31 also localizes to the yeast nuclear pore (Rout et al., 2000) and plays a role in mRNA export (Fischer et al., 2004). Similarly, human centrin 2 is critical for centriole duplication (Salisbury et al., 2002) and is a key component of the vertebrate nuclear pore, with roles in mRNA and protein export (Resendes et al., 2008).

Given the dual roles of centrin2 and importin beta in interphase at the nuclear pore and in mitosis at the spindle poles and centrosomes, we were interested to determine whether there was a physical link between centrin 2 and importin beta. In previous study, our data showed that GST-Xenopus importin beta pulls down centrin 2 from Xenopus egg extract (Lau and Forbes, 2009 submitted). Similarly, GST-human centrin 2 reciprocally pulled down human importin beta from Hela cell lysate (unpublished data). While this biochemical data showed an interaction between importin beta and centrin 2 in both Xenopus extract and human cells, it remained unclear whether importin beta binds centrin2 via the adaptor, importin alpha via other proteins with which each interact, such as the Nup107-160 complex, or whether importin beta directly interacts with centrin 2. Previous study showed that importin beta negatively regulates TPX2, an initiating spindle

assembly factor, through interaction with the adaptor, importin alpha binding to an NLS in TPX2 (Gruss et al., 2001). However, for centrin 2, sequence analysis using an online NLS predictor program (http://www.rostlab.org/services/predictNLS/), showed no evidence of a potential classical NLS that might bind importin alpha within the centrin 2 protein sequence, leading us to ask whether importin beta might directly interact with human centrin2.

Results and Discussions

To this end, we first carried out a direct binding assay in which GST-Xenopus importin beta and untagged recombinant human centrin 2 were incubated together, in the presence or absence of mutant RanQ69L loaded with GTP. Following incubation of these proteins, glutathione beads were then added to the binding reactions. We found that human centrin 2 directly interacted with Xenopus importin beta (Fig 9A, lane 4, upper panel). This interaction was less in the presence of Ran-GTP (Fig 9A, lane 5, upper panel). GST was used as negative control (Fig 9A, lane 3, upper panel). Thus, our data showed that Xenopus importin beta directly binds to human centrin 2.

Given this finding, it would be predicted that human importin beta should also directly interact with human centrin2. Thus, we carried out a reciprocal direct binding assay using GST-human centrin2 and untagged human importin beta together, in the presence or absence of mutant RanQ69L-GTP. GST-RanQ69L beads and untagged importin beta were used as a positive control for importin beta binding (Fig 9B lane 2, upper panel, while GST was used as a negative control (Fig 9B, lane 3, upper panel). The pulldown results showed that human importin beta directly interacted with human centrin2 (Fig 9B, lane 4, upper panel). As in previous experiment, the interaction was

diminished by addition of Ran-GTP (Fig 9B, lane 5, upper panel). We concluded that both Xenopus and human importin beta directly bind to human centrin 2 and the interaction is reduced in the presence of Ran-GTP.

To further dissect a potential importin beta binding domain in human centrin2, we carried out a similar direct binding assay using either full-length GST-centrin2 or a C-terminal half fragment of centrin2 (GST-C2C). To these, untagged human importin beta was added with or without RanQ69L-GTP. GST-Ran beads and untagged human beta was used as a positive control (Fig 9C, lane 2, upper panel). GST was used as negative control (Fig 9C, lane 3, upper panel). As previously seen, full-length human centrin2 directly interacted with human importin beta in a Ran-dependent manner (Fig 9C, lane 4 and 5, upper panel). However, the C-terminal half of centrin failed to bind importin beta (Fig 7C, lane 6, upper panel). This shows that while the C-terminal domain of centrin 2 is not sufficient for binding to importin beta. It is possible, however, that the C-terminal domain may be necessary for high-affinity binding of full-length centrin to importin beta. Taken together, the data show that both Xenopus and human importin beta can directly interact with human centrin 2 and this interaction can be reduced by the addition of RanQ69L. The results further show that the C-terminal domain of human centrin2 is not sufficient for the direct binding of full-length centrin2 to importin beta.

To understand the nature of importin beta's direct binding to centrin2, we looked at the protein sequence of centrin2 and compared it to known importin beta binding consensus motifs. It was previously shown that importin beta can directly bind proteins with arginine-rich NLS, independently of importin alpha (Palmeri et al., 1999; Gorlich et al., 1999). Interestingly, a sequence of five consecutive amino acids SQRKR is present

on the N terminal, but not the C terminal of centrin2. Thus, it is possible that importin beta directly binds full-length centrin 2 via the N terminal half of centrin 2. Given the known regulatory role of importin beta in mitosis and the function of centrin2 in the dynamic behavior of mitotic centrosome and spindle pole formation, we hypothesize that the function of centrin2 in interphase and mitosis might be temporally and spatially regulated by importin beta.

Four possible scenarios can be imagined . First, in interphase, it is possible that importin beta might function as a cytoplasmic chaperone, directly binding centrin2 to prevent its self-aggregation. Second, in a different scenario, it has been shown that LMB (leptomycinB) inhibition of Crm1-mediated export led to nuclear accumulation of GFP-centrin2 (Keryer et al., 2003) and that centrin2 also plays a role in protein and mRNA export (Resendes et al., 2008). Perhaps importin beta binds and re-imports centrin2 back into the nucleus. Even though, centrin2 is about 20kD and should be able to re-enter the nucleus by simple diffusion, importin-beta mediated import of centrin2 may contribute to the efficiency of centrin 2 accumulation in the nucleus in a timely manner rather than centrin 2 entering by random simple diffusion. This interphase function of importin beta in protecting highly basic proteins and importing them to the nucleus has been previously discussed (Jakel et al., 2000). Thus it is possible that centrin2 is just another target of importin beta.

In mitosis, importin beta negatively regulates nuclear pore assembly by binding to nuclear pore subunits required for assembly steps. It has been shown that ELYS, a pore-targeting protein which functions in the initiating step of nuclear pore assembly by docking to chromatin and subsequently recruiting Nup107-160 complex and other pore

subcomplexes to the forming pore (Rasala et al, 2008). Importin beta was found to bind the Nup107-160 complex and interfere with recruitment of Nup107-160 complex. Interestingly, in previous studies, we found centrin2 in immunoprecipitation of Nup107-160 complex (Art's data) and showed that centrin2 is present at the nuclear pore (Resendes et al., 2008). Thus, the third scenario is that importin beta regulation of the Nup107-160 complex is accomplished via its direct binding to centrin2. For example, importin beta binding directly to centrin 2 on the Nup107-160 complex might interfere with its binding to ELYS in pore assembly.

Lastly, importin beta interaction with centrin2 might be related to the mitotic function of centrin at the spindle poles. Previous studies showed that siRNA knockdown of centrin2 led to inhibition of the duplication of centrioles and aberrant multipolar spindle formation, which demonstrated a functional involvement of centrin2 in centriole duplication and proper centriole separation (Salisbury et al., 2002). Interestingly, it has been shown that importin beta localizes to the mitotic spindle poles and that overexpression of importin beta led to supernumerary spindle poles (Lavia et al., 2004).Thus, in this possibility, centrin2 might normally be regulated by importin beta to ensure proper separation of the centrioles. In the absence of centrin caused either by centrin RNAi knockdown or by directly being sequestered by excess importin beta, premature splitting of the centrioles results in multipolar mitotic spindles which then cause chromosomes missegregation and genetic instability. This eventually results in supernumerary centrosomes and aneuploidy phenotypes which are characteristic of many forms of cancer.

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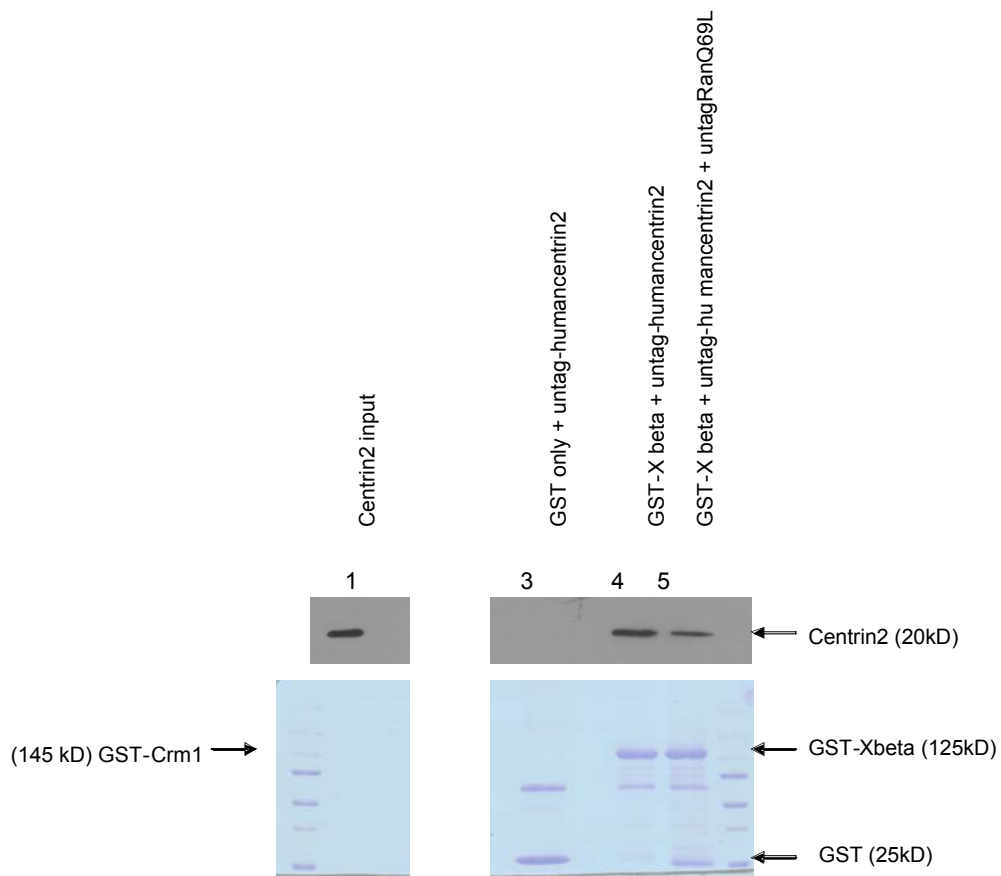
Figure 3.1 Xenopus importin beta directly interact with human centrin2. Xenopus-importin beta directly interacts with human centrin2 (lane 4) and the interaction is irreversible by Ran (lane 5). GST-Crm1 together with untagged human centrin2 was used as a positive control reaction (lane 2). GST was used as negative control (lane 3).
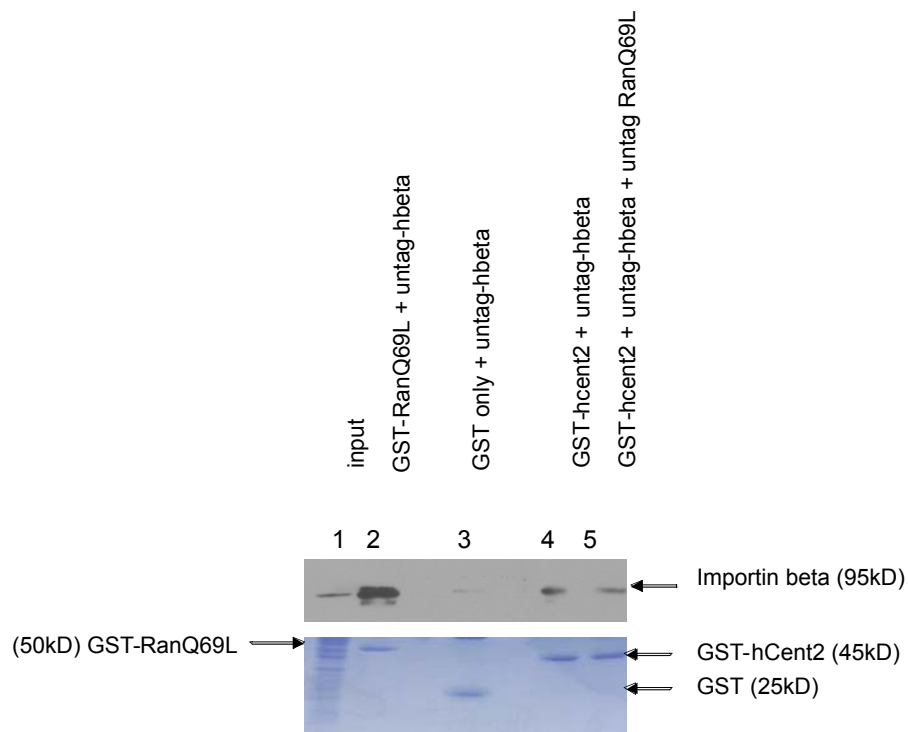
Figure 3.2 Human importin beta directly interact with human centrin2. Human importin beta directly interacts with human centrin2 (lane 4) and the interaction is also diminished by Ran-GTP (lane 5). GST-Ran together with untagged human beta was used as a positive control (lane 2). GST was used as negative control (lane 3).
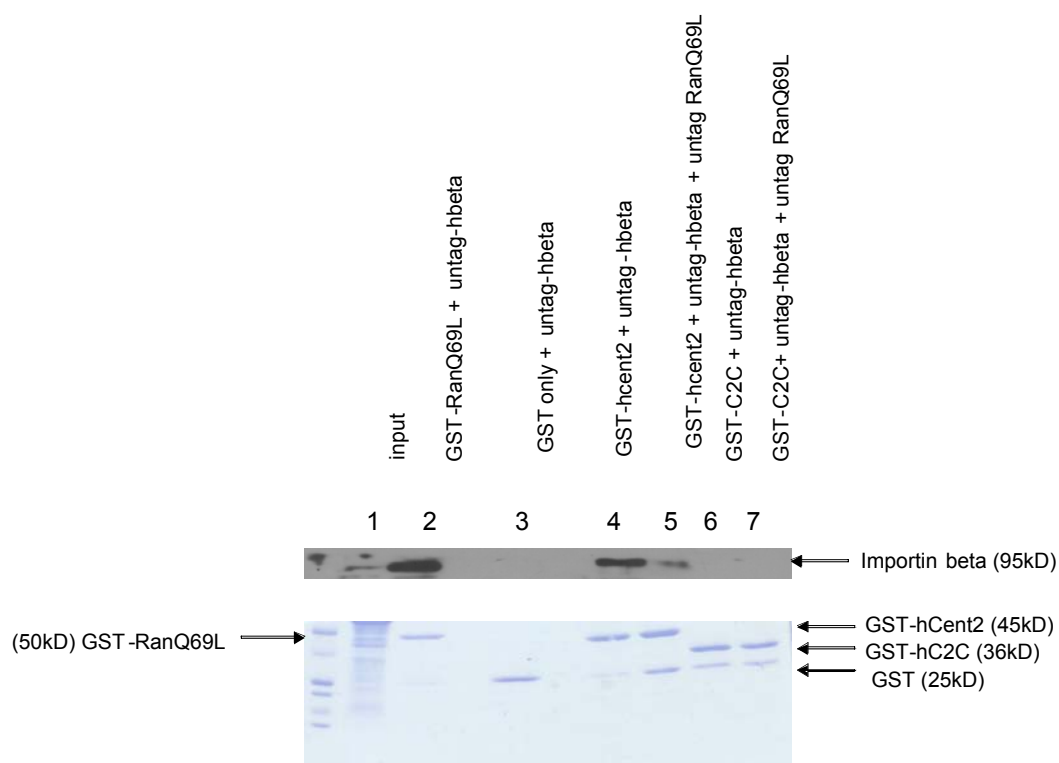
Figure 3.3 Xenopus and human importin beta directly interact with human centrin2
Human importin beta directly interacts with full-length human centrin2, but not via the C-
terminal of centrin2. Full-length human centrin2 directly interacts with human importin
beta in a Ran-dependent manner, as previously shown (lane 4 and 5); however, the C-
terminal half of centrin failed to bind importin beta (lane 6), suggesting that while the C-
terminal domain of centrin is not sufficient for binding to importin beta.

CONCLUSIONS and FUTURE DIRECTIONS

In summary, our goal in this study was to address the question of whether a potential TREX 2 complex exists in vertebrates. In yeast, the TREX2 complex, which is comprised of four protein subunits, including Sac3, Thp1, Sus1, and Cdc31, is essential for coupling transcription to mRNA export. The yeast centrin protein, Cdc31, was found not only at the spindle pole bodies but also localized to the yeast nuclear pore (Rout et al., 2000); it is and involved in mRNA export, but not nuclear protein export (Fischer et al., 2004). Recent work from our lab found that human centrin 2, the vertebrate homologue of yeast Cdc31, localizes to the mammalian nuclear pore and functions in mRNA and nculear protein export (Resendes et al., 2008). This finding led to our search for potential vertebrate homologues of other members of the yeast TREX2 complex. Of the three Sac3-related proteins identified in previous homology studies (Abe et al., 2000; Kuwahara et al., 2001), GANP and MCM3AP contain non-Sac3 domains with other unrelated enzymatic functions. Shd1, despite being smaller in size (only a third of yeast Sac3) and lacking the centrin-interacting-domain (CID), has been shown to be most similar to Sac3 in mitotic function essential for proper cell cycle progression (Khuda et al., 2004). Thus, we assessed the function of human Shd1 and found that overexpression of Shd1/Sac3 led to the nuclear accumulation of poly(A)+ RNA. This functional defect in mRNA export mirrored the effect of overexpression of dominant negative fragments of centrin 2 (Resendes et al., 2008). We next looked for a vertebrate homologue of yeast Sus1 and found a single Drosophila homologue, E(y)2, which had been reported to have an effect on mRNA export (Kurshakova et al., 2007). We found that overexpression of a human homologue, hEny2, also caused a nuclear accumulation of mRNA. Furthermore,

we cloned a human GST-Eny2 to perform pulldown experiments to determine potential interacting proteins. We found that human Eny2 biochemically interacts with multiple nuclear transport factors involved in mRNA export and nuclear protein import. Of the tested proteins, Eny2 was found to interact with the human mRNA export receptor, TAP/NXF1, and nucleoporins with a known functional involvement in mRNA export, Nup160 and Nup153. Major nuclear import factors also found to interact with Eny2 included importin alpha, importin beta, transportin, and the small regulatory GTPase Ran. GST-Eny2, however, failed to pulldown either the NES export receptor Crm1, consistent with the lack of effect on nuclear protein export, and human centrin 2.

Even though Eny2/Sus1 failed to bring down with centrin 2 by pulldown, we think that this can be due to technical limitations of the pulldown assay used in our study. It could be that the recombinant GST-Eny2 cannot disrupt the stable interaction of endogenous Eny2 with centrin 2 in the putative vertebrate TREX2 complex. It will be of future interest to perform immunoprecipitation with antibodies to Eny2, Shd1, or centrin 2 to determine what proteins are associated within the putative endogenous TREX complex, as this would reveal the molecular composition of the intact complex as it is derived from cells. Another approach that would prove useful will be perform similar experiments with human Thp1/PICD2, which when depleted by RNAi in Drosophila proved to cause a nuclear accumulation of poly(A)+ RNA (Farny et al., 2008). Productions of antibodies to human PICD2/Thp1 will be of great aid to the identification of a putative TREX 2 complex in vertebrates.

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MATERIALS and METHODS

Antibodies

Commercial antibodies used for Western blotting include: human centrin 2 (Cell Signaling Technologies, Danvers, MA), human centrin 1 (also recognize centrin 2) (Sigma, St. Louis, MO), anti-htransportin Ab (BD Biosciences, San Jose, CA), anti-Ran Ab (BD Biosciences), anti-hTAP mAb (BD Biosciences), anti-importin alpha (BD Biosciences), and MAb414 anti-FG Nups (Covance, Berkeley, CA). Other antibodies used were importin beta (Harel et al., 2003), hCrm1, Nup62 (a gift from Val Delmar); anti-hNup160 (Vasu et al., 2001), anti-hNup153 (Forbes et al., 1999). For visualizing HA-tagged constructs by immunofluorescence: mouse monoclonal anti-HA Ab (Abcam, Cambridge, MA) was used together with anti-mouse Oregon green or Texas red secondary antibodies. For immunofluoresence of myc-tagged proteins: FITC-labeled anti-myc Ab (Santa Cruz Biotechnology, Santa Cruz, CA) or TRITC-labeled anti-myc Ab (1:100; Santa Cruz Biotechnology, Santa Cruz, CA).

Constructs

HA-tagged Eny2 was obtained from Didier Devys (Zhao et. al.,2008). In addition, a full-length cDNA clone of HsENY2 (Accession No NM_020189.4) was obtained from Origene (Rockville, MD). Oligonucleotides were used to amplify the Eny2 coding region from the cDNA clone, which were subcloned as BanH1-Xho1 fragments into a pGEX6P3 (GE Healthcare, Piscataway, NJ) clevable GST vector . A full-length cDNA clone of HsShd1(Accession No NM_013299.2) was obtained from Origene (Rockville, MD).

Oligonucleotides were used to amplify the Shd1 coding region from the cDNA clone, which were subcloned as EcoRI-Kpn1 fragments into a CDNA3.1 myc-tagging transfection vector (Invitrogen, Carlsbad, CA). All sub cloning was performed by K. Resendes.

GST pulldowns. Recombinant GST, GST-Eny2 were incubated with Glutathione-Sepharose beads (GE Healthcare) without crosslinking for 1.5 hour with rotation at 4°C, then blocked with 20 mg/mL Bovine Serum Albumin (BSA) in PBS for 30 minutes. Meanwhile, cell lysates were added to BSA-blocked beads and were incubated for 30 min with rotation at 4°C to pre-clear the lysates. After pre-clearing, the lysates were then added to the beads and incubated for 2 hours at 4°C (25 uL cytosol in 500 uL PBS). After washing the beads with lysis buffer and PBS, the bound proteins were eluted with 0.1 M Glycine, pH 2.5, and neutralized with 1 M Tris, pH 8.0. The reaction was loaded for SDS-PAGE and processed for immunoblotting.

GST direct binding assay

Procedures were performed as previously described in Lau et al., 2009. The only modification was final concentration of recombinant proteins (GST-centrin 2, GST-Xenopus importin beta, untagged beta, untagged centrin 2) used at 2.5uM per reaction.

Poly(A)$^+$ RNA nuclear accumulation assay

Cells were grown on coverslips for 1 day and then transfected for ≃24 h with

control plasmids (HA-Lap2 or PK-myc) or plasmids encoding HA-Eny2 or Shd1-myc, using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). Cells were fixed (3% formaldehyde in PBS; 20 min on ice), permeabilized (0.5% Triton X-100 in PBS), incubated for 5 min with PBS plus 1 mM vanadyl ribonucleoside complexes (VRC) and then for 5 min with 2x SSC (1x SSC is 0.15 M NaCl plus 0.015 M sodium citrate) plus VRC (0.3 M NaCl, 0.03 M sodium citrate [pH 7]), and then prehybridized with 50% formamide, 2x SSC, 1 mg/ml bovine serum albumin, 1 mM VRC, and 10% dextran sulfate (1 h at 37°). The cells were hybridized with Cy3-oligo(dT)$_{50}$ (GeneLink, Hawthorne, NY) at 100 pg/μl in the same buffer (overnight at 37°C), washed three times in 2x SSC (at 37°C for 5 min each), and then refixed with 3% formaldehyde in PBS for 20 min on ice. The expression of transfected proteins was detected with FITC-labeled anti-myc Ab for Shd1-myc or PK-myc (1:100; Santa Cruz Biotechnology, Santa Cruz, CA) and with primary anti-HA Ab followed by secondary Oregon green goat-anti-mouse Ab for HA-Lap2 and HA-Eny2 .Coverslips were mounted on Vectashield (Vector Laboratories, Burlingame, CA) and visualized with a Zeiss Axioskop fluorescence microscope (Carl Zeiss, Thornwood, NY) at a magnification of x63, using an oil objective lens.

Nuclear protein import and export

RGG2.2 cells which were stably transfected with the Rev-glucocorticoid-green fluorescent protein (GFP) (pXRGG) plasmid (Hanover et al., 1998) were transiently transfected with either (i) the control plasmid encoding HA-Lap2 or PK-myc, or (ii) HA-Eny2 or Shd1-myc in pCDNA3.1, using Lipofectamine 2000 (Invitrogen, Carlsbad, CA).

24 hours post transfection, the cells were treated with dexamethasone (final concentration 1 µM) (Sigma-Aldrich, St. Louis, MO) for 60 min to induce RGG import. In parallel, an identical set of transfected cells were treated with dexamethasone for 60 min to induce RGG import, washed, and then incubated with medium lacking dexamethasone (for 2 h at 37°C) to promote RGG export. Cells were fixed (with 3% formaldehyde in PBS for 15 min on ice), permeabilized (with 0.5% Triton X-100 in PBS for 10 min), and blocked (with 5% FBS in PBS for 10 min). The transfected expressed proteins were detected with tetramethyl rhodamine isocyanate (TRITC)-labeled anti-myc Ab (1:100) (Santa Cruz Biotechnology, Santa Cruz, CA) or, in case of HA-tagged proteins, primary anti-HA antibodies were used followed by secondary Texas red antibody, and in the case of RGG, by its GFP moiety. Coverslips were mounted on Vectashield (Vector Laboratories, Burlingame, CA) and visualized with a Zeiss Axioskop fluorescence microscope (Carl Zeiss, Thornwood, NY) at a magnification of x63, using an oil objective lens.