UNIVERSITY OF CALIFORNIA SAN DIEGO

Analysis of Regulation of Major Mitotic Assembly Events *In Vitro* by Nuclear Import and Export Receptors

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

Doctor of Philosophy

in

Biology

by

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This Dissertation of Matthew S. Nord is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

Chair

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Dedication

Dedicated to my mother, Kelly,
who has demonstrated time and again what true perseverance looks like.
You have instilled in me the qualities necessary to complete my graduate training.
Thank you.

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Acknowledgements

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Chapter 2, in full, is a reprint as it appears in Molecular Biology of the Cell: Bernis, C., B. Swift-Taylor, **Matthew S. Nord**, S. Carmona, Y. M. Chook, and D. J. Forbes. 2014. "Transportin Acts to Regulate Mitotic Assembly Events by Target Binding Rather than Ran Sequestration." *Molecular Biology of the Cell* 25 (7): 992–1009. https://doi.org/10.1091/mbc.E13-08-0506. I am the tertiary author of this paper.

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ABTRACT OF THE DISSERTAION

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by Nuclear Import and Export Receptors

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Professor Douglass J. Forbes, Chair

This dissertation is an analysis of regulation of major mitotic assembly events by nuclear transport receptors, or karyopherins. Karyopherins, composed of both importins and exportins, are a large family of proteins that transport specific proteins and RNAs between the cytoplasm and nucleus of the eukaryotic cell. Each karyopherin has a distinct set of cargoes that it binds and transports through the nuclear pore complex. A high concentration of RanGTP inside the nucleus, created by the chromatin-bound enzyme RCC1, is harnessed by karyopherins to achieve

transport directionality. During mitosis, upon nuclear breakdown, RCC1 continues to produce a high concentration of RanGTP around the mitotic chromosomes. Away from the chromosomes, where RanGTP is low, Importins α and β inhibit assembly factors for spindle, nuclear membrane, and nuclear pore assembly. However, near the chromosomes, where RanGTP levels are high, Importins α and β release assembly factors with the result that mitotic structures only assemble around the chromosomes. Thus, evolution has co-opted the RanGTP/karyopherin system to spatially regulate the assembly of multiple critical structures in mitosis. Much of this has been determined in *Xenopus* egg extracts, which are unique in that one can reconstitute cellular events in cell cycle-specific states.

Chapter 2 extends the role of RanGTP/karyopherin spatial regulation by dissecting the mechanism of action by which the import receptor Transportin regulates mitotic assembly events. Using a variety of molecular tools and *Xenopus* egg extracts, we show that Transportin inhibits the assembly of major mitotic structures, not by titrating RanGTP, but by directly blocking proteins needed for assembly everywhere except in areas near mitotic chromosomes.

Chapter 3 presents an extensive published review of karyopherins as global regulators of mitotic assembly events, both *in vivo* and *in vitro*, including protein interactions, checkpoints, and critical proteins required for proper assembly.

Chapter 4 dissects whether export receptors, or exportins, play a role in mitotic assembly regulation, focusing on Crm1/Exportin-1, Exportin-tRNA, and

Exportin-5. Using *Xenopus* egg extracts, we find that exportins are potent inhibitors of spindle assembly, nuclear membrane fusion, and nuclear pore formation, and their inhibition can be, to an extent, counteracted by RanGTP.

Chapter 1: General Introduction

The eukaryotic cell is defined by its nucleus. A large, double-membraned organelle, the nucleus contains and separates the genetic material of the organism into its own compartment. Many cellular processes occur inside the nucleus, including the replication of DNA, transcription of mRNA, and the synthesis of ribosomal subcomplex components. The biochemical environment within the nucleus allows the cell to control these crucial processes, along with others, with exquisite precision in both a spatial and temporal manner. This allows the cells to tightly control when a gene is activated. The physical demarcation between the cytoplasm and the nucleoplasm is provided by the nuclear envelope, which consists of a double membrane, nuclear lamina, and nuclear pores. The inner nuclear membrane (INM), along with its integral membrane proteins, physically interacts with the underlying nuclear lamina, a structural support system that functions to maintain the integrity of the nucleus. The outer nuclear membrane (ONM) has a separate set of integral membrane proteins and is contiguous at points with the endoplasmic reticulum (ER).

The Nuclear Pore Complex

Because the nuclear envelope provides a physical barrier between the cytoplasm and nucleoplasm, there must be gateways to allow passage of material between the two distinct environments. Nuclear pore complexes (NPCs) have evolved to serve as these gateways. The NPC is an extremely large structure,

~120MDa in size in vertebrates, and is composed of a family of proteins collectively known as nucleoporins or Nups. Approximately 30 different Nups, present in 8-32 copies each, create the nuclear pore complex (Figure 1.1). The Nups that compose the NPC are arranged in eightfold-radial symmetry and range from the Nups forming cytoplasmic filaments, to scaffolding Nups supporting the inner channel, to the Nups that comprise the nuclear basket in the nucleoplasm.

Within the NPC, there exist different classes of Nups. Integral membrane Nups, including gp210, NDC1, and Pom121, anchor the NPC structure within the nuclear membranes (Figure 1.1, brown). Scaffold Nups, such as the Nup107-160 "Y" complex and the Nup205 complex, together form the massive architectural support of the NPC (Figure 1.1, green and yellow). Along the central axis of the NPC are phenylalanine-glycine or FG Nups, which comprise 1/3 of the total Nups in the NPC (Figure 1.1, red). These FG Nups contain multiple phenylalanine-glycine repeats in their amino acid sequence. The FG Nups specific to the central channel are responsible for creating a hydrogel mesh network at the center of the NPC. This network creates the main permeability barrier between the cytoplasm and nucleus, of which the Nup98 GLFG (glycine-leucine-phenylalanine-glycine) repeats form the most restrictive barrier (Labokha et al., 2013; Schmidt and Görlich, 2015). A cross-section of the barrel-shaped NPC is shown in Figure 1.1.

The central channel of the NPC, composed of the FG Nup hydrogel, is what allows for the passage of material between the cytoplasm and the nucleoplasm. While there are FG repeats found on a number of different Nup subunits, from the

cytoplasmic filaments to the nuclear basket, the Nup62 complex of FG Nups and specifically Nup98 are responsible for the selectivity of the central channel hydrogel (Chug et al., 2015; Hülsmann et al., 2012; Labokha et al., 2013; Schmidt and Görlich, 2015). Because the passage of material must be tightly controlled, the central channel of the NPC provides a selective barrier, which still allows free passage of molecules ranging from water and ions to proteins up to 20-40 kDa in size. The central meshwork of the NPC channel, however, prevents unescorted entry or exit of larger proteins or RNPs. Instead, proteins or RNPs larger than 20-40 kDa requiring transport must be actively transported through the NPC by a family of proteins collectively known as karyopherins (Barry and Wente, 2000; D'Angelo and Hetzer, 2008; Izaurralde and Adam, 1998).

Karyopherins: Nuclear Transport Receptors

Karyopherins, also known as nuclear transport receptors, vary in number in different organisms. Yeast have 14 karyopherins, while humans have 21 (Güttler and Görlich, 2011). The karyopherin family of proteins has evolved into two very different sides of the family: importins and exportins. Importins in the cytoplasm recognize nuclear localization signals, or NLSs, on cargoes and transport them into the nucleus (Conti et al., 1998; Dingwall and Laskey, 1991; Soniat and Chook, 2015). Different importins recognize different NLS's. Importin β was the first transport receptor discovered and often uses an adaptor, Importin α, to recognize an NLS. One

example of an NLS for Importins α and β is termed the "classical" NLS. First identified in the SV-40 large-T antigen, its NLS is a short stretch of amino acids that are positively charged, the amino acid sequence being PKKKRKV (Christophe et al., 2000; Kalderon et al., 1984a, 1984b; Lange et al., 2007; Nigg, 1997). Another example of an NLS is the "PY" containing NLS (proline-tyrosine), which the importin, Transportin, recognizes. While there is great variability in this NLS, a commonality is the proline-tyrosine found in the amino acid sequence of the cargo proteins of Transportin (Chook and Süel, 2011; Lee et al., 2006; Soniat and Chook, 2015). Conversely, exportins recognize nuclear export signals, or NESs, on export cargoes within the nucleus and transport them to the cytoplasm (Figure 1.2). The exportin Crm1 recognizes what is now called a "classical" NES, or a leucine-rich NES. Over a stretch of ~10-15 amino acids, a common amino acid found is leucine, or other amino acids of similar nature, such as isoleucine (Dong et al., 2009a; Fornerod et al., 1997; Kutay and Güttinger, 2005; Ossareh-Nazari and Dargemont, 1999).

Both importins and exportins interact with FG Nups and, by binding to sequential FG Nups, traverse the selective FG Nup meshwork through the NPC. Because all importins and exportins bear some similarity to Importin β, karyopherins are said to be members of the Importin β family (D'Angelo and Hetzer, 2008; Fried and Kutay, 2003; Güttler and Görlich, 2011).

The Ran Gradient and Directionality of Transport

The directionality of nuclear transport, whether import or export, relies on the biochemical distribution of the small GTPase Ran. Ran is converted to its active form, RanGTP, inside the nucleus by the RanGEF (guanine exchange factor) , RCC1. As RCC1 is a chromatin-bound enzyme, the concentration of RanGTP is very high in the nucleus (Bischoff and Ponstingl, 1991a, 1991b). On the cytoplasmic side of the NPC, the Ran GTPase-activating protein, RanGAP, stimulates Ran to hydrolyze its GTP to GDP, rendering Ran into its inactive form. Thus, the location of RCC1 and RanGAP on opposite sides of the nuclear envelope creates a steep biochemical gradient that can be utilized to direct nuclear transport (Hopper et al., 1990; Kalab et al., 2002) (Figure 1.3). Nuclear transport receptors then harness this gradient to transport their cargos across the nuclear envelope.

In general, importins bind cargo bearing a nuclear localization signal (NLS) in the cytoplasm, and travel inward. Often, import of a cargo requires an adaptor protein. For example, the import complex, Importin β/Importin α/NLS cargo, passes through the nuclear pore where it then encounters and binds to RanGTP within the nucleus. This causes a conformational change in Importin β, which promotes its dissociation from Importin α and its import cargo, completing import (Figure 1.4). Conversely, inside the nucleus, exportins differ from importins in that they simultaneously bind cargo bearing a nuclear export signal (NES) and RanGTP, forming a ternary export complex. This export complex travels out through the nuclear pore, then encounters RanGAP on the cytoplasmic filaments of the NPC.

RanGAP stimulates RanGTP to hydrolyze its GTP to a GDP, which causes a conformational change in the exportin that results in disassembly of the export complex (Figure 1.5) (Barry and Wente, 2000; D'Angelo and Hetzer, 2008; Fried and Kutay, 2003; Izaurralde and Adam, 1998; Weis, 2003).

Mitotic Roles for Nuclear Transport Receptors and the Ran Gradient

The Ran gradient is equally important to the cell during mitosis, when the nuclear envelope is broken down. Interestingly, the steep biochemical gradient of RanGTP is still present around the chromosomes, as the RanGEF RCC1 is chromatin-bound and remains active (Hutchins et al., 2004; Li and Zheng, 2004). This high concentration of RanGTP around the mitotic chromosomes has been shown to be utilized as a cellular "GPS" that has proven crucial for proper mitotic spindle formation (Figure 1.6) (Kalab and Heald, 2008). Early studies found RanGTP to be a critical regulator of mitosis: these studies showed that the high RanGTP concentration around the chromosomes induces microtubule polymerization and mitotic spindle formation, doing so by freeing spindle assembly factors (SAFs) from inhibition by Importin α/β (Carazo-Salas et al., 2001; Kalab et al., 1999; Ohba et al., 1999; Wilde and Zheng, 1999; Zhang et al., 1999). These studies showed that at locations away from the chromosomes, where RanGTP is low or absent, Importins β and α bind to certain spindle assembly factors (SAFs) in an NLS-dependent manner, which effectively inhibits them and shuts off their spindle-promoting functions (Du et al.,

2002; Ems-McClung et al., 2003; Gruss et al., 2001; Nachury et al., 2001; Ribbeck et al., 2006; Schatz et al., 2003). This RanGTP-mediated "GPS" enables the cell to assemble the spindle in the correct area of the cell during mitosis, i.e., around the mitotic chromosomes. Thus, the cell effectively utilizes the RanGTP gradient in a parallel manner in mitosis, when there is no nuclear envelope and no nuclear transport.

While the mechanism of Importin β inhibition was first discovered to be active during spindle assembly, it was later studied in downstream mitotic assembly processes, such as the reformation of the nuclear membrane (Hachet et al., 2004; Harel et al., 2003; Hetzer et al., 2000, 2002; Lu et al., 2012; Zhang et al., 2002) and the formation of the nuclear pores (Harel et al., 2003; Walther et al., 2003). It was found that Importin β negatively regulates both nuclear membrane fusion and nuclear pore formation *in vitro,* and that RanGTP is able to relieve the inhibition (Delmar et al., 2008; Harel and Forbes, 2004; Harel et al., 2003). This model of karyopherin regulation of mitosis was expanded further when it was found that Transportin, a close relative of Importin β, was also able to regulate the same mitotic assembly processes as Importin β. Moreover, Transportin's inhibition of assembly was found to be counteracted by RanGTP (Lau et al., 2009) (Figure 1.6). Furthermore, using a set of molecular tools described fully in Chapter 2, we determined that, mechanistically, Transportin was regulating the three mitotic assembly processes by directly binding and inhibiting protein targets required for proper assembly, in a manner parallel to the way in which Importin β functions (Bernis et al., 2014).

In Vitro Assays and *Xenopus* Egg Extracts

Much of the work that contributed to the discovery and analysis of the regulatory role of Importins α/β and Transportin in mitosis was accomplished with the aid of *in vitro Xenopus laevis* egg extracts. *Xenopus* eggs are relatively large (~1mM in diameter) and are arrested in metaphase of meiosis II (Tunquist and Maller, 2003). They are primed for rapid cell division after fertilization and, as such, store large amounts of the cellular components needed to support rapid cell growth (Forbes et al., 1983; Lohka and Maller, 1985; Lohka and Masui, 1984; Newport, 1987). This makes *Xenopus* egg extracts ideal for studying biochemical processes *in vitro.* Two different types of *Xenopus* egg extracts were used in this study: mitotic and interphase extracts. Mitotic (or CSF) extract is prepared in the presence of the Ca^{2+} chelator EGTA. When the eggs are lysed, the EGTA prevents Ca^{2+} release . This preserves the mitotic state of the extract, making it ideal for the study of spindle assembly *in vitro* (Desai et al., 1998; Maresca and Heald, 2006). In contrast, interphase extracts are used to mimic the telophase assembly events of nuclear membrane and nuclear pore assembly. Such extracts are formed by lysing *Xenopus* eggs in the presence of Ca^{2+}. This allows the extract to progress out of mitosis and into interphase. When chromatin is added to interphase extracts, nuclei form around the decondensing chromatin. Such nuclei have been shown to possess a functional nuclear import system (Bernis and Forbes, 2014; Chan and Forbes, 2006; Finlay and Forbes, 1990). With their capacity for biochemical manipulation by addition of exogenous proteins, small molecule inhibition, immunodepletion, and many other

methods of experimentation, the *Xenopus* egg extracts provide a powerful platform for studying processes that play different roles in physiologically distinct phases of the cell cycle, such as the role of Importin β in regulating nuclear import during interphase and in regulating mitotic assembly events, such as spindle formation, in mitosis.

Expanding the Roles of Karyopherins During Mitosis

In Chapter 2 of this dissertation, we greatly expand upon the mechanism of regulation of major mitotic events by importins. Transportin, an import karyopherin, had been shown previously to negatively regulate three major mitotic assembly events: spindle formation, nuclear membrane fusion, and nuclear pore formation (Lau et al., 2009). We sought to discern whether Tranportin acts mechanistically in the same way or in a different way from Importin β in regulating these mitotic assembly events. We asked whether, for example, Transportin sequesters RanGTP away from Importin β, thus preventing Importin β from releasing its bound target proteins. Conversely, does Transportin act in a manner parallel to Importin β, i.e., by directly binding and inhibiting a set of spindle assembly factors. If the latter, does Transportin bind a distinct set of target proteins? For this, we took advantage of molecular tools such as: (a) a mutant Transportin, termed TLB, that cannot release bound assembly factors regardless of RanGTP binding, and (b) M9M, a protein containing a super PY NLS chimeric protein that is capable of displacing any bound

assembly factors from Transportin. We determined that Transportin indeed regulates mitotic assembly events in a parallel pathway to Importin β, i.e., by binding directly to target assembly factors (Bernis et al., 2014).

In Chapter 3 of the dissertation, we present a comprehensive review of the state of karyopherin regulation of mitotic assembly events (Forbes et al., 2015). The faithful segregation and distribution of chromosomes during mitosis is one of the most critical cellular functions and must be executed with high fidelity by the cell. Any deviations from near-perfection can lead to a delay in mitosis, to aneuploidy, or even to apoptosis. As such, there is a high level of regulation the cell employs to ensure proper cell division. Large structures must be assembled, including the microtubule-based spindle, and the kinetochores, which are large protein complexes that provide an interface between the chromosomes and mitotic spindle and senses tension in the microtubules. These events are under surveillance by karyopherin family members in conjunction with RanGTP. Summarization of the field at the time led us in a direction that was undefined previously in the literature: the role of exportins in mitotic assembly events.

In Chapter 4 of the dissertation, we explored the "GPS" model of karyopherin regulation of mitotic events further, asking if *exportins* can regulate mitotic assembly events. We focused on Crm1/Exportin-1, Exportin-t, and to a lesser extent, Exportin-5. As all three are members of the karyopherin family, they all bear a certain resemblance to one another. All karyopherins possess a CRIME domain, or the "Crm1, Importin β, Etc." domain on their N-terminus that provides significant

binding sites for RanGTP (Cook et al., 2007; Ossareh-Nazari and Dargemont, 1999). In addition, the major structural building blocks of all karyopherins are HEAT (Huntington's, elongation factor 3, protein phosphatase 2a, and TOR1 kinase) repeats, which form helical structures (Yoshimura and Hirano, 2016). Crm1 has 21 HEAT repeats, organized overall to form an inner tube-like shape (Dong et al., 2009b; Güttler and Görlich, 2011; Monecke et al., 2009). RanGTP binds to the center of the Crm1 structure. In contrast, the leucine-rich NES of a given export cargo protein binds between HEAT repeats 11A and 12A (Fornerod et al., 1997). Because this NES-binding cleft is on the outside of Crm1, it can accommodate each of many different NES-bearing proteins for transport across the nuclear envelope without steric interference. Crm1 carries a diverse array of cargo. Crm1 export cargoes range from ribosomal subunits to transcription factors, from anti-tumor proteins to pro-apoptotic factors, among others (Xu et al., 2012). Crm1 also exports snRNPs and the Rev/unspliced HIV RNA complex (Booth et al., 2014; Yi et al., 2002). There have been several large-scale screens to identify NES-bearing proteins in the past few years; however, of the many proteins each identified, only a small subset of protein hits overlap in the respective screens (Kırlı et al., 2015; Thakar et al., 2013; Wühr et al., 2015). Additionally, there is an actively updated database of all experimentally confirmed NES-bearing Crm1 cargo (Xu et al., 2012).

Many of the above Crm1 cargoes are transcription factors that shuttle in and out of the nucleus for their function. It has been found that when Crm1 is overexpressed, as it is in several cancer types, it promotes excessive nuclear export of

anti-tumor proteins and pro-apoptotic factors, such as p53, FOXO, BRCA1, and BARD1 (Brodie and Henderson, 2012; Dickmanns et al., 2015; Freedman and Levine, 1998). Because the proteins are exported, they cannot perform their function inside the nucleus, which contributes to a cancerous phenotype. There are several chemical inhibitors, however, that specifically target Crm1 by covalently binding to Cysteine 528 inside the NES binding cleft, preventing Crm1from binding to its cargoes. The most well-known is Leptomycin B, which is fairly toxic to human cells by 24 hours (Kudo et al., 1999; Wolff et al., 1997). However, new drugs collectively known as Selective Inhibitors of Nuclear Export (SINEs) are less toxic and are showing great promise in clinical trials as chemotherapeutic agents, as they have been shown in culture to return cancer cells to a more normal state (Boons et al., 2015; Mendonca et al., 2014; Sun et al., 2016).

Exportin-t, in contrast to Crm1, has a much more clearly defined set of export cargo, tRNA. This makes Exportin-t a distinctly different candidate for further analysis in mitotic function due to its limited cargo range. Consisting of 19 HEAT repeats, Exportin-t binds tRNA and RanGTP in a cooperative manner (Arts, 1998; Kuersten et al., 2002; Kutay et al., 1998). Interestingly, Exportin-t overexpression is involved in several types of cancer, including breast and ovarian cancer and mesothelioma. While Exportin-t overexpression was found to correlate to a poor prognosis in these types of cancer, the mechanism of action is still unclear (Çağatay and Chook, 2018; Sun et al., 2018; Vaidyanathan et al., 2016).

Exportin-5 primarily exports pre-microRNAs (pre-miRNAs). It also has been found to export certain RNA/protein complexes, tRNAs, and the 60s ribosomal subunit (Bohnsack et al., 2004; Chen et al., 2004; Güttler and Görlich, 2011; Wild et al., 2010; Yi et al., 2003). Because Exportin-5 mediates the export of pre-microRNAs, any disruption of this export leads to problems with gene expression, as mRNA levels can no longer be correctly regulated by the corresponding anti-sense mature microRNA (miRNA). There is an interesting example of an Exportin-5 mutant that directly leads to cancer. In this case, Exportin-5 is expressed as a truncated mutant form, which prevents it from exporting its pre-microRNA cargo. The mutant Exportin-5 and its pre-microRNA cargo accumulate in the nucleus, ultimately leading to cancer (Melo et al., 2010). With such health-related significance, the study of Exportin-5 in different cell cycle events, such as mitosis, is highly warranted.

Chapter 4 of this dissertation directly examines whether there are potential regulatory roles for *exportins* in the three major mitotic assembly processes: spindle assembly, nuclear membrane fusion, and nuclear pore formation. We examine Crm1/Exportin-1, Exportin-t, and perform preliminary experiments on Exportin-5. We found that the exportins Crm1 and Exportin-t can inhibit these three assembly processes *in vitro*. Moreover, the inhibited phenotypes the exportins produce can be counteracted by the simultaneous addition of RanGTP. Crm1 and Exportin-t can be counteracted for both membrane fusion and nuclear pore formation by RanGTP. Also, when nuclear pore formation was examined with Exportin-5, we found it was

inhibited and could be counteracted by RanGTP. For spindle assembly, added Crm1 and Exportin-t both inhibit mitotic spindle assembly. Interestingly, Exportin-t shows distinctive intermediate spindle phenotypes that suggest some counteraction is possible. We have subsequently identified nucleoporin targets that could be under direct regulation by the exportins Crm1 and Exportin-t for assembly.

In summary, this is the first study to show that *exportins* can regulate the major mitotic assembly events from spindle assembly to nuclear pore formation. We provide direct evidence that the Exportins Crm1, Exportin-t, and Exportin-5 can negatively regulate one or more major mitotic events *in vitro*. This work is additionally significant because many of the studies implicate dysregulation of the exportins in cultured cancer cells or in human cancer. Inhibition of Crm1-mediated nuclear export by SINEs is being explored in clinical studies of multiple cancer types. However, the role that the exportins play in mitosis cannot, and should not, be ignored when considering them as therapeutic targets in disease treatment.

Figures

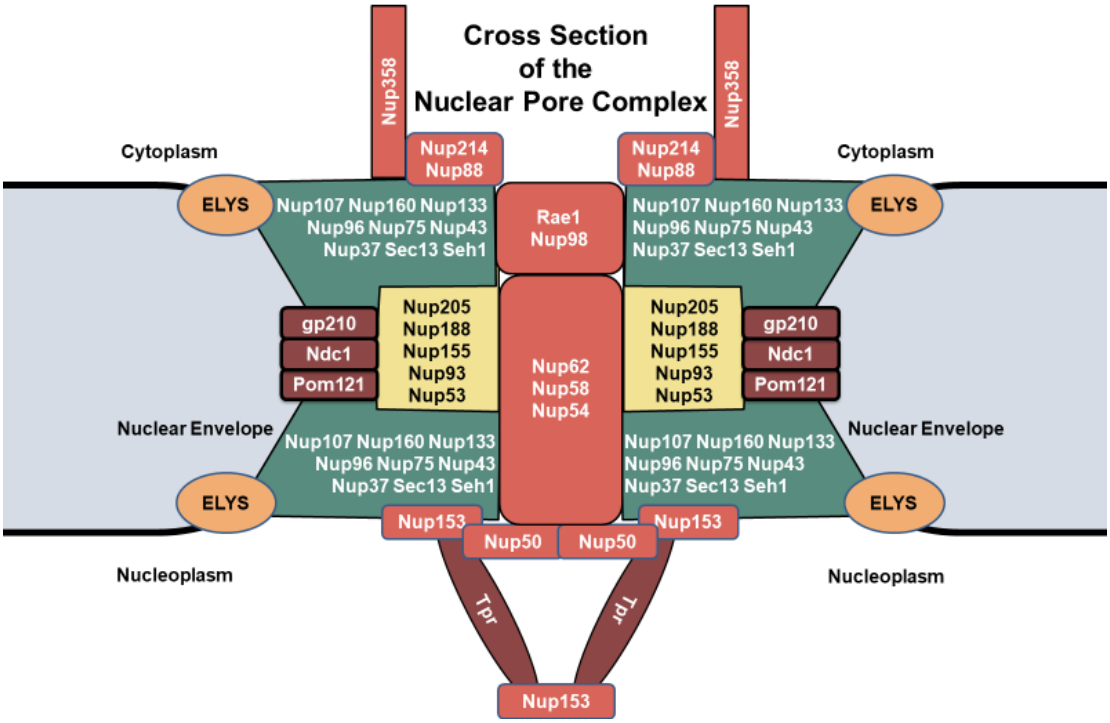

Figure 1.1: Schematic of the Nuclear Pore Complex. The nuclear pore complex perforates the nuclear envelope. Nucleoporins, or Nups, compose the nuclear pore and are present in multiple different copies. The shapes shown indicate specific nucleoporin subcomplexes. Mitosis in most higher eukaryotes results in disassembly into the subunits shown (Beck and Hurt, 2017; D'Angelo and Hetzer, 2008; Kabachinski and Schwartz, 2015; Wozniak et al., 2010).

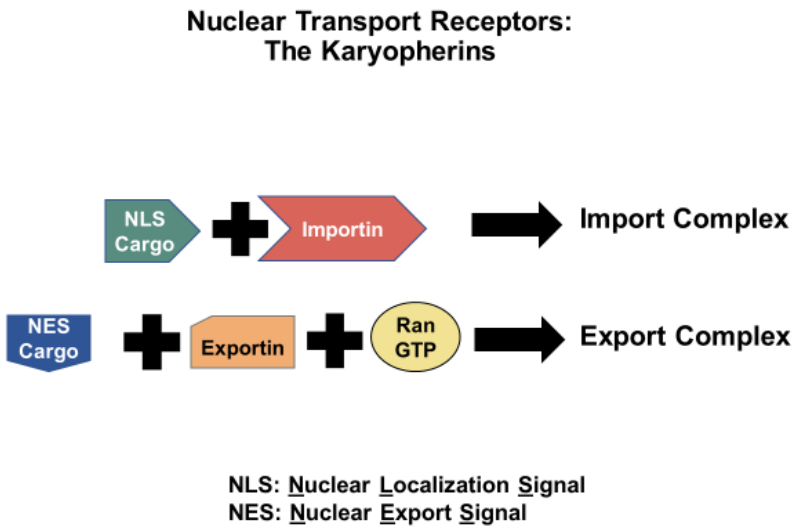

Figure 1.2: Nuclear Transport Receptors, also known as Karyopherins. An importin binds to cargo with a nuclear localization signal (NLS) and transports the cargo from the cytoplasm into the nucleus. Exportins bind to cargo bearing a nuclear export signal (NES), along with RanGTP, and transports the cargo from the nucleus into the cytoplasm.

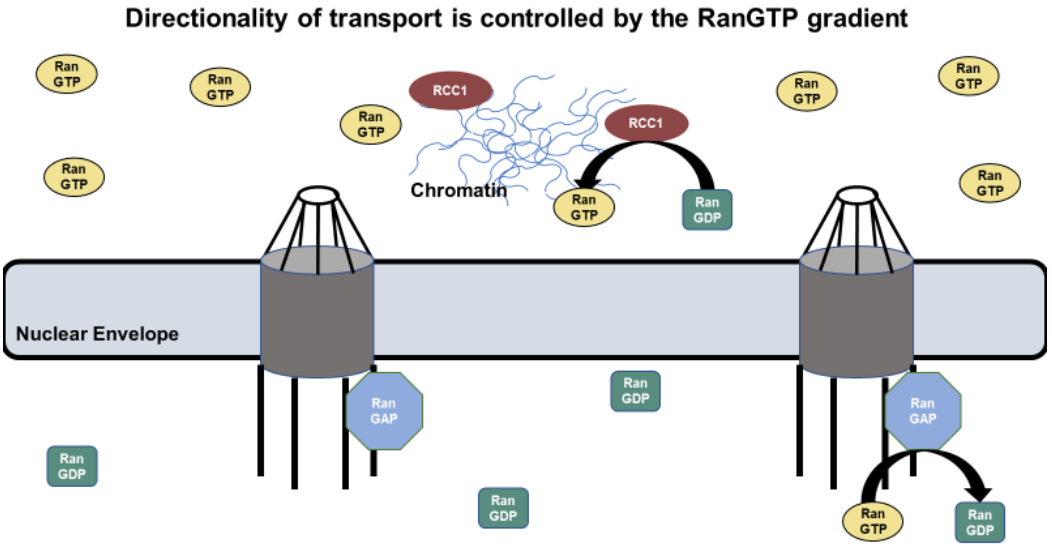

Figure 1.3: Directionality of transport is controlled by the RanGTP gradient. Inside the nucleus, RCC1 (RanGEF) is bound to chromatin. It activates Ran by inducing exchange of Ran's GDP for a GTP. Bound to the cytoplasmic filaments of the nuclear pore, RanGAP stimulates RanGTP to hydrolyze its GTP to a GDP. The localization of these two enzymes allows for the creation of a steep biochemical gradient of RanGTP across the nuclear envelope.

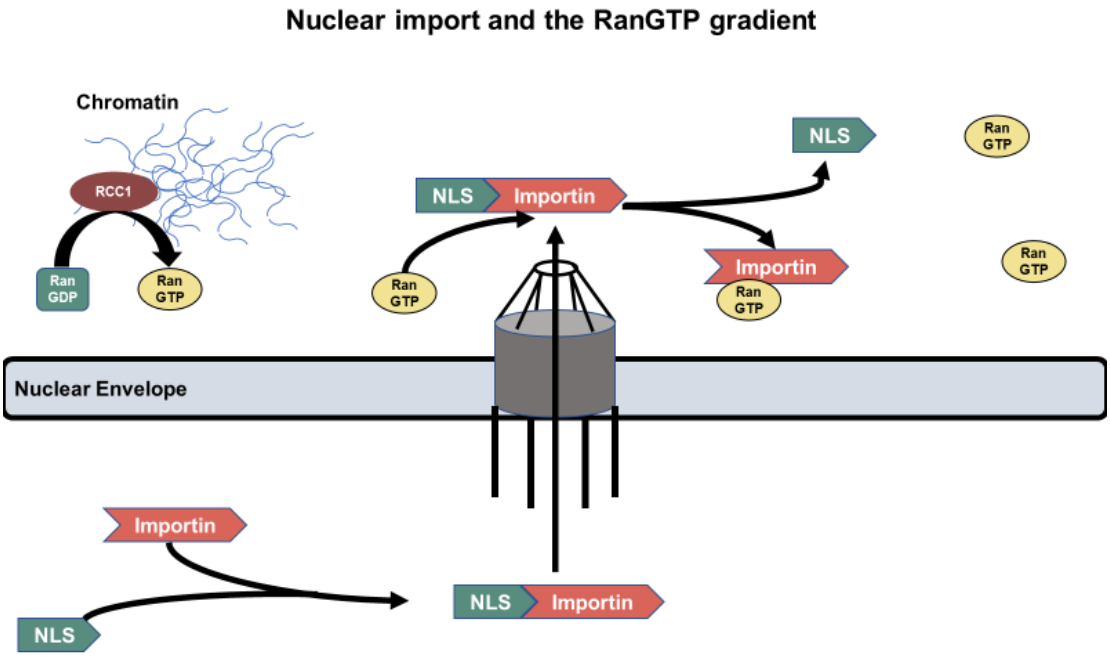

Figure 1.4: Nuclear import and the RanGTP gradient. An importin recognizes cargo with a nuclear localization signal (NLS) in the cytoplasm and forms a binary import complex. The import complex then travels through the nuclear pore and into the nucleoplasm. In the nucleus, RCC1 is generating large amounts of RanGTP, which the import complex encounters. Importins preferentially bind to RanGTP, and this binding generates a conformation change in the importin, which then dissociates from its cargo, completing import.

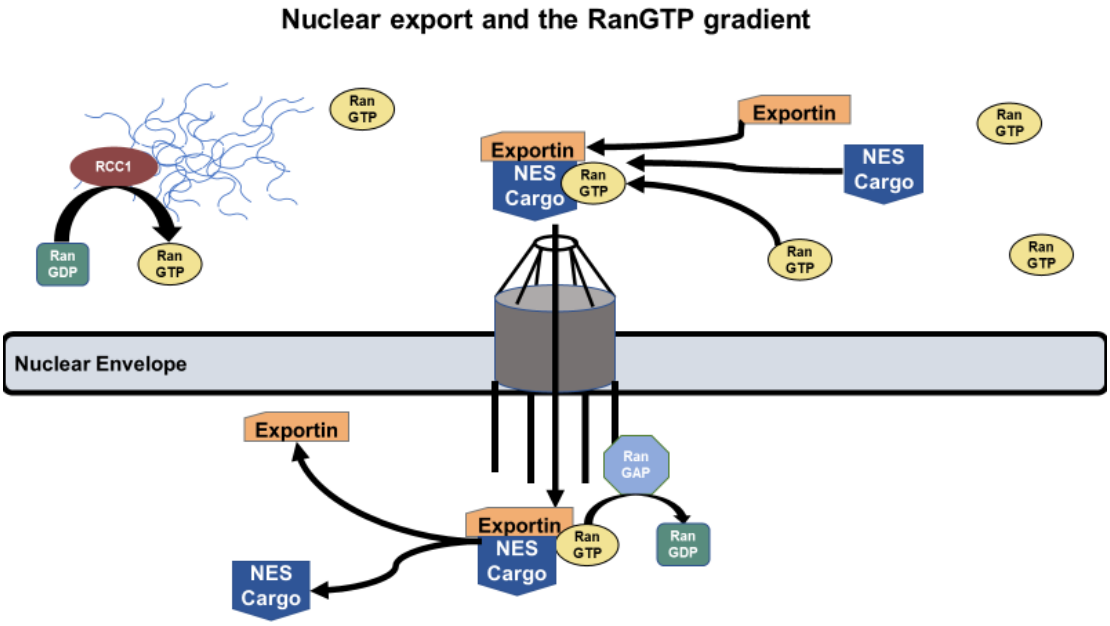

Figure 1.5: Nuclear export and the RanGTP gradient. In the nucleoplasm, an exportin forms a ternary export complex with a cargo bearing a nuclear exit signal (NES) and RanGTP. The ternary export complex then travels through the nuclear pore, where upon exit it encounter RanGAP bound to cytoplasmic filament of the nuclear pore. RanGAP stimulates Ran to hydrolyze its GTP to a GDP, and this hydrolysis causes a conformational change in the complex, which then dissociates in the cytoplasm, thus completing export.

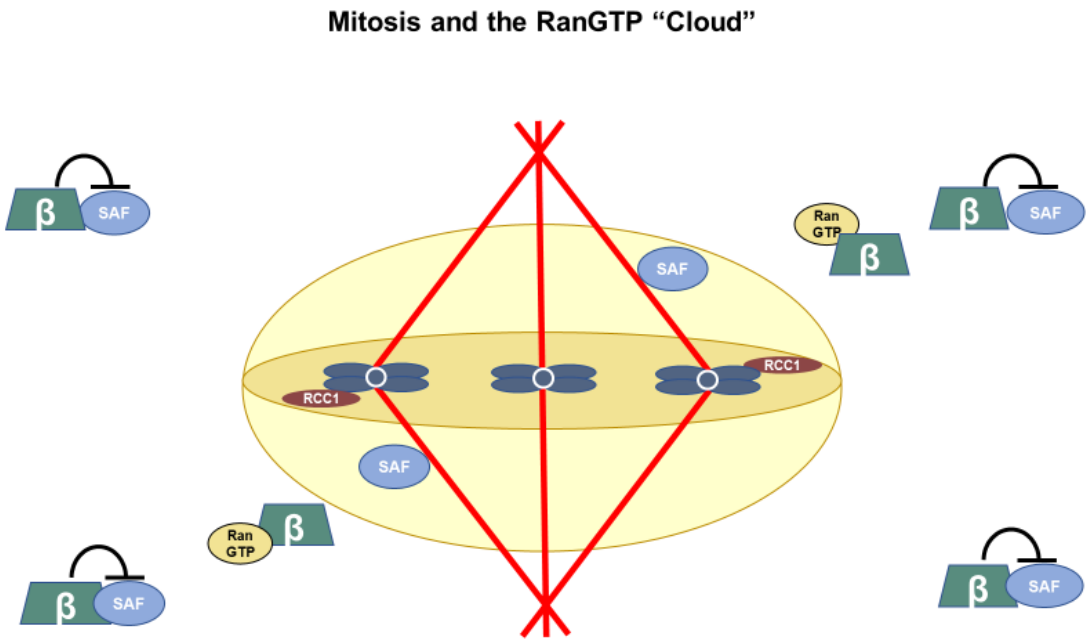

Figure 1.6: Mitosis and the RanGTP "Cloud." When the nuclear envelope breaks down in mitosis, RCC1, bound to chromatin, is still active and it is still producing RanGTP. This creates a "cloud" of highly concentrated RanGTP in the vicinity of the mitotic chromosomes (depicted in yellow, where the darker shades of yellow corresponds to higher levels of RanGTP). Away from the chromosomes, Importin β (β, green) and Transportin (Trn, red) bind to spindle assembly factors (SAFs). The binding prevents the SAFs from being activated in the wrong location, i.e., away from the chromosomes. However, as an importin /inhibited SAF complex diffuses closer to the chromosomes, the high local concentration of RanGTP induces the disruption of the inhibited importin/SAF complex. This frees the SAF and promotes spindle assembly in the proper location, i.e., around mitotic chromosomes.

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Chapter 2

MBoC | ARTICLE

Transportin acts to regulate mitotic assembly events by target binding rather than Ran sequestration

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ABSTRACT **The nuclear import receptors importin β and transportin play a different role in mitosis: both act phenotypically as spatial regulators to ensure that mitotic spindle, nuclear membrane, and nuclear pore assembly occur exclusively around chromatin. Importin β is known to act by repressing assembly factors in regions distant from chromatin, whereas RanGTP produced on chromatin frees factors from importin β for localized assembly. The mechanism of transportin regulation was unknown. Diametrically opposed models for transportin action are as follows: 1) indirect action by RanGTP sequestration, thus down-regulating release of assembly factors from importin β, and 2) direct action by transportin binding and inhibiting assembly factors. Experiments in *Xenopus* assembly extracts with M9M, a super-affinity nuclear localization sequence that displaces cargoes bound by transportin, or TLB, a mutant transportin that can bind cargo and RanGTP simultaneously, support direct inhibition. Consistently, simple addition of M9M to mitotic cytosol induces microtubule aster assembly. ELYS and the nucleoporin 107–160 complex, components of mitotic kinetochores and nuclear pores, are blocked from binding to kinetochores in vitro by transportin, a block reversible by M9M. In vivo, 30% of M9M-transfected cells have spindle/cytokinesis defects. We conclude that the cell contains importin β and transportin "global positioning system"or "GPS" pathways that are mechanistically parallel.**

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INTRODUCTION

Mitosis is a precisely controlled process that requires multiple mechanisms for that control. Mitotic kinases and phosphatases act to regulate the sequential changes between different mitotic events. For example, nuclear disassembly and chromatin condensation are set in motion at prophase by the mitotic kinase Cdk1/cyclin B. In contrast, mitosis-specific ubiquitination and proteolysis drive the transition from metaphase to anaphase. The foregoing enzymes all regulate the *timing* of mitotic events. However, the *spatial* regulation of assembly of mitotic structures involves unexpected players: the karyopherins and RanGTP. Importin β and importin α, together with the small GTPase Ran, act as dueling regulators to determine where mitotic spindle assembly occurs, causing this system to be referred to as a cellular "GPS" or "global positioning system" (Kalab *et al.*, 1999; Kalab and Heald, 2008; Gruss *et al.*, 2001; Nachury *et al.*, 2001; Wiese *et al.*, 2001; Askjaer *et al.*, 2002; Arnaoutov and Dasso, 2003; Di Fiore *et al.*, 2004; Ems-McClung *et al.*, 2004; Ciciarello *et al.*, 2007; Clarke and Zhang, 2008; Yokoyama *et al.*, 2008; Bird *et al.*, 2013). Late in mitosis, the same system also controls where nuclear membrane and nuclear pore assembly occur in the cell (Marshall and Wilson, 1997; Wiese *et al.*, 1997; Wilde *et al.*, 2001; Askjaer *et al.*, 2002; Hetzer *et al.*, 2002; Zhang *et al.*, 2002a,b; Harel *et al.*, 2003a; Harel and Forbes, 2004; Ryan *et al.*, 2003, 2007;

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Abbreviations used: BAPTA, 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*,*N* tetraacetic acid; FG, phenylalanine-glycine; GPS, global positioning system; HEAT, huntingtin-elongation factor 3-A subunit of protein phosphatase 2A-TOR1 lipid kinase; MBP, maltose-binding protein; Nup, nucleoporin; PY, proline–tyrosine; SAF, spindle assembly factor; TLB, truncated loop karyopherin β2.

Walther *et al.*, 2003b; Clarke and Zhang, 2004; Hachet *et al.*, 2004; Prunuske and Ullman, 2006; Rotem *et al.*, 2009). Importin β was believed to be the only karyopherin that plays this mitotic role, but subsequent work on the karyopherin transportin (Lau *et al.*, 2009) demonstrated that it too spatially regulates the same mitotic assembly events.

Integral to the above studies was the use of mitotic and interphase cytosolic extracts derived from *Xenopus laevis* eggs. These provided cell cycle phase–specific extracts in which one could reconstitute either the assembly of spindles in mitotic extracts or the assembly of nuclei with functional nuclear membranes and pores in interphase extracts, all in the space of an hour (Forbes *et al.*, 1983; Finlay and Forbes, 1990; Lohka and Masui, 1983, 1985; Newport, 1987; Newport and Spann, 1987; Wilson and Newport, 1988; Pfaller *et al.*, 1991; reviewed in Newmeyer and Wilson, 1991; Chan and Forbes, 2006; Maresca and Heald, 2006; Cross and Powers, 2008, 2009). Thus the extracts serve as excellent assays in which to test how karyopherin regulation of assembly occurs mechanistically.

To understand how karyopherins act in mitosis, knowledge of their action in interphase serves as an invaluable guide. Nuclear transport is governed by the small GTPase Ran and a family of nuclear transport receptors or karyopherins 90–130 kDa in size (Melchior *et al.*, 1993; Melchior and Gerace, 1998; Izaurralde *et al.*, 1997; Ben-Efraim *et al.*, 2009; Wente and Rout, 2010). Individual karyopherins, of which there are 22 in humans, are generally tasked either for nuclear import or nuclear export and often have specific cargoes (Aitchison *et al.*, 1996; Lee and Aitchison, 1999; Chook and Blobel, 2001; Marelli *et al.*, 2001a,b; Fried and Kutay, 2003; Chook and Suel, 2011). All karyopherins are composed primarily of HEAT repeats (huntingtin-elongation factor 3-A subunit of protein phosphatase 2A-TOR1 lipid kinase repeats), but in fact have low sequence homology to one another except for a common N-terminal RanGTP-binding domain.

The active form of Ran, RanGTP, is uniquely localized within the nucleus, and this sets up the directionality of transport (Izaurralde *et al.*, 1997; Kalab *et al.*, 2002, 2006). This is due to the fact that RCC1, the Ran guanine-exchange factor (Ran-GEF), is a chromatin-bound protein (Ohtsubo *et al.*, 1989; Bischoff and Ponstingl, 1991a,b; Ren *et al.*, 1993; Gorlich *et al.*, 1996; Gorlich and Kutay, 1999; Melchior and Gerace, 1998; Moore *et al.*, 2002; Li *et al.*, 2003; Clarke and Zhang, 2004). The inactive form, RanGDP, is the primary form found in the cytoplasm, as a result of the localization of RanGAP to the cytoplasmic face of the nuclear pore and the cytoplasm (Bischoff *et al.*, 1994; Gorlich *et al.*, 1996; Bischoff and Gorlich, 1997; Kehlenbach *et al.*, 1999, 2001). This asymmetry of RanGAP and RanGEF creates a sharp gradient of active and inactive Ran across the nuclear envelope (Kalab *et al.*, 2002, 2006; Di Fiore *et al.*, 2004).

Import karyopherins recognize cargoes in the cytoplasm via receptor-specific nuclear localization sequences (NLSs). The receptors then transport their cargoes into the nucleus by moving through the nuclear pore via interaction with a set of phenylalanine-glycine (FG) repeat–containing nuclear pore proteins (FG nucleoporins [Nups]; Powers *et al.*, 1997; Bayliss *et al.*, 2000b,c; Ben-Efraim and Gerace, 2001; Ben-Efraim *et al.*, 2009; Chook and Blobel, 2001; Chook and Suel, 2011; Blevins *et al.*, 2003; Strawn *et al.*, 2004; Conti *et al.*, 2006; Frey *et al.*, 2006; Walde and Kehlenbach, 2010; Xu *et al.*, 2010). Once inside the nucleus, import receptors bind to RanGTP, which causes the release of cargo and completion of import.

Importin β (karyopherin-β1) and transportin (karyopherin-β2) are by far the best studied of the import karyopherins. Each recognizes distinct cargoes in the cytoplasm, although some cargoes are recognized by both (Gorlich *et al.*, 1994, 1995; Gorlich and Kutay, 1999; Conti *et al.*, 1998; Harel and Forbes, 2004; Chook and Suel, 2011; Kimura *et al.*, 2013). Importin β, a 96-kDa protein, imports a wide range of proteins with positively charged "classical" NLSs. Often it uses the adaptor protein importin α to recognize these classical NLSs (Gorlich *et al.*, 1995; Goldfarb *et al.*, 2004; Mosammaparast and Pemberton, 2004).

Transportin, the subject of this study, functions without an adaptor. Transportin serves as the import receptor for a large class of mRNA-binding proteins, as well as for other proteins, including the medically relevant Fused in Sarcoma protein (Chook and Suel, 2011; Zhang and Chook, 2012; Dormann *et al.*, 2012). Transportin has additionally been shown to be the import receptor for exogenous DNA entry into the nucleus, an entry relevant to gene therapy (Lachish-Zalait *et al.*, 2009). Interestingly, two human papilloma viruses have evolved to inhibit transportin-mediated import of cellular cargoes; presumably this frees up nuclear materials for viral replication (Nelson *et al.*, 2002).

The NLSs recognized by transportin vary in sequence and length but often contain a proline–tyrosine (PY) dipeptide motif. Two subclasses of PY-NLSs, hydrophobic (h) and basic (b), have been defined. The hPY-NLS contains a hydrophobic motif preceding an R/H/K/$X_{(2\text{-}5)}$PY motif, whereas the bPY-NLS contains basic amino acid residues preceding the R/H/K/$X_{(2\text{-}5)}$PY motif (Lee *et al.*, 2006; Cansizoglu and Chook, 2007). Other transportin-binding cargoes contain dipeptide motifs homologous to the PY, such as PG, PL, or PV (Lee and Aitchison, 1999; Rebane *et al.*, 2004; Suel *et al.*, 2008; Chook and Suel, 2011; Lau *et al.*, 2009). PY-like NLSs are not found in all transportin cargoes, however, as some cargoes contain instead a recently characterized BIB motif (a Lys/Arg-rich segment), which can bind either to transportin or importin β (Kimura *et al.*, 2013).

Crystal structure analysis of transportin revealed that transportin undergoes structural changes during the import cycle. Transportin consists of two perpendicular arches of 20 HEAT repeats total (H1–H20; Figure 1B; Andrade and Bork, 1995; Chook and Blobel, 1999; Cansizoglu and Chook, 2007; Groves *et al.*, 1999; Conti *et al.*, 2006). The N-terminal arch of transportin contains the RanGTP-binding site, and the C-terminal arch contains the NLS/cargo-binding site. A long 62–amino acid acidic loop termed the H8 loop is inserted in HEAT repeat 8 and connects the two halves. The H8 loop normally protrudes and is disordered in either empty or NLS-bound transportin (Chook *et al.*, 2002; Cansizoglu and Chook, 2007; Lee *et al.*, 2006). When RanGTP binds to transportin, however, the H8 loop undergoes a conformational change, moving into the cargo-binding site and causing the release of the cargo (Figure 1B; Chook and Blobel, 2001; Chook *et al.*, 2002; Cansizoglu and Chook, 2007; Lee *et al.*, 2006).

A novel and potent molecular tool that can counteract this process was created by combining parts of the two types of PY-NLSs to form a chimeric peptide termed M9M (Figure 1A; Cansizoglu *et al.*, 2007). M9M was generated by fusion of the N-terminal hydrophobic segment of the heterogeneous nuclear ribonucleoprotein (hnRNP) A1 NLS and the C-terminal R/H/K/$X_{(2\text{-}5)}$PY motif from the basic-PY NLS of hnRNP M (Figure 1A). Representing the binding "hotspots" of their respective PY-NLSs, the avidity effect of combination of the two hotspots resulted in the chimeric M9M peptide, which has a significantly increased affinity for transportin that is ~200-fold tighter than its affinity for natural PY-NLSs or RanGTP (Chook *et al.*, 2002; Cansizoglu *et al.*, 2007). As a result, M9M acts as a Ran-resistant inhibitor of transportin. M9M prevents transportin from binding its endogenous cargoes by replacing native cargoes already bound to transportin (Cansizoglu *et al.*,

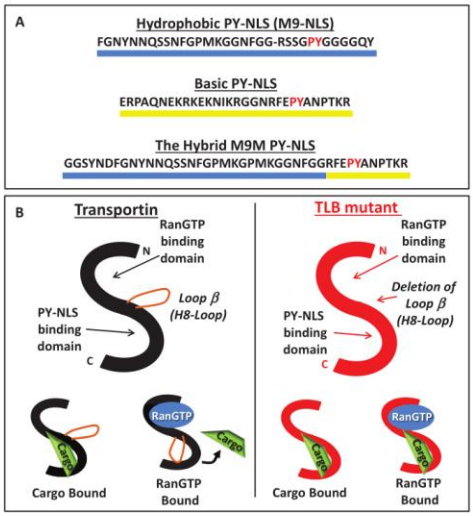

FIGURE 1: Molecular tools for probing the mechanism of action of transportin in mitosis. (A) M9M is a synthetic hybrid PY-NLS peptide capable of binding to transportin with 200-fold binding strength relative to the M9 NLS (Cansizoglu *et al.*, 2007). M9 is the NLS found in hnRNP A1 (Siomi and Dreyfuss, 1995; Nakielny *et al.*, 1996; Pollard *et al.*, 1996; Iijima *et al.*, 2006; Lee *et al.*, 2006); it is in the class of hydrophobic PY-NLSs. Also shown is the basic PY-NLS found in hnRNP M. M9M is a hybrid constructed from these hydrophobic and basic PY-NLSs. In all experiments, M9M is used in a recombinant form of MBP-M9M, where M9M is fused to MBP for ease of purification and use. (B) Wild-type transportin recognizes and binds cargo containing a PY-NLS; when the transportin:cargo complex encounters and binds RanGTP, the H8 loop moves to displace the cargo. The transportin TLB mutant lacks the H8 loop, and, as such, when RanGTP interacts with the cargo-laden complex, the cargo is not displaced. This allows TLB to be bound to cargo and RanGTP simultaneously. This schematic representation is an adaptation of the description provided in Chook *et al.* (2002).

2007; Dormann *et al.*, 2012). Small-molecule inhibitors of karyopherins have previously proven valuable for inhibiting karyopherins, including leptomycin B, which inhibits cargo binding to exportin1/Crm1, and karyostatin 1A and importazole, which inhibit RanGTP binding to importin β (Fornerod *et al.*, 1997; Ambrus *et al.*, 2010; Hintersteiner *et al.*, 2010; Soderholm *et al.*, 2011; Bird *et al.*, 2013). In this study, we use the M9M peptide to probe the role of transportin in mitosis.

During mitosis, importin β and transportin play an interesting and entirely different role from that of nuclear import. In mitosis, formation of a spindle and, later, a nuclear envelope is clearly desirable but only around chromosomes. Importin β has been coopted by evolution to act as a critical negative regulator for these mitotic assembly events. How does this work mechanistically? Owing to the presence of the RanGEF RCC1 on chromatin, a RanGTP "cloud" exists only around chromatin (Kalab and Heald, 2008). It has been found that importin β acts to bind and mask required spindle assembly factors (SAFs) everywhere except in the vicinity of chromatin. There RanGTP is produced and disrupts any nearby importin β:SAF complexes, freeing the SAFs for spindle assembly but only around the mitotic chromosomes (Nachury *et al.*, 2001; Wiese *et al.*, 2001; Askjaer *et al.*, 2002; Harel and Forbes, 2004; Kalab and Heald, 2008). Among the SAFs regulated by importin β are the nuclear mitotic apparatus protein NuMA (Nachury *et al.*, 2001), the microtubule-binding protein TPX2 (Gruss *et al.*, 2001), and lamin B, which serves as part of the matrix that organizes the spindle (Tsai *et al.*, 2006; Kalab and Heald, 2008). Proceeding into telophase, nuclear membrane assembly and nuclear pore assembly also employ importin β and RanGTP as dueling regulators (Askjaer *et al.*, 2002; Zhang *et al.*, 2002b; Clarke and Zhang, 2004; Harel *et al.*, 2003a; Walther *et al.*, 2003b; Ryan *et al.*, 2007; Delmar *et al.*, 2008; Kutay and Hetzer, 2008; Rasala *et al.*, 2008; Rotem *et al.*, 2009; Wandke and Kutay, 2013; reviewed in Dasso, 2001; Quimby and Dasso, 2003; Di Fiore *et al.*, 2004; Harel and Forbes, 2004; Mosammaparast and Pemberton, 2004; Ciciarello *et al.*, 2007; Kalab and Heald, 2008). Together importin β and RanGTP act to ensure that nuclear membranes and pores form only around chromatin, doing so in the correct place and in the correct amount.

We found in a previous study that the distantly related karyopherin transportin also functions as a negative regulator of spindle, nuclear membrane, and nuclear pore assembly (Lau *et al.*, 2009). The mode of transportin's action has been unknown. Two diametrically opposed hypothetical models could explain transportin's mechanism of action. In the first, transportin could act to down-regulate importin β by physically sequestering RanGTP. In this mode, transportin, by binding and decreasing the concentration of available RanGTP, would thereby reduce importin β's ability to release assembly factors around chromatin. In an alternate mechanism, transportin could act directly by inhibiting one or more structure-specific assembly factors everywhere except in the vicinity of chromatin where RanGTP is produced. The focus of the present study is to use potent mutant forms of the transportin NLS and of transportin itself to distinguish between these different regulatory mechanisms for spindle assembly, nuclear membrane assembly, and nuclear pore assembly.

RESULTS

Transportin and importin β are present in equivalent concentrations

Mitotic and interphase cytosolic extracts derived from *X. laevis* eggs provided a convenient way to test the Ran competition and direct inhibition models (Newmeyer and Wilson, 1991; Chan and Forbes, 2006; Maresca and Heald, 2006; Cross and Powers, 2008, 2009). In addition, the effects of recombinant proteins and potential inhibitors can easily be tested. Importin β exists in *Xenopus* egg extracts in micromolar concentration (Gorlich and Rapoport, 1993). The concentration of endogenous transportin was unknown. If transportin were, for example, 10-fold lower in concentration than importin β, a Ran competition mode by which transportin effectively modulates RanGTP would be less likely. Thus comparative quantitation was done by comparing concentrations of endogenous *Xenopus* importin β and transportin in egg extracts to a dilution series of recombinant importin β and transportin purified from *Escherichia coli* using immunoblot analysis. The concentration of endogenous importin β in interphase *Xenopus* egg extracts was found to average 6.5 μM (Supplemental Figure S1A), whereas that of endogenous transportin averaged 7 μM (Supplemental Figure S1B). We conclude that endogenous importin β and transportin are present in comparable concentrations in interphase *Xenopus* egg extracts.

The super NLS M9M shows high specificity for *Xenopus* transportin in interphase and mitotic extracts

M9M, the human chimeric PY-NLS peptide, has such high binding affinity ($K_d \approx 100$ pM) that it acts as a potent and specific inhibitor of human transportin function in vivo. To ask whether M9M shows a similar high binding affinity for *Xenopus* transportin, as well as a lack of affinity for importin β, we performed direct pull downs using recombinant NLS baits. As baits, maltose-binding protein (MBP), MBP fused to the hnRNPA1-derived NLS M9 (MBP-M9), or MBP fused to the transportin inhibitor M9M (MBP-M9M) were each bound to beads (Cansizoglu and Chook, 2007). Recombinant *Xenopus* glutathione *S*-transferase (GST)–transportin, *Xenopus* GST-importin β, or GST (100 μg) was incubated with each set of beads and then pulled down. On comparing the input samples of GST-transportin, GST-importin β, and GST (Supplemental Figure S1C, lanes 10–12) to the experimental bead pull downs (lanes 1–9), the only interaction we observed was GST-transportin and MBP-M9M (Supplemental Figure S1C, lane 3). No interaction of MBP-M9M was seen with importin β (Supplemental Figure S1C, lane 6). This demonstrated that M9M both specifically and directly binds to *Xenopus* transportin.

To test the interaction of M9M with endogenous *Xenopus* transportin in the context of interphase or mitotic *Xenopus* egg extracts, we again bound MBP, MBP-M9, or MBP-M9M (130 μg) as bait to beads. We then added 100 μl of interphase or mitotic egg extract to the beads. After removing any unbound proteins, we analyzed the binding of transportin or importin β by immunoblotting (Supplemental Figure S1D). The MBP control beads showed no affinity for endogenous *Xenopus* transportin or importin β in either interphase or mitotic extracts (Supplemental Figure S1D, lane 1). The original hnRNP A1–derived M9-NLS present in MBP-M9 *comparatively* pulled down only a very small amount of endogenous transportin from both extracts but did not pull down importin β (Supplemental Figure S1D, lane 2). In contrast, MBP-M9M strongly pulled down endogenous transportin from both interphase and mitotic extracts (Supplemental Figure S1D, lane 3). M9M beads did not pull down importin β. We conclude that M9M interacts strongly and specifically with both mitotic and interphase *Xenopus* transportin, mirroring its action in human cells. These experiments are the first to show affinity of M9M for transportin in mitosis.

Inhibition of transportin in HeLa cells induces defects in spindle assembly and cytokinesis

Transfection of M9M into interphase HeLa cells causes a functional block to nuclear import mediated by transportin (Cansizoglu *et al.*, 2007). We wanted to ask whether M9M would cause defects in mitotic events. We transfected the M9M super-NLS into HeLa cells and monitored the effect on events in mitosis and cytokinesis. HeLa cells were transfected overnight with myc-tagged control MBP or MBP-M9M in pCS2+ myc-tagged vectors. Transfected cells were detected with a tetramethylrhodamine isothiocyanate (TRITC)–anti-myc antibody. Microtubule structures were visualized with fluorescein isothiocyanate (FITC)–anti-tubulin antibody, and DNA was visualized with 4′,6-diamidino-2-phenylindole (DAPI). Microtubule structures in untransfected cells were assessed as an additional control.

Strikingly, ~30% of the cells transfected with the transportin inhibitor M9M showed defects in either mitosis or cytokinesis. These results are summarized in Figure 2, A–E, and quantitated in Figure 2F. In both control conditions (MBP-transfected as well as untransfected cells), ~3% of the cells contained microtubule midbodies (Figure 2F, midbodies, gray bar). This indicated that normally 3% of the HeLa cells under our control conditions are in cytokinesis. A midbody is a normal microtubule-containing structure spanning the region at the junction of cells as they are completing cytokinesis (arrow, Figure 2A, tubulin; Hu *et al.*, 2012). Strikingly, ~18% of M9M-transfected cells contained midbodies (Figure 2, A and F, midbodies, red bar). Moreover, ~72% of these M9M-transfected, midbody-containing cells had DNA bridges underlying their microtubule midbody (13% of total transfected cells; Figure 2, B, DNA, and F, DNA bridges, red bar). None of the control cells contained DNA bridges (Figure 2F, DNA bridges, gray bar).

In addition to the above, another 6% of M9M-transfected cells were multinucleate, whereas <1% of control cells (MBP-transfected or untransfected) showed a multinucleate phenotype (Figure 2, C and F, multinucleate cells, red bar). The observation of a multinucleate cell indicates that a cell attempted to divide and failed, and the two daughter cells were never able to separate. Together these aberrant structures—that is, increased midbodies, the presence of DNA bridges, and increased multinucleate cells—indicate that cells transfected with M9M have problems completing cytokinesis.

Another striking defect observed in M9M-transfected cells was an alteration not in the number, but in the morphology of their mitotic spindles. Normally, ~6% of control cells show a mitotic spindle (MBP-transfected or nontransfected cells). Fewer than 1 in 100 of these control cells with spindles have any significant problem with their spindle morphology (Figure 2, F, CTL, red and gray bars, and G). However, in M9M-transfected cells, although again 6% had a mitotic spindle, ~80% of these were defective spindles (Figure 2, F, abnormal spindles, +M9M, red bar, and G). Instead of a normal bipolar spindle, these cells had chaotic, disorganized microtubule structures around the DNA (Figure 2E, green; compare to normal spindle, Figure 2D, green).

In summary, cells that were transfected with M9M showed clear defects in both mitosis and cytokinesis. These defects indicate that, when M9M inhibits transportin, cells are unable to carry out mitosis normally. The defects could be due to loss of transportin's ability to regulate spindle assembly during *mitosis*. However, with this in vivo experiment we could not exclude that the mitotic defects result from an inhibition of transportin-mediated *import* of essential spindle assembly factors or chromosomal proteins required later for correct mitosis or even from the activation of the abscission checkpoint (i.e., where a defect in nuclear pore assembly activates the Aurora-B abscission checkpoint, leading to increased midbodies; Mackay *et al.*, 2010; Mackay and Ullman, 2011). Therefore, to examine the mitotic effects of transportin inhibition independent of any interphase or import role, we needed to use *Xenopus* cell cycle–specific mitotic extracts, as described next.

Addition of M9M to an in vitro spindle assembly assay: M9M mimics RanGTP in inducing larger spindles, multipolar spindles, and chromatin-free asters

Previous studies in our lab showed that transportin can function as a negative regulator of spindle assembly. That is, addition of excess transportin to mitotic *Xenopus* egg extracts inhibits spindle assembly (Lau *et al.*, 2009). Above, we found that inhibition of transportin by M9M in HeLa cells also caused striking defects in mitosis and cytokinesis. To begin to address the question of whether transportin functions in spindle assembly *indirectly* (by sequestering Ran) or *directly* (by binding and masking spindle assembly factors), we used interphase and mitotic extracts derived from *Xenopus* eggs.

For this analysis, *Xenopus* sperm chromatin was incubated in an interphase extract for 2 h to allow nuclei to form and the chromatin to be replicated. After this, 10 μl of the interphase reaction containing nuclei was mixed with 15 μl of freshly prepared mitotic extract to induce mitosis. The reaction was also supplemented

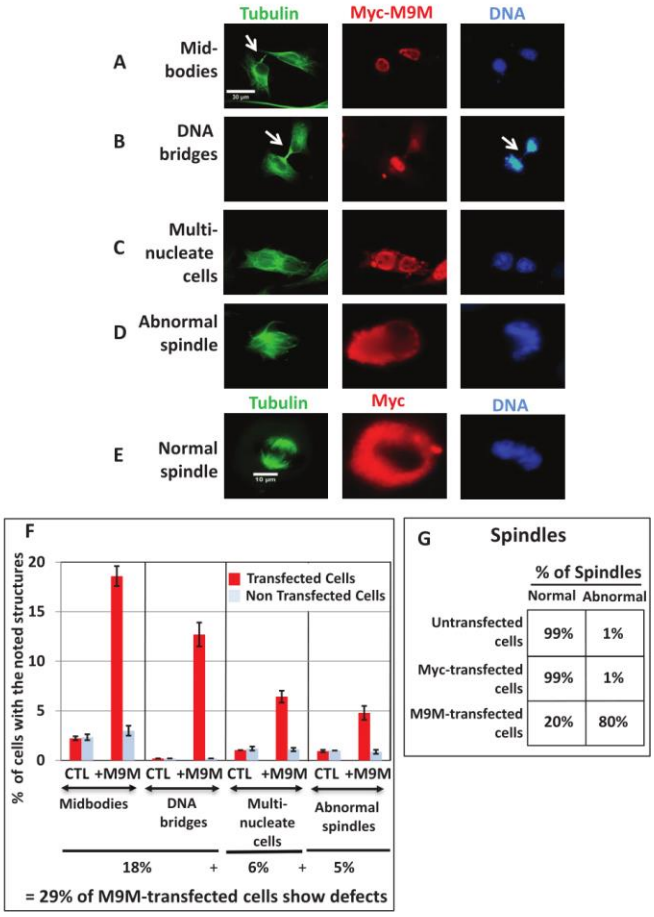

FIGURE 2: M9M inhibition of transportin in HeLa cells causes defects in mitosis. HeLa cells were transfected for 24 h with mammalian expression vectors containing the myc-tagged MBP constructs MBP, MBP-M9, and MBP-M9M. The transfection of the M9M construct caused clear defects in cytokinesis. Abnormal structures observed included (A) excess midbodies, (B) DNA bridges between cells accompanying said midbodies, and (C) multinucleate cells. Few of these defects were observed in cells that were untransfected or transfected with myc-tagged MBP (see F). In addition, in cells showing mitotic spindles, a large fraction had defective spindles after MBP-M9M plasmid transfection (D), whereas those transfected with MBP constructs showed normal spindles (E). Tubulin was detected with a FITC-labeled anti-tubulin antibody (green), myc-construct transfected cells were visualized with a TRITC-labeled anti-myc antibody (red), and DNA was visualized with DAPI (blue). (F) Quantification of the different aberrant structures observed in HeLa cells transfected with MBP constructs was performed and graphed. In cells transfected with MBP-M9M, 18% showed midbodies (+M9M, red bar), as compared with <3% of nontransfected cells on the same coverslip (+M9M, gray bar) or cells transfected with MBP (CTL, red bar, transfected; gray bar, untransfected on same coverslip). Thirteen percent of the MBP-M9M–transfected cells showing midbodies also showed DNA bridges (DNA bridges, +M9M, red bar), as opposed to no detectable DNA bridges in the MBP-transfected control or the nontransfected cells. Another 6% of the MBP-M9M–transfected cells were multinucleate (multinucleate cells, +M9M, red bar), as opposed to <1% of the MBP-transfected control or nontransfected cells. In all of our transfections, 6% of the total cells had mitotic spindles. (G) However, whereas only ~1% of the spindles in control myc-MBP–transfected cells and 1% of the spindles in nontransfected cells were abnormal, we found that 80% of the spindles in MBP-M9M–transfected cells were abnormal (see also abnormal spindles in F).

with rhodamine-labeled tubulin at this time, and this was designated $t = 0$ min for the mitotic reaction. After 5 min, an aliquot of the reaction was checked to verify that mitotic breakdown of the nuclear envelopes, chromosome condensation, and initiation of aster formation adjacent to the chromatin had occurred (Figure 3, 5-min time point). At this time, the reactions were supplemented with control protein, MBP-M9M, or RanQ69L-GTP and incubated for 1 h. An aliquot (2 µl) of each spindle assembly reaction was fixed on glass slides and examined by fluorescence microscopy. Microtubules were visualized via rhodamine-labeled tubulin, and chromatin was visualized by Hoechst DNA dye.

Following addition of control protein, the dominant structures observed in the reaction were normal bipolar spindles (77%; Figure 3A, +MBP/Ctl, top). Also observed but in lesser amounts in the control reaction were weak spindles (13%), half-spindles (5%), and condensed chromatin lacking any associated microtubules (4%). This range of structures is commonly observed in in vitro spindle assembly assays (see, for example, Orjalo *et al.*, 2006; Lau *et al.*, 2009).

When MBP-M9M (10 µM) was added to a spindle assembly assay, 45% normal bipolar spindles were observed (Figure 3B, +M9M, top). However, three unusual structures that formed in the M9M reaction differed greatly from those formed in the control. Very large spindles, assembled around a larger than normal amount of chromatin, were observed as 15% of the structures (Figure 3B, +M9M, second from top). Clusters of asters, which are groups of spontaneous microtubule structures nucleated without associated chromatin, represented 26% of the total (Figure 3B, +M9M, second from bottom). In addition, 8% of the M9M-induced structures were multipolar spindles (Figure 3B, +M9M, bottom), whereas 6% were chromatin not associated with microtubules (unpublished data).

Surprisingly, these M9M results mirrored those that occur upon addition of excess RanQ69L-GTP to mitotic *Xenopus* extracts. RanQ69L is a constitutively active mutant form of Ran, able to bind but not

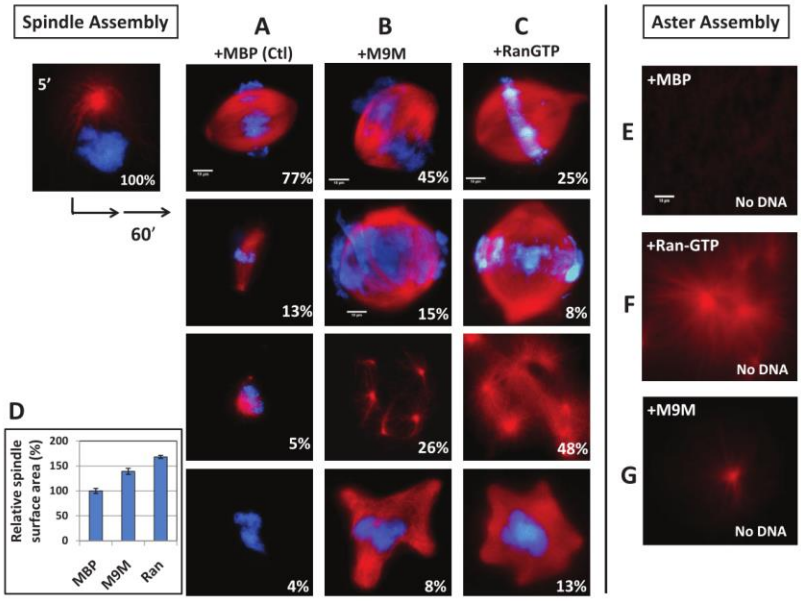

FIGURE 3: M9M addition to mitotic extracts mirrors Ran GTP in causing large spindles, free asters, and multipolar spindles. (A–C) Freshly prepared interphase *Xenopus* egg extract was mixed with sperm chromatin; nuclei were allowed to form and the DNA to replicate for 2 h. Mitotic *Xenopus* extract was then added to convert it to a mitotic state. Rhodamine-labeled tubulin was added with recombinant MBP, MBP-M9M, or RanGTP (Ran Q69L-GTP) 5 min later. The resulting microtubule structures (red) were examined at 60 min using fluorescence microscopy; chromatin was visualized with Hoechst DNA dye (blue). (A) Control condition: after MBP addition (10 μM), the majority of condensed chromatin structures (77%) showed strong bipolar spindles. Rarer structures included weak bipolar spindles (13%), half-spindles (5%), and chromatin with no associated microtubules (4%). (B) When MBP-M9M (10 μM) was added, 45% of the structures observed were normally shaped bipolar spindles of slightly increased size. However, now we also observed very large bipolar spindles with a larger than normal complement of DNA (15%), clusters of asters with no associated chromatin (26%), multipolar spindles (8%), and chromatin with no associated microtubules (6%; not shown). (C) When RanQ69L-GTP (10 μM) was instead added, we observed normally shaped bipolar spindles of slightly larger size (25%), very large spindles with much more associated DNA (8%), clusters of large asters with no associated chromatin (48%), and multipolar spindles (13%). Finally, 6% were chromatin with no associated microtubules (not shown). We note that the aforementioned M9M-induced asters are smaller than those induced in the RanGTP condition. (D) M9M, like RanGTP, promoted larger spindles in vitro. For A–C, representative images of the observed structures are shown. The percentage indicated on an image indicates the average percentage of that structure under that specific condition summarized from five replicate experiments; ~150 structures were counted for each condition for each experiment. The surface area of the normally shaped bipolar spindles from the MBP, M9M, and RanGTP conditions in A–C (top) were measured using ImageJ software (~40 spindles were measured per condition in three independent experiments). Overall, the bipolar spindles were ~40% larger with MBP-M9M addition and ~70% larger with RanQ69L-GTP addition than with MBP addition. (E–G) In the absence of chromatin, M9M, like RanGTP, induced aster assembly in vitro. *Xenopus* mitotic extract plus Energy Mix, but without added chromatin, was incubated with rhodamine-labeled tubulin and 10 μM MBP (control), 10 μM RanQ69L-GTP, or 10 μM MBP-M9M. No microtubule-containing structures were observed in the control (E; +MBP). RanGTP addition induced formation of microtubule asters (F; +Ran). RanGTP releases spindle assembly factors such as NuMa and TPX2 from importin (Zhang and Chook, 2012), causing impromptu microtubule nucleation (Gruss *et al.*, 2001; Nachury *et al.*, 2001). Microtubule asters also are seen here to form when MBP-M9M is added in the absence of chromatin (G; +M9M).

hydrolyze GTP. Addition of RanQ69L-GTP to mitotic extracts is known to cause widespread aster assembly, as well as larger bipolar spindles and multipolar spindles (Carazo-Salas *et al.*, 1999; Kalab *et al.*, 1999; Ohba *et al.*, 1999; Wilde and Zheng, 1999; Zhang *et al.*, 1999; Clarke and Zhang, 2008; Lau *et al.*, 2009; Gruss *et al.*, 2001; Nachury *et al.*, 2001; Ribbeck *et al.*, 2006; Cross and Powers, 2011). When excess RanQ69L-GTP was added (10 μM, Figure 3C), we observed ~25% bipolar spindles, ~8% very large bipolar spindles associated with excess chromatin, and ~47% large clusters of asters. In addition, ~13% of the structures were multipolar spindles (Figure 3C, +RanGTP) and ~6% were chromatin not associated with microtubules (unpublished data). These RanGTP results are consistent with previous RanGTP findings cited earlier.

An additional notable effect of either M9M or RanQ69L-GTP addition was that the bipolar mitotic spindles that had a normal amount of DNA appeared to have a larger surface area (Figure 3, B and C, +M9M or +RanGTP, top). We quantified the surface area of ~40 spindles formed in the control condition (Figure 3A, +MBP, top) or without addition (unpublished data) and defined this as the baseline of 100% for surface area quantitation in Figure 3D. The normal bipolar spindles in the MBP-M9M condition, when similarly quantitated, were found to be 40% larger, whereas those formed in excess RanQ69L-GTP were 70% larger (Figure 3D).

We next added RanGTP or M9M to a *Xenopus* mitotic extract lacking chromatin. As stated earlier, Ran-Q69L-GTP causes spontaneous nucleation of microtubule asters in the absence of chromatin. A mitotic extract was incubated for 1 h with rhodamine-labeled tubulin and 10 µM MBP, RanQ69L-GTP, or MBP-M9M. In the control MBP condition, no microtubule structures were ever induced (Figure 3E, +MBP). With added Ran Q69L-GTP, aster formation was induced, as expected (Figure 3F, +Ran; Carazo-Salas *et al.*, 1999; Kalab *et al.*, 1999; Ohba *et al.*, 1999; Wilde and Zheng, 1999; Zhang *et al.*, 1999; Clarke and Zhang, 2008; Gruss *et al.*, 2001; Nachury *et al.*, 2001; Ribbeck *et al.*, 2006; Lau *et al.*, 2009; Cross and Powers, 2011). Strikingly, added MBP-M9M similarly caused aster formation (Figure 3G, +M9M), although fewer and of smaller size. Because M9M is known to displace cargo bound specifically to transportin (Cansizoglu *et al.*, 2007), it can be concluded that transportin was actively masking SAFs that were then freed by M9M and subsequently initiated microtubule aster formation. The striking M9M result strongly favors the direct inhibition model of transportin action.

We conclude that M9M, like RanGTP, acts as if it were a positive regulator of spindle assembly, inducing larger spindles, multipolar spindles, and even microtubule nucleation into asters.

Inhibition of spindle assembly by the mutant transportin TLB is rescued by the potent NLS M9M but not by RanGTP

To address transportin's mechanism of action from a different direction, we performed spindle assembly in the presence of a mutant form of transportin, referred to as truncated-loop karyopherin β2 (TLB). TLB was previously constructed to have a deletion of the H8 loop (Chook *et al.*, 2002). TLB binds both cargo and RanGTP with similar affinities as wild-type transportin, but because the H8 loop has been deleted, the binding of RanGTP does not displace bound cargo (Figure 1B, lower right). Nuclei were formed in *Xenopus* interphase egg extract and the DNA allowed to replicate. An aliquot of mitotic extract was then added to convert the system to a mitotic state, together with added rhodamine-labeled tubulin. Five minutes later, upon verification of mitotic conversion, we added different recombinant proteins and assessed the resulting microtubule structures 1 h later. When control proteins GST or MBP were added, ~80% of the structures observed around chromatin were strong bipolar spindles (Figure 4, A and B; quantitated in Figure 4I, bipolar spindles, gray and dark green bars). Addition of excess wild-type transportin prevented spindle assembly around condensed chromatin, as we observed previously (Lau *et al.*, 2009; Figure 4, C and I, no microtubules, yellow bar). Notably, addition of the mutant transportin TLB also prevented spindle assembly (Figure 4, D and I, no microtubules, red bar).

Addition of RanGTP with wild-type transportin rescued spindle assembly (10 and 20 µM, respectively), with 55% of the condensed chromatin packages showing strong bipolar spindles (Figure 4, E and I, bipolar spindles, purple bar; see also Lau *et al.*, 2009). However, the addition of RanGTP with the mutant transportin TLB (10 and 20 µM) did not rescue spindle assembly. The great majority of condensed chromatin packages (≥95%) had no associated microtubules at all (Figure 4, F and I, no microtubules, dark blue bar). It is important to note that the 10 µM RanGTP concentration was chosen because 15–20 µM RanGTP addition was so potent that asters formed everywhere in the reaction and normal spindle formation was rare (unpublished data); this is presumably due to such a large-scale release of SAFs that microtubule nucleation occurs everywhere, not only around chromatin.

Indeed, the most illustrative addition proved to be that of M9M to either form of transportin. M9M binds both transportin and TLB with extremely high affinity to release bound cargoes from both proteins (Chook *et al.*, 2002; Cansizoglu *et al.*, 2007). The addition of M9M (10 µM) together with excess transportin (20 µM) was able to rescue spindle assembly; > 65% of the condensed chromatin was now present in strong bipolar spindles (Figure 4, G and I, bipolar spindles, light green bar). Most tellingly, M9M (10 µM) also rescued inhibition by TLB (20 µM). Greater than 70% of the condensed chromatin was in strong bipolar spindles (Figure 4, H and I, bipolar spindles, pink bar). The reversal of TLB inhibition by M9M strongly implies that the recovery of spindles around condensed chromatin had to be caused by M9M reversing TLB's sequestration of specific SAFs.

TLB and M9M support a direct inhibition model for transportin in nuclear membrane assembly

Transportin and importin β negatively regulate nuclear membrane assembly by blocking necessary membrane fusion, such that the assembly never progresses beyond membrane vesicle binding. Inhibition is prevented by the inclusion of RanGTP, such that RanGTP promotes vesicle fusion and formation of a continuous nuclear envelope (Zhang *et al.*, 1999, 2002a,b; Clarke and Zhang, 2004; Hetzer *et al.*, 2002; Harel *et al.*, 2003a; Walther *et al.*, 2003b; Lau *et al.*, 2009). However, the mechanism by which transportin negatively regulates nuclear membrane assembly has been unknown. Does transportin act by titrating RanGTP, thus limiting its ability to counteract importin β, or does transportin act more directly by masking factors needed for nuclear membrane vesicle fusion and formation?

To address this, we performed an in vitro nuclear membrane assembly assay in conjunction with the M9M and TLB tools used earlier. To initiate nuclear assembly, we added sperm chromatin and membranes to high-speed interphase egg cytosol. Under control conditions, complete fusion was observed, leading to the presence of fused nuclear membranes around the chromatin, as shown by a smooth, continuous membrane-staining pattern (CTL, Figure 5A, green). Fusion was also observed when RanQ69LGTP (+Ran, Figure 5D) or 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*,*N* tetraacetic acid (BAPTA) was added to the reaction (+BAPTA, Figure 5B). BAPTA, a Ca^{2+} chelator, allows nuclear membrane assembly around DNA but prevents nuclear pore formation (Macaulay and Forbes, 1996), whereas RanQ69L-GTP promotes extensive nuclear membrane fusion and nuclear pore formation (Harel *et al.*, 2003a; Lau *et al.*, 2009). As a negative control for lack of fusion, 2 mM GTPγS was added, which prevents vesicle–vesicle fusion, as indicated by a discontinuous membrane appearance (Newport and Dunphy (1992; +GTPγS, Figure 5C). Addition of transportin also blocked vesicle–vesicle fusion, as seen from the discontinuous green stain (+TRN, Figure 5E), and this inhibition was reversed by RanQ69L-GTP (+TRN+Ran, Figure 5F; Lau *et al.*, 2009).

Strikingly, the addition of the super-NLS M9M prevented transportin's block of nuclear membrane assembly (+TRN+M9M, Figure 5G). When the transportin mutant TLB was added, we found that, like transportin, it blocked nuclear membrane assembly; a discontinuous membrane stain was observed (+TLB, Figure 5H).

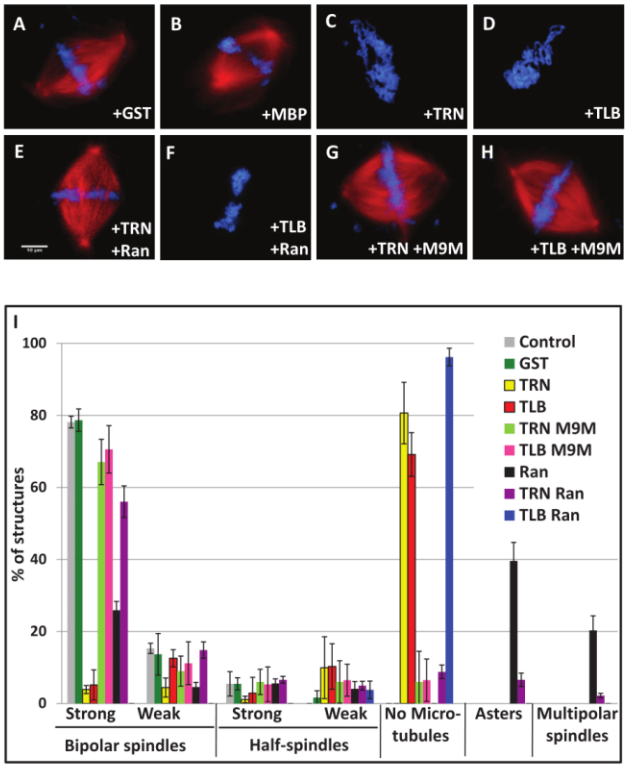

FIGURE 4: TLB inhibition of spindle assembly can be reversed by M9M addition but not by RanGTP addition. (A–H) Interphase *Xenopus* egg extract was mixed with sperm chromatin and rhodamine-labeled tubulin; nuclei were allowed to form, and the DNA was allowed to replicate for 1 h. A portion of this reaction was added to mitotic *Xenopus* extract to convert it to a mitotic state. Recombinant proteins were added as noted and the resulting structures examined by immunofluorescence microscopy; microtubules are red and chromatin is blue due to Hoechst DNA dye. Representative images. (A, B) Here 20 μM GST and 10 μM MBP are controls and show normal bipolar spindles. (C, D) Addition of 20 μM GST-transportin or 20 μM GST-TLB produced almost no microtubule formation over chromatin. (E) As shown in Lau *et al.* (2009), 10 μM RanQ69L-GTP plus 20 μM GST-transportin induced rescue of normal bipolar spindle assembly. (F) Use of 10 μM RanQ69L-GTP plus 20 μM GST-TLB produced essentially no microtubule formation over chromatin. (G) Use of 10 μM MBP-M9M plus 20 μM GST-transportin gave robust bipolar spindle assembly. (H) Use of 10 μM MBP-M9M plus 20 μM GST-TLB also produced robust bipolar spindle assembly. (I) Quantitation of the effects of addition of TLB, transportin, M9M, and RanGTP on spindle assembly. Five replicates of the experiment in A–H were done. Coverslips from each condition were examined and the different structures quantified; ~150 structures were examined for each condition in each experiment. Analysis of the combined results are shown in the graph. As shown by the error bars, little variability was observed between the five different experiments. Control conditions (10 μM MBP or 20 μM GST; gray, green bars) showed almost 80% normal bipolar spindles. Addition of 20 μM GST-TLB or 20 μM GST-transportin to the reaction caused dramatic loss of spindle formation, down to 5% or less in these experimental conditions (yellow, red bars). Addition of 10 μM RanQ69L-GTP with 20 μM GST-transportin (purple bar) rescued this inhibition to ~55% normal spindles. However, addition of 10 μM RanQ69L-GTP to the 20 μM GST-TLB condition showed no increase in spindle formation (blue bar; <5% normal bipolar spindles). Surprisingly, addition of 10 μM MBP-M9M to 20 μM GST-transportin (light green) rescued normal bipolar spindles >65%. Addition of 10 μM MBP-M9M to 20 μM GST-TLB pink bar) was also rescued to >70% normal bipolar spindles.

However, unlike the case of transportin, RanQ69L-GTP could not rescue TLB inhibition of membrane assembly (+TLB+Ran, Figure 5I). In contrast, M9M efficiently counteracted TLB inhibition, allowing nuclear membrane assembly (+TLB+M9M, Figure 5J).

To verify the aforementioned fusion, we tested the *permeability* of the nuclear envelopes formed in each condition using 70-kDa rhodamine-labeled dextran, which cannot pass through fully fused nuclear envelopes (whether or not they have functional nuclear pores; Lau *et al.*, 2009). In all conditions in which vesicle–vesicle fusion was inhibited (+ GTPγS, +TRN, +TLB, or +TLB+Ran), the membranes surrounding the chromatin were indeed permeable to 70-kDa rhodamine-labeled dextran, such that the panels were evenly red in appearance (Figure 5, C, E, H, and I, red). In contrast, in all the conditions in which the membrane stain had indicated the nuclei possessed fully fused nuclear envelopes (CTL, +BAPTA, +TRN+Ran, +TRN+M9M, +TLB+M9M), the nuclei were impermeable to 70-kDa rhodamine-labeled dextran (black holes in red backgrounds, Figure 5, A, B, D, F, G, and J). We conclude that transportin regulates nuclear membrane assembly, not through Ran competition, but through a direct mechanism that can be reversed by M9M.

TLB and M9M confirm a direct inhibition model for transportin in nuclear pore assembly

The nuclear pore complexes consist of ~30 different Nups in multiple copies (Reichelt *et al.*, 1990; Hinshaw *et al.*, 1992; Allen *et al.*, 2000; Rout *et al.*, 2000; Cronshaw *et al.*, 2002; Lim *et al.*, 2008). In higher organisms, the nuclear pores disassemble into ~13 subunits at mitotic entry. There are two times in the cell cycle when nuclear pores assemble from their subunits: 1) in interphase, when pore number doubles to provide for future division, and 2) in telophase, when all the pores that were disassembled in prophase need to reassemble in the two new daughter nuclei. It is the telophase assembly that transportin is known to negatively regulate. Excess transportin addition prevents nuclear pore assembly independently of nuclear membrane assembly. RanGTP counteracts this negative regulation (Lau *et al.*, 2009). Nuclear pore assembly can be isolated and studied as a step independent of nuclear membrane assembly by performing assembly in the presence of the calcium chelator BAPTA. The resulting pore-free

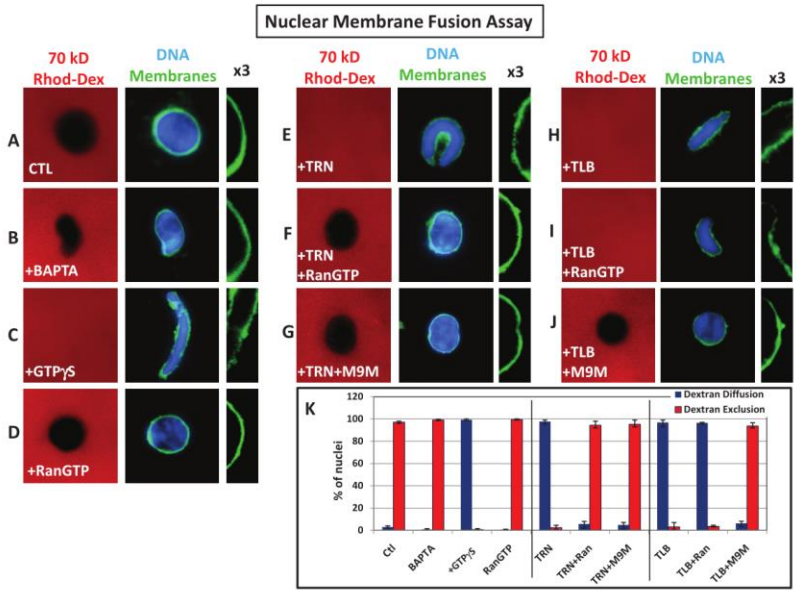

FIGURE 5: Transportin inhibition of nuclear membrane fusion is rescued by M9M addition. (A–H) High-speed interphase *Xenopus* egg extract was mixed with sperm chromatin and membranes and allowed to incubate at room temperature for 60 min. Recombinant proteins were added at the concentrations detailed in *Materials and Methods*. The resulting nuclear structures were examined for integrity using 70-kDa rhodamine–dextran exclusion and for membrane fusion with the membrane dye DHCC (green). Chromatin was stained with Hoechst DNA stain (blue). (A) As a control, addition of 15 μM GST showed smooth nuclear envelope staining and impermeability to 70-kDa dextran. (B, D) The same phenotype was observed when 8 mM BAPTA or 15 μM RanQ69L-GTP was added to the reaction. (C) Addition of 2 mM GTPγS prevented vesicle–vesicle fusion, shown by the rough nuclear envelope staining around the DNA and a permeable nuclear envelope. (E) Addition of 15 μM GST-TRN prevented vesicle–vesicle fusion, inducing a permeable nuclear envelope. (F, G) Addition of 15 μM RanQ69L-GTP or 15 μM MBP-M9M to 15 μM GST-TRN rescued membrane integrity and continuous envelope staining. (H) Vesicle–vesicle fusion was also inhibited by 15 μM GST-TLB. (I) Addition of 15 μM RanQ69L-GTP could not rescue inhibition by 15 μM GST-TLB. (J) Addition of 15 μM MBP-M9M was able to restore the integrity of the nuclear envelope when added with 15 μM GST-TLB. (K) Quantitation of five different experiments; between 70 and 100 nuclei were examined for each condition for each experiment. Little variability was observed between the five experiments for each condition (±1–5%). Blue bars, percentage of nuclei showing dextran diffusion; red bars, percentage of nuclei excluding dextran; representative images in A–J.

nuclear membrane–enclosed intermediates (Figure 6A) can then be diluted into fresh interphase cytosol plus or minus specific inhibitory proteins in order to assess the effect of those proteins on the nuclear pore assembly step (Macaulay and Forbes, 1996; Harel *et al.*, 2003a; Delmar *et al.*, 2008). To examine nuclear pore assembly, we generated BAPTA pore-free nuclear intermediates containing fully fused nuclear membranes (Figure 6A). When such intermediates were diluted 1:10 into fresh *Xenopus* egg cytosol plus the control protein MBP, nuclear pore assembly occurred (+MBP, Figure 6B, red). This rescue of normal pore assembly was apparent from the appearance of punctuate FG nucleoporin staining on the nuclear rim, as depicted in the +MBP control condition.

Addition of transportin completely inhibited the incorporation of FG-nucleoporins into the nuclear envelopes of the pore-free intermediates (+TRN, Figure 6C). As in the case of spindle assembly and nuclear membrane formation, addition of RanGTP rescued the block to nuclear pore assembly by transportin (+TRN+Ran, Figure 6D). Of note, whereas the transportin mutant TLB also blocked nuclear pore assembly (+TLB, Figure 6F), the addition of RanGTP did *not* reverse the block to pore assembly (+TLB+Ran, Figure 6G). Strikingly, however, addition of the super-NLS M9M with either transportin or the mutant transportin TLB rescued nuclear pore assembly (+TRN+M9M, Figure 6E; +TLB+M9M, Figure 6H). These results strongly support transportin acting as a negative regulator of nuclear pore assembly not by Ran sequestration, but instead by direct inhibition of proteins involved in NPC assembly.

Transportin binds a subset of nucleoporins in mitosis in an M9M-sensitive manner

The foregoing evidence indicates that transportin negatively regulates multiple mitotic assembly events by "assembly factor" binding. When considering potential targets, we examined what is already known about transportin's known nucleoporin binding

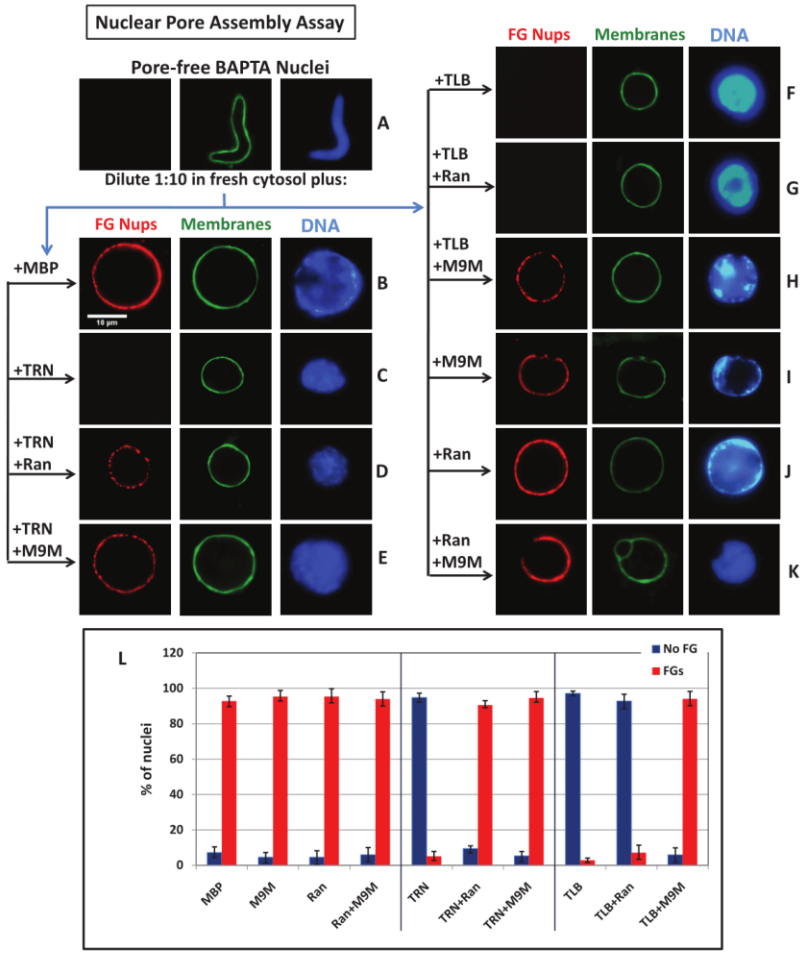

FIGURE 6: Nuclear pore assembly inhibition by excess transportin is reversed by M9M. (A) To assemble pore-free nuclear intermediates, we incubated sperm chromatin, interphase egg extract, and membranes in the presence of 8 mM BAPTA for 60 min. Pore-free nuclei containing fused nuclear membranes were formed (Macaulay and Forbes, 1996). Nuclear pore assembly was tested under different conditions by diluting an aliquot of these pore-free nuclei 1:10 into fresh cytosol and incubating for another 60 min. The presence of nuclear pores was detected by staining for FG-nucleoporins (mAb414-Alexa555; red), whereas membranes were stained with DHCC (green) and the DNA with Hoechst dye (blue). (B) Addition of 15 µ M MBP as a control allowed nuclear pore assembly; red. (C) Addition of 15 µM GST-TRN inhibited nuclear pore assembly. (D, E) Addition of 15 µM RanQ69L-GTP or 15 µM MBP-M9M with 15 µM GST-TRN rescued nuclear pore assembly. (F) The presence of 15 µM GST-TLB abolished nuclear pore assembly. (G) Addition of 15 µM RanQ69L-GTP with GST-TLB did not rescue nuclear pore assembly; however, (H) addition of 15 µM MBP-M9M to 15 µM GST-TLB did rescue nuclear pore assembly. (I–K) As controls, we found that the addition of 15 µM MBP-M9M, 15 µM RanQ69L-GTP, or a combination of both did not interfere with nuclear pore assembly. (L) Quantitation of five different experiments; between 70 and 100 nuclei were examined for each condition for each experiment. Little variability was observed between experiments within a condition (±1–6%). Only two types of results were observed: blue bars indicate the percentage of nuclei without FG Nups, and red bars indicate the percentage of nuclei with abundant FG-containing nuclear pores. Representative images in A–K.

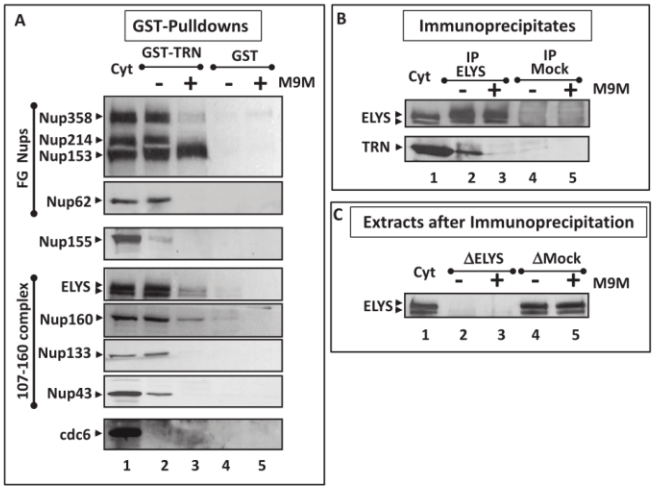

FIGURE 7: Multiple FG nucleoporins and the Nup107–160 complex are transportin-binding targets that are released by M9M. (A) GST or GST-transportin beads were incubated with *Xenopus mitotic* cytosol for 1 h at room temperature. The bound proteins were analyzed by immunoblotting. An aliquot of mitotic cytosol is shown in lane 1 (Cyt). FG-nucleoporins (Nup358, Nup214, Nup153, and Nup62), members of the Nup107–160 complex (Nup160, Nup133, and Nup43), and ELYS were seen to interact with transportin (GST-TRN; lane 2; Lau *et al.*, 2009). Neither Nup155 nor the non-Nup cdc6 protein, tested as a negative control, associated with transportin (GST-TRN; lane 2). GST-bound beads did not interact with any of the tested nucleoporins (GST; lane 4). When M9M was added in the reaction in lane 3 (GST-TRN+M9M), only Nup153 remained bound to the beads. (B) To determine whether the interaction of endogenous *Xenopus* ELYS with endogenous transportin was sensitive to M9M, immunoprecipitation from mitotic *Xenopus* egg extract was performed using anti-xELYS antibodies. This was followed by immunoblotting with anti-ELYS or anti-transportin. Immunoprecipitation of ELYS (lane 2, top) shows that transportin coimmunoprecipitates (lane 2, bottom). Lane 3 shows that addition of M9M to the extract prevents ELYS from binding to transportin. Neither ELYS nor TRN is present in mock immunoprecipitations using control immunoglobulin G (IgG) plus and minus M9M (lanes 4 and 5). (C) The anti-ELYS antiserum is specific. The ELYS doublet was present in mitotic extract mock depleted with IgG beads (ΔMOCK; lanes 4 and 5) but was absent from mitotic cytosol previously subjected to immunodepletion of ELYS (ΔELYS; lanes 2 and 3).

partners. Minimally, these include import cargoes and nucleoporins. In interphase, a transportin–cargo complex has to bind FG-nucleoporins as the import complex passes through the intact nuclear pore (Powers *et al.*, 1997; Bayliss *et al.*, 2000c; Chook and Blobel, 2001; Chook and Suel, 2011; Blevins *et al.*, 2003; Fried and Kutay, 2003; Mosammaparast and Pemberton, 2004; Strawn *et al.*, 2004; Frey *et al.*, 2006; Walde and Kehlenbach, 2010). In addition, we have demonstrated biochemically that transportin binds to a subset of nucleoporins in a Ran-regulated manner (Lau *et al.*, 2009). These include not only FG-nucleoporins, but also the scaffold nucleoporins of the large Nup107–160 complex and its associated partner ELYS (Harel *et al.*, 2003b; Walther *et al.*, 2003a; Loiodice *et al.*, 2004; Galy *et al.*, 2006; Orjalo *et al.*, 2006; Rasala *et al.*, 2006; Franz *et al.*, 2007; Boehmer *et al.*, 2008; Mishra *et al.*, 2010; Bilokapic and Schwartz, 2012, 2013). However, we did not know whether these nucleoporins were interacting with transportin via its cargo-binding site or through a different binding site. To address this, we performed GST pull downs from *Xenopus* mitotic cytosol using GST or GST-transportin, in the presence or absence of M9M.

We found that in *mitotic* cytosol, GST-transportin binds to the FG nucleoporins Nup358, Nup214, Nup153, and Nup62. Strikingly, addition of M9M prevented this interaction, except in the case of Nup153 (Figure 7A, FG-Nups, compare lanes 2 and 3). We also found that transportin bound to the major scaffold subunit of the pore, the Nup 107–160 complex and its binding partner ELYS. This binding to transportin in mitotic extract was lost upon M9M addition (Figure 7A, 107–160 complex, compare lanes 2 and 3). We further noted that the same Nups and Nup subcomplexes bind to transportin in *Xenopus* egg *interphase* cytosol and that those interactions too are disrupted by M9M (unpublished data).

Not all nucleoporin subunits bind to transportin: Nup155, a member of a different pore scaffold subunit, did not bind, indicating that this subunit is not a target of transportin (Figure 7A, Nup155, lanes 2 and 3). As another negative control, we tested the non-Nup protein cdc6, a member of the DNA prereplicative complex, for interaction with transportin. Cdc6 is carried into the nucleus via importin α/β in *Saccharomyces cerevisiae* (Hahn *et al.*, 2008) and, consistent with this, we found that cdc6 did not interact with transportin (Figure 7A, cdc6).

To test whether the endogenous protein ELYS is normally found bound to endogenous transportin in mitotic *Xenopus* egg extracts, we immunoprecipitated ELYS and probed the immunoprecipitate with anti-transportin antibodies. We found that endogenous ELYS and transportin do coimmunoprecipitate (Figure 7B, lane 2, ELYS and TRN) and that this interaction is abolished by M9M addition to the *Xenopus* mitotic cytosol used for the immunoprecipitation (Figure 7B, compare lanes 2 and 3, TRN). As a control, we showed that M9M does not interfere in any way with the antibody immunoprecipitation of ELYS itself, since ELYS can be completely depleted from mitotic extract even in the presence of M9M (Figure 7C, lane 3, ΔELYS). Together these results strongly demonstrate that transportin binds to a subset of potential targets, such as specific nucleoporin subunits in their disassembled mitotic state. This is consistent with transportin preventing pore subunit assembly in membranes that are distant from chromatin.

Transportin regulates kinetochore binding of the Nup107–160 complex and ELYS nucleoporins

Next we tested whether the addition of excess transportin blocks Nup107–160 complex recruitment to kinetochores during mitosis in vitro. Of importance, it is known that a pool of the Nup107–160 complex and ELYS localizes to the kinetochores during metaphase in both mammalian cells and *Xenopus* egg extracts (Belgareh *et al.*, 2001; Loiodice *et al.*, 2004; Galy *et al.*, 2006; Orjalo *et al.*, 2006; Rasala *et al.*, 2006; Franz *et al.*, 2007; Zuccolo *et al.*, 2007; Mishra

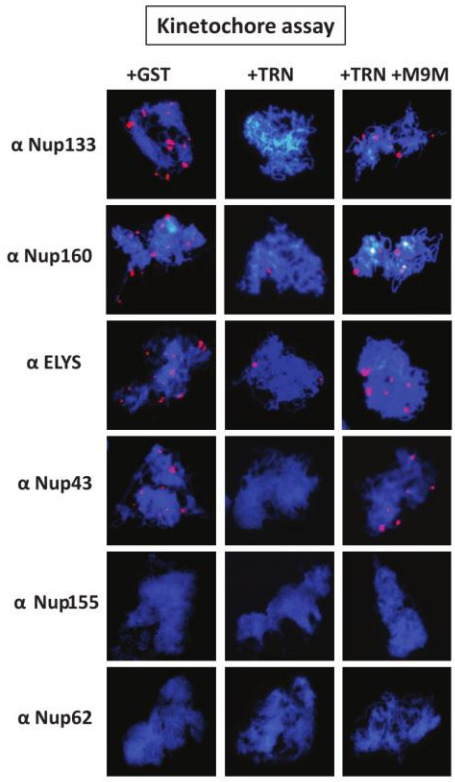

FIGURE 8: Transportin regulates kinetochore binding of ELYS and the Nup107–160 complex. Mitotic *Xenopus* egg extract was mixed with sperm chromatin, and the chromatin was allowed to remodel and condense for 60 min. Where indicated, GST-transportin (+TRN) or GST-transportin plus MBP-M9M (TRN+M9M) was added at 20 μM at $t = 0$ min. The presence of nucleoporins on kinetochores was detected by immunofluorescence with Nup-specific antibodies (red dots). In control conditions (+GST), nucleoporins of the Nup107–160 complex and ELYS each showed a dot-like staining on kinetochores (αNup133, αNup160, αELYS, αNup43), as expected (Belgareh *et al.*, 2001; Orjalo *et al.*, 2006; Rasala *et al.*, 2006). Addition of GST-transportin (+TRN; 20 μM) caused almost-complete disappearance of the nucleoporin staining on kinetochores (αNup133, αNup160, αNup43, αELYS). However, inclusion of 20 μM MBP-M9M with 20 μM GST-transportin (+TRN+M9M) restored the kinetochore localization of these particular Nups. Antibodies to the nucleoporins Nup155 and Nup62 did not show any staining on kinetochores under any condition (as previously shown; Orjalo *et al.*, 2006), serving as a negative control. This experiment was performed three times; ~70 mitotic chromosome packages were observed per condition. Representative images are shown for each condition and antibody probe.

et al., 2010). Moreover, this localization to the outer kinetochore region is critical for mitotic spindle assembly and kinetochore function. For example, mitotic extracts depleted of the Nup107–160 complex cannot form a mitotic spindle (Orjalo *et al.*, 2006). We added sperm chromatin to *Xenopus* mitotic egg extracts supplemented with GST, transportin (20 μM), or transportin plus M9M (20 μM+20 μM). We tested the recruitment of the Nup107–160 complex and other nucleoporins to kinetochores on the resulting condensed chromatin by immunofluorescence (Orjalo *et al.*, 2006). When the control protein GST was added, Nup133, Nup160, and Nup43 showed kinetochore localization (Figure 8, +GST). The same kinetochore localization was found for ELYS (αELYS, +GST). As a negative control, we analyzed Nup155 and Nup62, which are known not to be localized at kinetochores during mitosis (Orjalo *et al.*, 2006), and observed no such presence (Figure 8, +GST, αNup155, and αNup62). Addition of 20 μM GST-transportin to the assay greatly reduced the presence of Nup133, Nup160, Nup43, and ELYS on chromatin (Figure 8, +TRN). However, addition of GST-transportin with MBP-M9M (20 μM each) restored Nup107–160 complex and ELYS localization to chromatin (Figure 8, +TRN+M9M). These results are consistent with transportin being able to regulate the kinetochore localization of the Nup107–160 nucleoporins.

DISCUSSION

Transportin negatively regulates three major mitotic assembly events: spindle assembly, nuclear membrane assembly, and nuclear pore assembly (Lau *et al.*, 2009). To probe mechanisms of action, we took advantage of the synthetic NLS-peptide M9M, which has been shown to be a potent transportin inhibitor of nuclear import in human cells (Cansizoglu *et al.*, 2007). We determined that M9M interacts specifically with both recombinant and endogenous *Xenopus* transportin but not with importin β (Supplemental Figure S1). Upon examination, M9M-transfected HeLa cells showed striking defects, including an increased number of cells with microtubule midbodies, DNA bridges, and multiple nuclei. In those cells that were in metaphase, the great majority showed abnormal spindles. Indeed, almost 30% of all M9M-transfected cells showed a defect in either completion of cytokinesis or spindle assembly (Figure 2). Thus inhibition of transportin clearly results in significant mitotic defects in vivo. Switching to an in vitro approach allowed us specifically to study the effect of inhibiting transportin on the mitotic spindle. We found that addition of M9M to spindle assembly extracts containing chromatin produced not only larger bipolar spindles, but also giant spindles, multipolar spindles, and free asters (Figure 3B). Strikingly, even in chromatin-minus conditions, M9M induced aster assembly (Figure 3, E–G).

Previous studies revealed that the addition of excess RanGTP to *Xenopus* mitotic extracts caused the release of specific spindle assembly factors from a strong masking inhibition by importin β or importin α/β (Carazo-Salas *et al.*, 1999; Kalab *et al.*, 1999; Ohba *et al.*, 1999; Wilde and Zheng, 1999; Zhang *et al.*, 1999; Clarke and Zhang, 2008; Gruss *et al.*, 2001; Nachury *et al.*, 2001; Di Fiore *et al.*, 2004; Ribbeck *et al.*, 2006; Lau *et al.*, 2009; Cross and Powers, 2011). Moreover, when this increased unmasking by excess RanGTP occurred in the vicinity of chromatin, the spindles produced were larger than normal because spindle size was no longer limited by the reach of the RanGTP created only by chromatin-bound RCC1 (Bischoff and Ponstingl, 1991a,b; Clarke and Zhang, 2008; Kalab and Heald, 2008).

If transportin binds and inhibits SAFs, then the addition of M9M might well induce the same microtubule structures induced by excess RanGTP. This was what we saw (Figure 3, B and C). Indeed, with a direct target inhibition model, in which transportin binds to and regulates spindle assembly factors, either RanGTP *or* M9M addition should free SAFs. RanGTP would do so by

releasing the cargo SAFs via an H8-loop allosteric mechanism. M9M would do so by binding to the cargo site of transportin with extreme affinity and thus displacing the cargo SAFs. In both cases, the freed SAFs would then be available to induce larger spindles than normal, as well as free asters. Would there be any difference expected between RanGTP and M9M observed phenotypes? Because RanGTP would free both transportin- and importin β–bound SAFs, whereas M9M would free only transportin-bound SAFs, the excess RanGTP condition would be expected to show more and larger asters, as well as slightly larger spindles than the M9M condition. This was what we found (Figure 3, B, C, and E–G). Thus we conclude that the M9M results support a direct target inhibition mechanism for transportin action.

Another route to determining the mechanism by which transportin regulates mitotic events was to use the mutant transportin, TLB. Ran binding and cargo binding are uncoupled in TLB, such that the mutant TLB transportin fails to undergo cargo release upon Ran binding. The mutant still binds RanGTP in its N-terminal domain, but this does not release bound cargo from the C-terminal domain (Chook *et al.*, 2002). We found that addition of TLB strongly inhibited spindle assembly in vitro, just as transportin does (Figure 4, C and D). However, unlike transportin, TLB inhibition was not reversed by RanGTP (Figure 4F). If transportin regulates through a mechanism by which it *depletes* RanGTP, then TLB should also still deplete RanGTP, and TLB's inhibitory activity should be reversed by providing the same amount of RanGTP that reverses transportin inhibition. Because this did not occur, the TLB results independently point toward a direct target model.

The most definitive evidence for direct target inhibition, however, came from the addition of M9M in combination with TLB. Adding M9M with TLB caused robust rescue of spindle assembly (Figure 4H). This means that M9M, when binding with high affinity to the cargo site of transportin, must be releasing one or more factors that restore spindle assembly. Put another way, rescue of spindle assembly by M9M must be due to M9M keeping TLB from binding and inhibiting spindle assembly factors in its cargo site. Thus we conclude that transportin acts during spindle assembly by masking spindle assembly factors.

Evidence supporting a direct inhibition mechanism for transportin in both nuclear membrane assembly and nuclear pore assembly was similarly found. RanGTP could not rescue TLB inhibition of nuclear membrane or nuclear pore assembly, but M9M could (Figures 5 and 6). The targets inhibited by transportin for nuclear membrane assembly are unknown, but here we show that in *mitotic* extracts, multiple nuclear pore proteins bind to transportin and do so in an M9M-reversible manner (Figure 7). Transportin does not bind to all Nups. For example, it fails to bind to the pore scaffold component Nup155 (Figure 7; Lau *et al.*, 2009). However, of those Nups that transportin does bind, with the exception of Nup153, all show M9M-reversible binding. This includes the FG nucleoporins Nup358, Nup214, and Nup62, the Nup107–160 complex (as tested with anti-Nup160, -Nup133, and -Nup43 antibodies), and the pore-targeting protein ELYS. In consequence, the data strongly suggest that transportin regulates the nuclear pore assembly event that occurs in late mitosis by *directly* binding and inhibiting multiple soluble nuclear pore proteins needed for pore assembly everywhere except in the vicinity of chromatin.

On the basis of these data, one can speculate that in *mitosis* the aforementioned nucleoporins bind to transportin in its cargo-binding site and are displaced from the site by M9M. If true, this would mean that the Nups bind to transportin in mitosis via a *different* transportin-binding site than they do in interphase. Of note, importin β does indeed bind to FG nucleoporins during nuclear import using binding sites distinct from its cargo-binding site (Bayliss *et al.*, 2000a,c; Bednenko *et al.*, 2003).

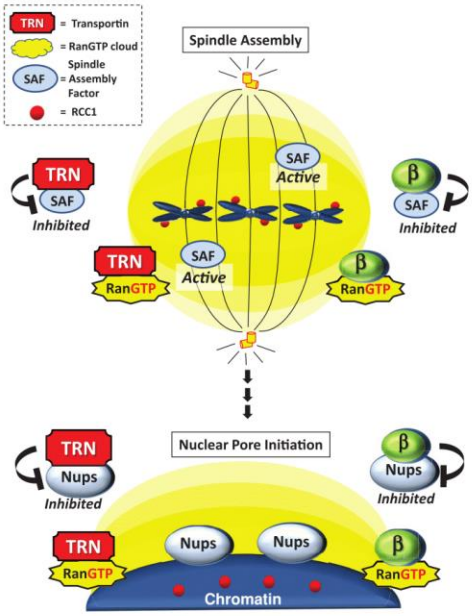

FIGURE 9: Mechanistically parallel transportin and importin β GPS pathways. As depicted in the model shown, transportin (TRN) acts by a mechanism parallel to that of importin β, that is, by direct factor inhibition. Spindle assembly at metaphase requires spindle assembly factors or SAFs (blue spheres; top half). As the cell cycle proceeds, a subsequent mitotic assembly event that occurs is the initiation of nuclear pore assembly in telophase. This is characterized by the recruitment and binding of an early set of Nups, the Nup107–160 complex and ELYS, to the surface of the decondensing chromatin (Nups; gray-blue spheres; bottom half). In metaphase, transportin mechanistically acts by binding to and inhibiting spindle assembly factors in regions far from the chromatin; in contrast, SAFs near chromatin are released from transportin by the RanGTP cloud (yellow), produced by the chromatin-bound RanGEF RCC1 (small red spheres), and release promotes spindle assembly. Later, after the spindle has disassembled and telophase begins, nucleoporins such as the Nup107–160 complex and ELYS are inhibited by transportin in regions far from chromatin, but those near chromatin are released from transportin by the RanGTP cloud and initiate nuclear pore assembly on the surface of chromatin. Importin β was shown previously to pursue a similar mechanism of inhibition counteracted by localized RanGTP production, which acts as a GPS system to tell where the mitotic assembly events should take place (see text for references). From the data in Figures 3–8, we conclude that transportin and importin β have mechanistically parallel regulatory mechanisms for the major mitotic assembly events. Of importance, not only is the Nup107–160 complex vital for nuclear pore initiation, but it is in fact also a SAF essential to metaphase kinetochore function. Thus, by regulating this one protein complex, transportin can regulate both spindle and nuclear pore assembly.

Returning once again to spindle assembly, the spindle has been predicted to contain ~800 proteins (Sauer *et al.*, 2005). Although it would be unlikely that all are needed for the act of spindle assembly, assuredly many are. Importin β has been shown to negatively regulate at least 13 or 14 different SAFs in a Ran-dependent manner (Kalab and Heald, 2008). To seek transportin-regulated SAFs, one could look for PY sequences on known SAFs; however, because of the variability of the PY-NLS, simple sequence analysis is unlikely to reveal these targets (Suel *et al.*, 2008; Xu *et al.*, 2010). Moreover, a recent study found that transportin also recognizes a second type of NLS signal, the BIB domain, which is LYS-ARG rich and capable of also binding importin β (Kimura *et al.*, 2013). This would make such an undirected approach laborious and of uncertain outcome.

The most meaningful finding, however, is that transportin clearly binds to the Nup107–160 complex and ELYS, both of which localize to kinetochores in mitosis in vivo and in vitro (Belgareh *et al.*, 2001; Loiodice *et al.*, 2004; Galy *et al.*, 2006; Orjalo *et al.*, 2006; Rasala *et al.*, 2006; Franz *et al.*, 2007; Zuccolo *et al.*, 2007; Lau *et al.*, 2009; Mishra *et al.*, 2010). Significantly, the presence of the Nup107–160 complex at kinetochores has been shown to be absolutely essential for spindle assembly; its removal by depletion can cause insurmountable spindle and kinetochore defects (Orjalo *et al.*, 2006; Zuccolo *et al.*, 2007; Mishra *et al.*, 2010). We further show in this study that the Nup107–160 complex and ELYS are demonstrable transportin targets in *mitotic* extracts, since they are found to be bound by transportin and freed by M9M addition (Figure 7). Furthermore, they are prevented from assembling onto mitotic kinetochores by transportin addition in vitro (Figure 8). By showing that these outer kinetochore components are modulated by transportin, we have gone as far toward finding the essence of transportin spindle inhibition as we can without going further back into the inception of the kinetochore, such as searching for a potential transportin inhibition of an inner kinetochore protein.

In conclusion, our data argue that transportin acts directly to bind and mask assembly factors used in spindle assembly, nuclear membrane assembly, and pore assembly to ensure that these structures each assemble only in the vicinity of chromatin (Figure 9). Transportin therefore acts as a "global positioning system" or GPS (Kalab and Heald, 2008) to direct where such structures should assemble. Thus we conclude that the cell contains mechanistically parallel importin β and transportin GPS pathways.

MATERIALS AND METHODS

Antibodies and recombinant proteins

Antibodies used for immunoblotting included mouse anti–human transportin (BD Biosciences, San Jose, CA), anti–*Xenopus* importin β (Delmar *et al.*, 2008), anti–FG Nup mAb414 (Covance, Berkeley, CA), anti-Nup155 (Lau *et al.*, 2009), anti-xELYS (Rasala *et al.*, 2006), anti-Nup160 and anti-Nup133 (Harel *et al.*, 2003b), anti-Nup43 (Orjalo *et al.*, 2006), and anti-cdc6 (Hua and Newport, 1998). For immunofluorescence on HeLa cells, antibodies included a TRITC-labeled anti-myc antibody (Santa Cruz Biotechnology, Santa Cruz, CA) and a FITC-labeled anti-tubulin antibody (DM1A clone, Sigma Aldrich, St. Louis, MO).

GST-human transportin, GST-RanQ69L, and the recombinant form of the transportin mutant TLB, GST-TLB (Chook *et al.*, 2002), were expressed in Rosetta *E. coli* cells from pGEX vectors (Chook *et al.*, 2002; Harel *et al.*, 2003a; Lau *et al.*, 2009). One-liter LB-ampicillin cultures were grown at 37°C until an OD of 0.3–0.6 and induced for 4 h with 1 mM isopropyl-β-D-thiogalactoside (IPTG). GST-*Xenopus* importin β was expressed in Rosetta *E. coli* cells from pGEX6P vectors (Delmar *et al.*, 2008). One-liter LB-ampicillin cultures were grown at 37°C until an OD of 0.3, moved to a 16°C water bath, and induced with 0.3 mM IPTG for 4 h and then with 0.7 mM IPTG overnight (~12 h). All GST-tagged proteins were purified on glutathione Sepharose beads (BioWorld, Dublin, OH) per manufacturer's specifications and were eluted from the beads using a glutathione solution (50 mM, pH 7.5). The GST tag was removed from transportin for the quantification studies (Supplemental Figure S1) and from RanQ69L by proteolytic digestion for all experiments using GST-PreScission Protease (GE Healthcare, Piscataway, NJ) overnight at 4°C according to the manufacturer's protocol as described (Lau *et al.* (2009). RanQ69L was loaded with GTP as described (Harel *et al.*, 2003a).

MBP, MBP-M9, and MBP-M9M were expressed in Rosetta *E. coli* cells from pMAL vectors (Cansizoglu *et al.*, 2007). One-liter LB-ampicillin cultures of these were grown at 37°C until an OD of 0.3–0.6 and induced for 4 h with 1 mM IPTG. All MBP-tagged proteins were purified on amylose resin (New England Biolabs, Beverly, MA) according to manufacturer's specifications and eluted with maltose elution buffer (Tris 50 mM, NaCl 50 mM, maltose 50 mM, pH 7.5). These proteins, as well as transportin, importin β, and TLB, were dialyzed into *Xenopus* buffer (XB; 50 mM sucrose, 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid [HEPES], 100 mM KCl, 1 mM $MgCl_2$, 0.1 mM $CaCl_2$) and stored in 5-µl aliquots at –80°C for later use.

HeLa cell transfection

To assess the effect of transportin inhibition on mitosis in vivo (Figure 2), HeLa cells (200,000/well) were seeded in six-well plates containing coverslips (12 mm) and grown overnight 37°C in 5% CO_2. Cells were then transfected using JetPI according to manufacturer's protocol (PolyPlus Transfection, Illkirch, France) with 2 µg of mammalian expression vector pCS2+MT MBP (as a control) or pCS2+MT MBP-M9M (Cansizoglu *et al.*, 2007). The cells were allowed to grow at 37°C and 5% CO_2 for 24 h and then fixed on the coverslips with cold (–20°C) 100% methanol for 20 min and rehydrated with 1× phosphate-buffered saline (PBS) for 1 h. After this, the coverslips were blocked with 5% fetal bovine serum in PBS for 30 min at room temperature and incubated with TRITC-labeled anti-myc and FITC-labeled anti-tubulin antibodies (1:100 dilution in PBS for 1 h at room temperature) before the coverslips were mounted on slides using 2 µl of Vectashield with DAPI mounting media (Vector Labs, Southfield, MI) and visualization with a Zeiss Axioskop 2 microscope and 63× objective. Approximately 200 cells were observed for each condition (MBP or MBP-M9M) and the structures noted and quantified.

Xenopus egg extracts

Newly laid *X. laevis* eggs are naturally arrested in the second metaphase of meiosis by cytostatic factor (CSF; Lohka and Maller, 1985; Sagata *et al.*, 1989; Murray, 1991). This arrested stage is biochemically and physiologically related to a mitotic state. Extracts that are considered either mitotic (CSF) or interphase extracts can easily be generated (Murray, 1991; Chan and Forbes, 2006; Maresca and Heald, 2006; Bernis *et al.*, 2007; Cross and Powers, 2008,). Preparation of the fresh low-speed (crude) mitotic and interphase *Xenopus* egg extracts used for the spindle assay was as described (Lau *et al.*, 2009). For nuclear pore and nuclear membrane assembly assays (see later description), high-speed interphase *Xenopus* egg extracts were prepared as described (Harel *et al.*, 2003a; Chan and Forbes, 2006; Lau *et al.*, 2009).

Xenopus spindle assembly assay

To observe spindle assembly, the protocol used was essentially as in Lau *et al.* (2009). *Xenopus* sperm chromatin was added to *Xenopus* egg interphase extract to allow nuclei to form and the chromatin to replicate. For this, interphase extract was incubated with Energy Mix (10 mM phosphocreatine, 80 μg/ml creatine kinase, 1 mM ATP, 2 mM $MgCl_2$, and 100 mM ethylene glycol tetraacetic acid [EGTA]) and sperm chromatin (70,000 SpC/20-μl interphase extract) at room temperature for 2 h to allow DNA replication. To induce mitosis, 10 μl of this reaction, which now contained nuclei, was mixed with 15 μl of CSF extract, 1 μl of rhodamine-labeled tubulin (Cytoskeleton, Denver, CO), and 2 μl of Energy Mix. On verification of mitotic conversion after 5 min (i.e., visual loss of nuclear membranes, appearance of chromatin condensation and early microtubule structures; as seen in Figure 3, 5-min time point), when indicated, the following proteins were added: 20 μM GST, 10 μM RanQ69L-GTP, 20 μM GST-transportin, 20 μM GST-TLB, 10 μM MBP, or 10 μM MBP-M9M (Figures 3 and 4). The reaction was incubated in the dark at room temperature. At 60 min, 2.5 μl were removed and mounted with 1 μl of fixation buffer (48% glycerol, 11% formaldehyde, 10 mM HEPES, 5 μg/ml Hoechst, pH 7.5) on glass slides. For each condition, between 50 and 100 microtubule structures or condensed chromatin packages were analyzed using a Zeiss Axioskop 2 microscope and a 63× objective (Figures 3 and 4). To measure spindle size (Figure 3D), the surface area of ~40 individual spindles from the conditions MBP, MBP-M9M, and RanQ69L-GTP was measured in three independent experiments using ImageJ software according to the instructions found at http://rsbweb.nih.gov/ij/.

Nuclear membrane fusion and nuclear pore assembly assays

Interphase *Xenopus* high-speed cytosol and membranes and *Xenopus* sperm chromatin were prepared as described (Harel *et al.*, 2003b). The membrane fusion assay (Figure 5) and nuclear pore assembly assay (Figure 6) were performed as described (Harel *et al.*, 2003a). Nuclear membrane assembly was visualized using the fluorescent membrane dye 3,3-dihexyloxacarbocyanine iodide (DHCC; Eastman Kodak, Rochester, NY). Images were processed using ImageJ and Photoshop (Adobe, San Jose, CA). For dextran analysis of membrane integrity, in vitro nuclear reconstitution reactions were carried out as in Harel *et al.* (2003a), except that at the beginning of the reaction, additions were made as follows, where noted: GST as control (15 μM), GTPγS (2 mM), BAPTA (8 mM), RanQ69L-GTP (15 μM), GST-transportin (15 μM), GST-TLB (15 μM), MBP-M9M (15 μM), or combinations thereof. Assembly was allowed to proceed for 45 min, and then wheat germ agglutinin (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-kDa dextran; Lau *et al.*, 2009). Reactions were then stopped 5 min on ice, and 1 μl of rhodamine-labeled dextran (70 kDa, 2.5 μg; Molecular Probes, Eugene, OR) was added to a 25-μl reaction, followed by a second incubation for 15 min on ice. The reactions were fixed by dilution 1:1 with egg lysis buffer containing 7.4% paraformaldehyde, and dextran exclusion was visualized.

For nuclear pore assembly, pore-free nuclear intermediates were assembled by adding 8 mM BAPTA (EMD Chemicals, San Diego, CA) to the nuclear formation reaction at $t = 0$ min as described (Macaulay and Forbes, 1996; Harel *et al.*, 2003a). At $t = 60$ min, extract containing BAPTA-arrested pore-free nuclei was diluted 1:10 into fresh cytosol in the presence or absence of recombinant proteins (MBP, MBP-M9M, RanQ69L-GTP, transportin, TLB, or combinations thereof). Reactions were allowed to proceed for additional 60 min. Membranes were visualized with DHCC, and nuclear pore assembly (Figure 6) was visualized by staining for FG nucleoporins with Alexa 555–labeled mAb414 antibody (Covance, Berkeley, CA; Invitrogen, Eugene, OR). For the latter, Alexa 555–mAb414 (1 μg/μl) was diluted 1:50 in the nuclear assembly reaction and allowed to incubate for 15 min before fixation and visualization.

GST pull downs

To assess the M9M sensitivity of nucleoporin interactions with transportin (Figure 7A), magnetic beads containing GST-transportin or GST were prepared. The beads were prepared by incubating 100 μg of GST-transportin or 100 μg of GST with 30 μl of magnetic glutathione beads in 150 μl of 1× PBS for 1 h at room temperature. The protein-coupled beads were then washed twice with 1× PBS and resuspended in 150 μl of PBS. When indicated in Figure 7, 100 μg of MBP-M9M was added and incubated for 1 h at room temperature. The beads were then washed twice with 1× CSF-XB (100 mM KCl, 0.1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, 50 mM sucrose, and 7 mM EGTA). Each set of beads was then incubated with 50 μl of *Xenopus* mitotic egg extract supplemented with 1× Energy Mix and gently shaken for 1 h at room temperature. The beads were washed two times with RIPA-SDS (50 mM Tris, 150 mM NaCl, 1 mM EDTA, 1% NP40, 1% sodium deoxycholic acid, 1 mM NaF, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, pH 7.4), three times with 1× CSF-XB + 0.1% NP40, and two times with 1× CSF-XB before final resuspension in 50 μl of protein sample buffer. Each sample was heated for 5 min at 65°C (to preserve ELYS; Rasala *et al.*, 2006), loaded onto a 5–20% gradient SDS–PAGE gel, and transferred to nitrocellulose. Nucleoporin binding to the protein-coupled magnetic beads was assessed by cutting the blot into strips and immunoblotting with the individual antibodies shown; in some cases of similarly sized nucleoporins, the blot was stripped, verified to be negative, and reprobed with a different antibody. Each type of experiment was performed three to five times.

Determination of ELYS/transportin interaction

To determine whether transportin interacts with endogenous *Xenopus* ELYS protein in an M9M-sensitive manner (Figure 7, B and C), we performed coimmunoprecipitations. Three micrograms of purified anti-xELYS antibody or control immunoglobulin G was incubated with 30 μl of magnetic Protein G beads in 100 μl of 1× CSF-XB for 1 h at room temperature. The beads were then washed twice with 1× CSF-XB. After this, the beads were incubated with 30 μl of *Xenopus* mitotic egg extract at room temperature for 1 h, supplemented where noted with 20 μM M9M. The beads were then washed three times with RIPA-SDS and two times with 1× CSF-XB. The washed beads were resuspended in 40 μl of protein sample buffer and the eluate subjected to gradient 5–20% SDS–PAGE. Immunoprecipitated proteins were analyzed by immunoblotting.

Kinetochore localization of nucleoporins

To analyze mitotic chromatin and kinetochore staining of nucleoporins (Figure 8), 20 μl of *Xenopus* mitotic extract (supplemented with 1× Energy Mix) was incubated with sperm chromatin (70,000 SpC/20-μl mitotic extract) at room temperature as described (Orjalo *et al.*, 2006). Where indicated, 20 μM GST, 20 μM GST-transportin (+TRN), or 20 μM GST-transportin plus 20 μM MBP-M9M (+TRN+M9M) was added at $t = 0$ min. After 60 min of incubation, each reaction was diluted five times in dilution buffer (0.8× XB-EGTA buffer, 250 mM sucrose) and then mixed with 500 μl of a fixation buffer specific to this assay (0.8× XB-EGTA, 250 mM sucrose, 4% formaldehyde) and incubated for 30 min at room temperature (Orjalo *et al.*, 2006). Each reaction was then overlaid onto a glycerol

cushion (40% glycerol, 0.8× XB-EGTA, 0.1% Triton X-100) and spun down onto coverslips for 10 min at 6000 × *g* at 18°C. The coverslips were washed one time with 1× PBS, fixed with −20°C methanol for 30 min, washed twice with 1× PBS, rehydrated with 1× PBS + 0.1% Triton X-100 for 60 min, and blocked with 5% bovine serum albumin in 1× PBS for 60 min. Immunofluorescence assays were performed with anti-nucleoporin antibodies as described (Wood *et al.*, 1997; Arnaoutov and Dasso, 2003; Harel *et al.*, 2003b; Orjalo *et al.*, 2006).

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Chapter 3

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Current Opinion in Cell Biology

Nuclear transport factors: global regulation of mitosis☆

Douglass J Forbes, Anna Travesa, Matthew S Nord and Cyril Bernis

The unexpected repurposing of nuclear transport proteins from their function in interphase to an equally vital and very different set of functions in mitosis was very surprising. The multi-talented cast when first revealed included the import receptors, importin alpha and beta, the small regulatory GTPase RanGTP, and a subset of nuclear pore proteins. In this review, we report that recent years have revealed new discoveries in each area of this expanding story in vertebrates: (a) The cast of nuclear import receptors playing a role in mitotic spindle regulation has expanded: both *transportin*, a nuclear import receptor, and *Crm1/Xpo1*, an export receptor, are involved in different aspects of spindle assembly. Importin beta and transportin also regulate nuclear envelope and pore assembly. (b) The role of nucleoporins has grown to include recruiting the key microtubule nucleator – the γ-TuRC complex – and the exportin Crm1 to the mitotic kinetochores of humans. Together they nucleate microtubule formation from the kinetochores toward the centrosomes. (c) New research finds that the original importin beta/RanGTP team have been further co-opted by evolution to help regulate other cellular and organismal activities, ranging from the actual positioning of the spindle within the cell perimeter, to regulation of a newly discovered spindle microtubule branching activity, to regulation of the interaction of microtubule structures with specific actin structures. (d) Lastly, because of the multitudinous roles of karyopherins throughout the cell cycle, a recent large push toward testing their potential as chemotherapeutic targets has begun to yield burgeoning progress in the clinic.

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Karyopherins and RanGTP in mitosis: an evolutionary tour-de-force of repurposing

Each dividing eukaryotic cell cycles with elegant choreography between interphase and mitosis. Mitosis in higher eukaryotes, the focus of this review, involves the set up and breakdown of multiple 'scenes', each with a different purpose or theme. First, there is assembly of a large mitotic spindle with duplicated chromosomes aligned *via* their kinetochores (metaphase). Then a poignant separation of the duplicated chromosomes occurs (anaphase), and finally a triumphant reassembly of nuclear envelopes and nuclear pores around the sets of separated chromosomes (telophase). At the molecular level, each structure involves the choreographed assembly of hundreds (kinetochores and NPCs) to thousands (spindles and nuclei) of individual proteins. Early on in cell cycle research, it was found that kinases and phosphatases determine the *timing* of the above events. This set of regulatory phosphorylations was further aided by key ubiquitination and proteolytic events in order to convey irreversibility, such as in the shift from metaphase to anaphase. But what *spatial* regulation directs each of the large mitotic structures to assemble in the correct place?

The answer was unexpected. If the first act of cell cycle proliferation is interphase, the second act is mitosis. Instead of changing the protagonists, in an ingenious tour-de-force, evolution kept the main actors of nuclear transport – karyopherins and RanGTP – and assigned them new roles for mitosis. Other cast members came on stage for the second act. These different *assembly factors*, regulated by karyopherins and RanGTP, proved to be proteins directly involved in forming the mitotic structures to be assembled: *spindle assembly factors* (SAFs) for the mitotic spindle, *nucleoporins* for nuclear pore assembly, and quite a few surprises.

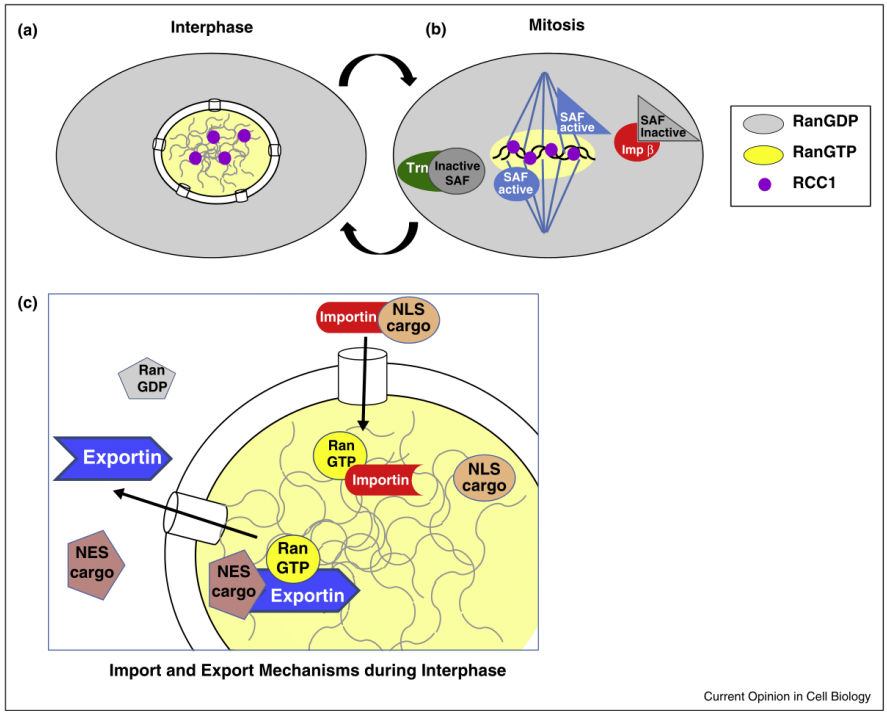
Initial studies: *in vitro* spindle and nuclear reconstitution

Prior to 1999, little thought of a mitotic role for karyopherins was envisioned. Then, an impressive set of seminal studies by multiple labs broke upon the scene from 1999 to 2002. Using *in vitro* mitotic *Xenopus* egg extracts, long known to be capable of spindle assembly [1], multiple groups discovered that importin beta and RanGTP together determine *where* spindle assembly occurs [2•,3–8]. Importin beta, often with the aid of its NLS-binding adaptor protein, importin alpha, binds to and masks spindle assembly factors (SAFs) in cytoplasmic areas distant from the

chromatin. By such binding, importin beta prevents spatially inappropriate spindle formation. In the vicinity of the mitotic chromosomes, however, importin β releases its bound SAFs, which sets spindle assembly in motion. Why only around chromosomes? Strikingly, a localized RanGTP 'cloud' is produced around the mitotic chromosomes. This localized RanGTP cloud results from the fact that active RCC1, the RanGEF that stimulates the production of RanGTP, is a chromatin- and DNA-binding protein [9–11]. In addition, RanGAP is cytoplasmic and converts any RanGTP that diffuses away from the chromatin into RanGDP. Thus, importin beta (or α/β-) bound SAFs are released from inhibition by the high levels of RanGTP near the mitotic chromosomes and the mitotic spindle in all its complexity and beauty forms *solely* in that locale [9,10•] (Figure 1B).

The flurry of initial studies showed that RanGTP and importin beta act as dueling positive (RanGTP) and negative (importin beta) regulators. At heart, RanGTP acts as an all-powerful 'GPS' or 'genome-positioning signal' for mitotic assembly, counteracting the overall micromolar concentrations of karyopherins, albeit in a very localized area. In this manner, the production of RanGTP acts as a spatial cue that directs the major mitotic structures to assemble around the chromosomes – and not elsewhere (Figure 1B) [9]. Depending on the phase of mitosis, the dueling karyopherin/RanGTP team

Figure 1



Karyopherins and RanGTP in interphase and mitosis.(A) In interphase, RanGTP (yellow) is primarily found only in the nucleus, due to the localization of the RanGEF, RCC1 (purple), to chromatin. RanGDP (light gray) is found in the cytoplasm where the RanGAP and its accessory protein RanBP1 (both not shown) are also localized and induce RanGTP hydrolysis. **(B)** In mitosis, RCC1 continues to be bound to the chromatin of the mitotic chromosomes and produces a 'cloud' of RanGTP, which dissociates any adjacent importin beta/SAF or transportin/SAF pairs. The freed spindle assembly factors or SAFs thus promote spindle assembly only around the mitotic chromosomes. At a distance from the chromosomes, the SAFs are held inactive by the binding of the transport receptors importin beta and transportin [9,49,52••]. Thus, overall RanGTP appears to act as a spatial cue for assembly of the mitotic spindle and, later in mitosis, for assembly of the nuclear membranes and nuclear pores around chromatin. **(C)** For reference, the schematic shows the details of nuclear import of an NLS cargo protein by a generic importin receptor. The importin/NLS cargo complex is dissociated by nuclear RanGTP. Also shown is the export of an NES cargo protein by a generic exportin receptor. In this case, the export complex requires RanGTP as a co-factor in its formation. After export, the exportin/NES cargo/RanGTP complex is dissociated upon RanGTP hydrolysis by cytoplasmic RanGAP/RanBP1 (not shown).

regulates assembly of the mitotic spindle, the nuclear envelope, and the nuclear pores (see left panels, Figure 3A-C). Indeed, it was the easy manipulability of *Xenopus* egg extracts that facilitated the *in vitro* study of all these assembly events [10•,12–16].

Spindle assembly factors: extensive regulation

The first molecular targets of karyopherin/Ran regulation were in the area of spindle assembly. The identification of a number of importin beta- (and alpha/beta-) inhibited spindle assembly factors burst upon the scene (Figure 1B). In our review, the definition of SAF is taken to mean any factor that promotes the assembly (or nucleation) of microtubules into a bipolar spindle at mitosis. Many of the SAFs identified (Table 1) were found, quite logically, to regulate microtubule nucleation, growth, stability and organization. *Microtubule-associated proteins* (MAPs) with specific spindle assembly functions include the MAPs TPX2, NuMA, Xnf7, HURP, Maskin, and NuSAP. Other SAFs were kinesins (XCTK2, Kid), which affect spindle bipolarity and chromosome orientation, and Cdk11, a cyclin-L-dependent kinase, responsible for microtubule stabilization and microtubule-kinetochore interaction [9,17]. Still other relevant players were found to regulate RanGTP production and modulation. These include the RanGEF RCC1, the RanGAP, its activating partner RanBP1, and RanBP2 (Figure 2A) [18,19•]. Interestingly, some proteins that are nuclear in interphase and SAFs in mitosis, such as lamin B and Rae1, are part of a mitotic spindle matrix, an entity that includes all proteins encompassed in the cytoplasmic region of the spindle [9,20–22].

Since 2008, more SAFs controlled by importin beta and Ran have been identified (Table 1). These include: (a) the tumor suppressor Adenomatous polyposis coli (Apc), which helps assemble and bundle microtubules [23], (b) two chromatin remodeling ATPases, CHD4 and ISWI, that act as Ran-dependent microtubule stabilizers [24,25], (c) a k-fiber stabilizer, MCRS1 [26], and (d) a set of nucleoporins (see below) [27,28]. Each of the above SAFs, both old and new, are hypothesized to be bound and inhibited by importin beta (or α/β) away from chromatin and released by RanGTP near chromatin.

Nuclear pore proteins act in spindle microtubule nucleation

A very unexpected group of spindle assembly factors were revealed to be nuclear pore proteins or *nucleoporins* (*Nups*). It turns out that a subset of nucleoporins, like importin beta and Ran, lead a double life during mitosis. In interphase, the nucleoporins in question reside largely at the nuclear pore (and to a lesser level at certain chromatin sites) [29–31]. In mitosis, however, this set of Nups transits broadly to the kinetochores, centrosomes, and/or fibers of the mitotic spindle to carry out mitotic functions [27,28].

In spindle assembly in animal cells, it is known that microtubules can grow from the centrosomes and from the kinetochores. Depending on the cell type or situation, one or the other source of microtubule growth can dominate [32–35]. It is now clear that Ran regulates both types of microtubule assembly [36].

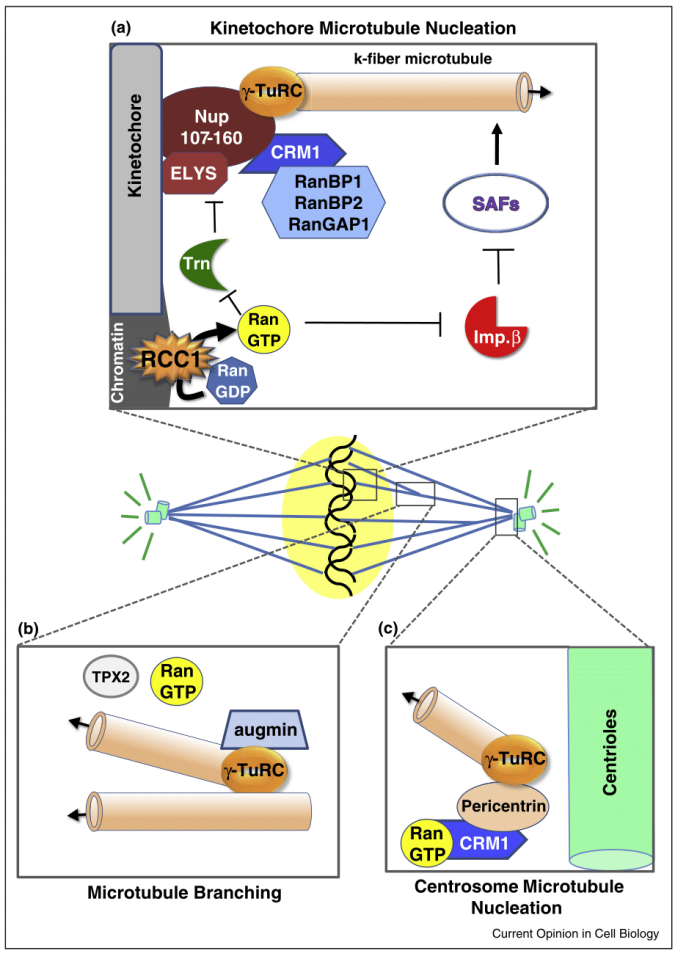
An expanding list of nucleoporins has been found to regulate multiple aspects of mitosis [27–29]. A full description is not possible here, but among them the most intriguing is the 9-member nuclear pore subunit termed the Nup107-160 or Y complex and its closely associated partner, ELYS. These Nups clearly localize to the kinetochore at mitosis and have proven essential for functional kinetochores and for microtubule nucleation into spindles (Figure 2A) [37–43,44•]. In fact, depletion of the Y complex was shown to allow initiation of centrosomal

Table 1

	Examples	*Refs.*
Spindle Assembly Factors (SAFs)		
MAPs	TPX2, NuMA, Xnf7, HURP, Maskin, NuSAP	[9]
Kinesins	[a] XCTK2, [b] Kid	[9]
Spindle matrix proteins	Lamin B, Rae1	[9,20–22]
K-fiber stabilization	MCRS1*	[26]
Tumor suppressor	Adenomatous polyposis Coli (Apc)*	[23]
Chromatin remodeling ATPases	CHD4*, ISWI*	[24,25]
Multiple cellular functions	Nucleophosmin, Survivin, Cdk11	[9,17]
NPC proteins	Nup107-160 complex, Nup98*, ELYS/Mel28*	[38,39,42••,44•]
NPC Assembly Factors		
Importin beta and/or transportin regulated Nups	Nup107-160 complex, ELYS, FG Nups (Nup358, Nup214, Nup153, Nup98, Nup62, Nup50)	[39,40,50,51,52••]
Ran Modulation Factors		
	RCC1, RanGAP, RanBP1*, RanBP2, Sumo	[9,19•,63•]

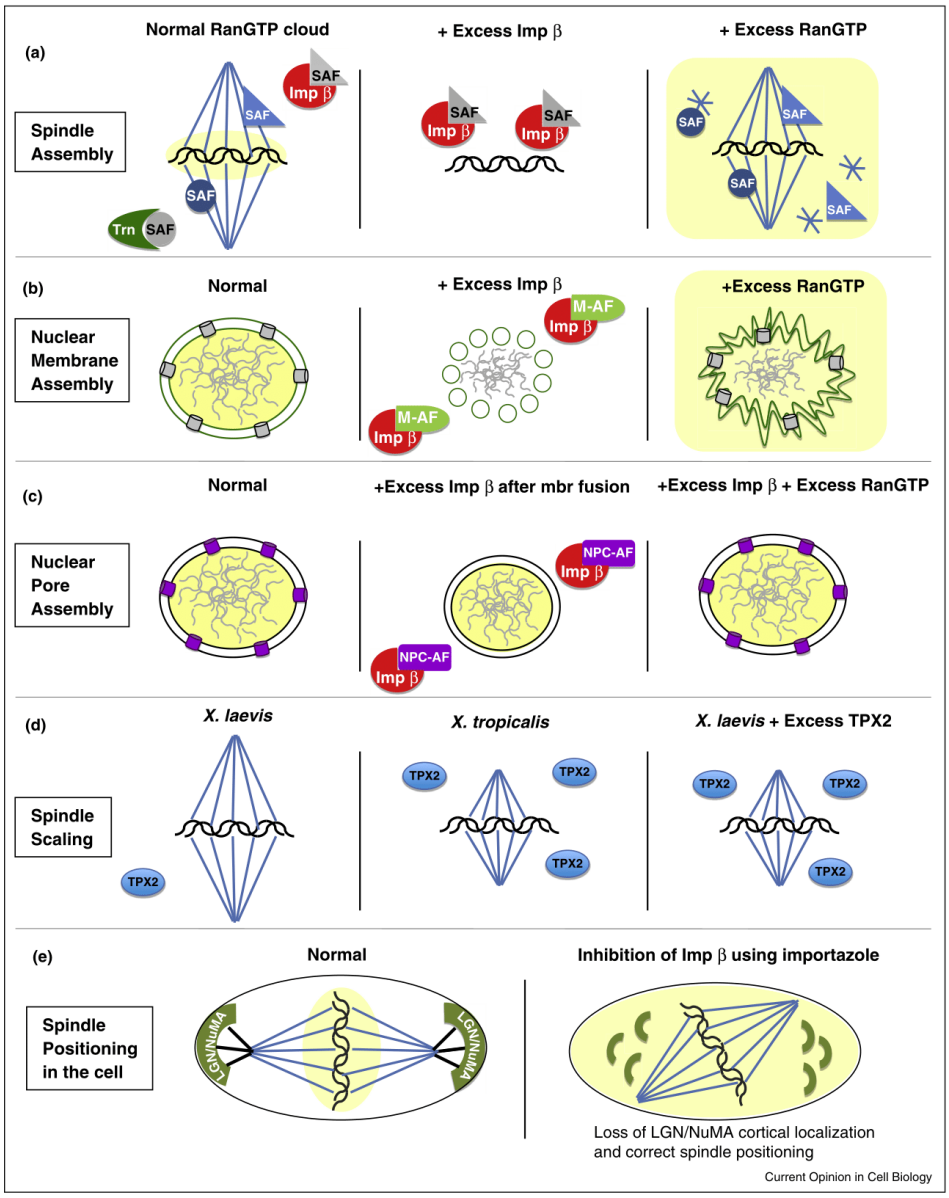
[a] Spindle-Kinesin.
[b] Chromokinesin.
* Recently characterized Ran- and/or Karyopherin-regulated SAFs.

Figure 2



Microtubule nucleation occurs at multiple locales within the spindle and is regulated by RanGTP in each.(A) In mitosis, microtubules have been shown to grow from γ-TuRC complexes recruited to the kinetochore by the Nup107-160 nucleoporin complex [42••]. In humans, the export receptor Crm1 is also present at the kinetochore with its binding partners RanGap1, RanBP1, and RanBP2. The RanGEF RCC1 is present on mitotic chromosomes and, as described in Legend 1B, produces a gradient ('cloud') of RanGTP around the chromosomes. RanGTP frees local SAFs from inhibition by importin beta (multiple SAFs) and transportin (the Nup107/160 complex and Elys SAFs) [9,10•,49,52••], such that k-fiber microtubules can grow from the kinetochores. **(B)** A newly discovered branching mechanism acting in spindle microtubule assembly is depicted. Here assembly can occur not only from centrosomes and kinetochores, as has long been known, but also from the sides of existing spindle microtubules. Augmin and γ-TuRC complexes are involved in the initiation of branching; RanGTP and TPX2 are also involved, but their exact mechanistic roles remain unknown [53••,54,55]. **(C)** Spindle microtubules also initiate strongly from the centrosomes in many instances [35], nucleated by γ-TuRC complexes. Exportin Crm1/RanGTP aids in the recruitment of pericentrin to the centrosome region, which then recruits the γ-TuRC complexes [91].

Figure 3



Multiple different arenas are regulated by karyopherins and RanGTP.(A) *Spindle assembly*. During normal mitotic conditions (left panel), as also described in Legend 1B, importin beta (Imp β, red) and transportin (Trn, green) bind to and inhibit Spindle Assembly Factors (inactive SAFs; gray) in areas far from chromatin. This inhibition prevents mitotic microtubule assembly at a distance from the chromosomes. A RanGTP cloud (yellow area), produced by the RanGEF RCC1 (not shown), around the mitotic chromosomes causes localized release of the SAFs from adjacent

microtubules in *in vitro* spindle reconstitution extracts, but proper connection to the chromosomes was not possible, and the microtubules disassembled with time [38].

Surprisingly, it was later discovered that the Nup107-160 subcomplex acts to recruit γ-TuRCs (gamma-tubulin ring complexes) to the kinetochore at mitosis, which initiate 'k-fiber' or kinetochore-initiated spindle microtubules [42••]. This startling discovery made symmetrical sense from a nucleation point of view in that γ-TuRC ring complexes were first discovered at the poles of the spindle and act there as the microtubule-nucleating components of the centrosomes. RanGTP has also been found to be present at both kinetochores and centrosomes [34,36,42••,45,46]. Importantly, the presence of RanGTP at the kinetochore is vital for Nup107-160/γ-TuRC-microtubule nucleation and counteracts the effects of importin beta (Figure 2A) [42••,45,47]. In different studies, it was shown that importin β binds to the Nup107-160 protein complex and the nucleoporin ELYS/Mel-28 and prevents their interaction both with chromatin [40,43,48–51] and with kinetochores [52••] in *Xenopus in vitro* extracts.

Microtubule branching occurs in spindles and is regulated by RanGTP

A newer discovery was in the works: it had long been thought that microtubule assembly occurs by the *linear* addition of tubulin α/β hetero-dimers to existing microtubules to produce entirely linear microtubules. A recent surprising study has shown that during *in vitro* spindle assembly in *Xenopus* egg extracts, a branching microtubule assembly mechanism occurs [53••,54,55]. Petry *et al.* (2013), using total internal fluorescence (TIRF) microscopy, visualized microtubules initiating on the sides of existing microtubules: branch points could clearly be seen. This branching requires the microtubule nucleating protein augmin, γ-tubulin and TPX2, and is stimulated by RanGTP (Figure 2B), the latter of which was shown to act by freeing the spindle assembly factor TPX2, presumably from importin α/β [53••,54,56]. The branching leads to an increased density of bundles of spindle microtubules and the authors suggest this may be for the purpose of amplifying the effect of the RanGTP gradient around the mitotic chromosomes. Nup98 also can induce a phenotype of excess microtubules in mitotic spindle assays, *via* excess Nup98 C-terminal fragment addition. The Nup98 fragment appears to inhibit MCAK (the microtubule-depolymerizing mitotic centromere-associated kinesin) [57]. It will be interesting to determine whether Nup98 or MCAK in some way influence the hitherto unsuspected branching microtubule mechanism that enhances spindle assembly.

RanGTP and its modifiers: new findings and variations on the theme

It is now well established that a gradient of RanGTP is crucial for spatially assembling the right mitotic structures at the right place: microtubule nucleation occurs where RanGTP is sufficiently high, which in most *in vivo* instances is around chromatin (Figure 3A, left panel). Indeed, addition of excess RanGTP to *Xenopus* mitotic extracts causes starbursts of microtubule nucleation (*i.e.*, asters) to occur throughout the extract, as karyopherin inhibition is released everywhere (Figure 3A, right panel). Another study further showed that simply tethering RanGTP-generating machinery to the plasma membrane causes organized microtubule arrays to form at that locale [58]. The discovery that RanGTP is found at other sites in the cell, such as at the centrosomes and kinetochores where microtubules nucleate (see Crm1 section below) or

(Figure 3 Legend Continued) importins; the released SAFs are now active and promote spindle assembly (active SAF; blue) in the correct location, *i.e.*, around the chromosomes. In extracts, as in cells, the initial balance of RanGTP, karyopherins and SAFs determines the influence of karyopherins and Ran on spindle assembly. If excess importin beta is added (middle panel), the importin beta (red) overwhelms the amount of RanGTP produced (not shown for clarity) and sequesters even the SAFs close to the chromosomes preventing formation of a mitotic spindle. In contrast, if excess RanGTP is added (right panel, yellow), the release of SAFs from importin beta and transportin (not shown) occurs throughout the extract, independently of their position with respect to chromatin. Thus, microtubules nucleate throughout the reaction, generating not only a mitotic spindle, but abundant microtubule asters (stars). **(B)** *Nuclear membrane assembly*. Normally the coordinated action of RanGTP and importin beta promote the formation of a double nuclear membrane surrounding the chromatin during telophase (green lines; left panel). If excess importin beta is added (middle panel), this is found to prevent the fusion of the ER membrane vesicles and tubules that normally form the double nuclear membrane, resulting in unfused vesicles (green circles). This occurs presumably by inhibiting one or more 'membrane assembly factors' (M-AFs), whose nature is still unknown and could be either soluble or membrane-bound. If instead too much RanGTP is added (right panel), this causes excess nuclear membrane production, which appears as invaginated nuclear membranes replete with nuclear pores (light gray) around the chromatin. **(C)** *Nuclear pore assembly*. In normal conditions, cells possess a double nuclear membrane studded with nuclear pore complexes (purple cylinders; right panel). When excess importin beta is added to a pore-free nuclear assembly intermediate (containing fused nuclear membranes but no nuclear pores that has been induced by BAPTA [52••]; middle panel), the excess importin beta binds to and inhibits nuclear pore complex assembly factors (NPC-AFs: purple color), and thus the nucleus remains devoid of nuclear pores. These NPC-AFs are disassembled nuclear pore proteins that act as NPC assembly factors at the end of mitosis. If, instead, excess RanGTP is included with the excess importin beta (left panel), the balance between the two is restored and nuclear pore assembly occurs as normal. **Note**: In (A), (B) and (C), an excess of transportin would cause the same effect that an excess of importin beta does (middle panels). **(D)** *Spindle scaling*. In nature, mitotic spindle size mirrors organismal size. For example, the spindle of the large frog, *X. laevis* (left panel), is larger than the spindle of a smaller frog, *X. tropicalis* (middle panel). This spindle scaling has been shown to be dependent on TPX2 levels. Indeed, adding an excess of TPX2 to *X. laevis* spindle assembly reactions reduces the size of the *X. laevis* spindle to that of *X. tropicalis* (right panel). Note: Karyopherins and RanGTP are present, but are dominated by higher TPX2 concentrations [65]. **(E)** *Spindle positioning in the cell*. The mitotic spindle orients lengthwise in a normal cell (left panel) due to the pulling forces that cortically-bound LGN and NuMA (green) generate on the astral microtubules (black lines). Inhibition of importin β, using the inhibitor importazole, causes misoriented spindles due to a loss of LGN and NuMA from the cortex (right panel).

inside of primary or motile cilia [59,60••]), leads one to believe that we have not heard the last of Ran's molecular talents.

Probing the regulatory mechanism for generating local RanGTP in mitosis is thus of great interest. Localization of RCC1 on chromatin has been seen to increase during mitosis [61,62], an increase tightly regulated by RanBP1 [19•]. RanBP1, already known to increase the activity of the RanGAP, has been shown to bind to RCC1 and modulate RCC1's enzymatic activity, thus controlling the spatial organization and amplitude of the mitotic spindle [19•]. Other experiments using beads coated with purified RCC1 in *Xenopus* egg extracts demonstrate that RCC1 is one of the minimal chromosome components able to generate a spindle [63•].

This is perhaps a good place to bring forth the fact that a set of recent studies find that certain cell types and developmental stages appear to have become less reliant on spatial control by Ran/karyopherin gradients [35,64]. One example is in spindle size scaling [65–67]. *Xenopus* frog species differ in overall size, a difference mirrored in their mitotic spindles. Eggs of the small frog *X. tropicalis* contain 3-fold as much SAF TPX2 as eggs of the larger *X. laevis*. Interestingly, artificially increasing TPX2 concentration in *X. laevis* egg extracts (without increasing importin alpha/beta) produces smaller spindles [65] (Figure 3D). It may be that simply increasing SAF concentration in some cell types, *via* evolutionary change, could decrease normal control by karyopherin/RanGTP if the latter's concentrations remained lower. However, an in-depth analysis of diverse adaptations such as this can be better obtained from more extensive reviews [35,64].

Nuclear membrane and nuclear pore assembly: Karyopherin/RanGTP regulation in *in vitro* reconstitution systems

Equally compelling were discoveries that the importin beta/Ran pair of dueling regulators also spatially controlled the major assembly events of late mitosis, *i.e.*, nuclear membrane assembly and nuclear pore assembly [10•,68–72]. Following recruitment of membrane vesicles to mitotic chromatin in interphase nuclear reconstitution extracts, importin beta/RanGTP were found to regulate the vesicle-vesicle fusion reaction required to form a double nuclear membrane. Excess importin beta inhibited fusion, while excess RanGTP promoted it. The *balance* of their activity was essential: excess importin beta resulted in unfused membrane vesicles, while excess RanGTP resulted in excessive, invaginated nuclear membranes around the chromatin [10•,69] (Figure 3B). One recent study implicates the Lamin B receptor, LBR, as an importin beta-regulated target involved in nuclear membrane formation [73].

A similar regulatory scheme controls nuclear pore assembly in late mitosis: excess importin beta blocks nuclear pore assembly, while added RanGTP allows NPC assembly [10•,69–72,74] (Figure 3C). For example, importin beta regulates the seeding of initiation sites for NPC assembly on chromatin *via* binding to and inhibiting ELYS and Nup107-160 complex, the initiators of telophase NPC assembly (aka 'postmitotic' assembly), from anchoring to chromatin *in vitro* [40,48,50,75]. Importin beta regulation of NPC assembly is also observed to take place *in vivo* in interphase pore assembly [76]. Further description of these events and more complete references are reviewed in [10•,77•,78].

An expanding karyopherin network: Transportin

With 21 importin beta family members in humans [79], a key question is whether karyopherins other than importin beta regulate mitotic assembly events. It is widely thought that importin beta binds the SAFs it regulates in mitosis by binding to their nuclear localization sequences (NLSs). Transportin, the first identified relative of importin beta, recognizes a different class of NLS that, although varying in length and sequence, is often characterized by a Proline-Tyrosine (PY) dipeptide [80,81]. PG, PV or PL NLS motifs as well as very different Lys/Arg basic NLSs are also recognized by transportin [82,83•]. An extensive review of transportin in normal and disease contexts is available [84].

Transportin has been found to negatively regulate spindle assembly, nuclear membrane assembly, and nuclear pore assembly [49,52••], by directly binding and inhibiting targets, in a manner parallel to importin beta. Addition of a super-affinity Transportin NLS (M9M) causes aster assembly throughout the extract cytoplasm, indicating that simply freeing transportin's cargo is enough to initiate spindle and aster assembly. It was found that transportin also regulates nuclear envelope and NPC assembly *via* a direct inhibition model [52••]. What are transportin's assembly factor targets? For spindle assembly, a major likely target is the Nup107/160 Y complex, given that mitotic extracts depleted of the Nup107/160 Y complex fail to form bipolar spindles, and transportin blocks the binding of the Y complex to kinetochores *in vitro* [52••] [27,38,41,85]. Thus, the network of regulatory importins is expanded to include both transportin and importin beta.

The exportin Crm1 is a cell cycle regulator and chemotherapy target

Crm1, also known as Exportin-1 or Xpo-1, is the major nuclear export receptor for protein cargos in interphase. Crm1 recognizes leucine-rich nuclear export signals (NESs) on a multitude of gene regulatory and nucleocytoplasmic shuttling proteins. Crm1 can only export its cargos in the form of a ternary complex, Crm1/NES-cargo/RanGTP. Upon reaching the cytoplasmic face of the pore (where in mammals RanGAP is bound), RanGAP

together with RanBP1 and RanBP2 stimulate RanGTP hydrolysis to disassemble the export complex (Figure 1C) [86].

In the past decade, a growing body of evidence has revealed that Crm1 plays essential roles in mitosis. However, instead of releasing assembly factors in areas of high RanGTP as do importin β and transportin, Crm1 binds to both RanGTP and key mitotic proteins to target those proteins to specific areas of the spindle.

Crm1 and kinetochores

One key area is the kinetochore of mitotic chromosomes. Surprisingly, Crm1/Xpo1, localizes to the mitotic kinetochores of both yeast and humans [18]. Such Crm1 presence was shown to be needed for functional kinetochore nucleation of microtubules in humans [18,34,87]. Specifically, Crm1 targets a complex of RanBP2, RanGAP1 and RanGTP to the kinetochores of human cells (Figure 2A) [18,87,88]. With its partners, Crm1 is proposed to stabilize the connection of microtubule kinetochore fibers (k-fibers) to the kinetochore and, by doing so, promote proper chromosome segregation. In addition, Crm1 in human cells has been implicated in tethering the Chromosomal Passenger Complex (CPC) to the centromeric region of chromosomes, *via* the CPC Survivin protein's NES domain [89]. A recent study delineating antagonistic roles of importin beta and Crm1 at human kinetochores reveals that overlapping karyopherin regulatory webs exist [90•]. Lastly, it should be noted that *Xenopus* kinetochores have been mentioned to lack Crm1 and/or RanGAP presence (mentioned, but not shown in ref 87), but it is not known whether this apparent lack is due to antigen inaccessibility, less stable k-fibers, or actual absence of Crm1 (M. Dasso, personal communication).

Crm1 and centrosomes

Interestingly, Crm1 is also observed to be present at the centrosomes throughout the cell cycle (Figure 2C) [91]. It is proposed that Crm1 binds to RanGTP present in the centrosome, then recruits the major centrosomal scaffold protein, pericentrin. Pericentrin in turn is known to recruit γ-TuRC complexes and together these act to nucleate the centrosome-initiated spindle microtubules. Either RNAi depletion of Crm1 or overexpression of the N-terminal RanGTP-binding domain of Crm1 causes reduction in both pericentrin and γ-TuRCs at centrosomes and disrupts the mitotic spindles in cultured cells [91]. Also, prior to its targeting, Crm1 is mitotically phosphorylated by the mitotic kinase CDK1/cyclinB (Ser391), which enhances its ability to target RanBP2/RanGAP1 to the mitotic spindle [92].

Crm1 has also been observed to be involved in the targeting of NES-bearing proteins to the centrosome. BRCA1 and BARD1, an E3 ubiquitin ligase when heterodimerized, are both targeted to the centrosome by Crm1 independently of one another [93,94]. Normally, the BRCA1/BARD1 protein complex plays roles in DNA damage response and centrosome duplication, thus ensuring proper centrosome duplication. Correct centrosomal targeting of these proteins is critical, since perturbations to centrosome duplication can lead to inherited genetic defects and aneuploidy. For example, inhibition of Crm1 in early metaphase results in excess, acentriolar spindle poles [95].

Crm1, cancer and chemotherapy

Crm1 is the major nuclear export receptor for many DNA damage monitors and tumor repressor proteins, including p53, Rb, and FOXO [96,97]. The observed overexpression of Crm1 in many cancers results in preferential localization of these tumor suppressors to the cytoplasm where it is thought they are unable to function to subvert DNA damage and inappropriate cell proliferation [97]. Newly developed Small Inhibitors of Nuclear Export or SINEs represent a promising treatment for many cancer types. Modeled on an older inhibitor of Crm1 (Leptomycin B/LMB), SINEs prevent NES-cargo binding to vertebrate Crm1 by covalently binding to Cysteine 528 in the NES-binding cleft. SINEs are less toxic than LMB, as they are more highly specific to Crm1 [98••]. Currently it is thought that the fact that SINEs block the export of many tumor suppressor proteins and other functionally relevant cell cycle inhibitors from the nucleus explains how SINEs act as cancer inhibitors in cultured cell studies, mouse studies, and an increasing number of human clinical trials. However, it would appear from the above considerations equally possible that SINEs could interfere with the *mitotic* roles of Crm1 delineated above, and thereby affect their observed block to cell proliferation in cancer trials.

In vivo evidence

Strong corroborative evidence for the karyopherin/Ran control of spindle assembly comes from a number of *in vivo* studies, only a few of which can be mentioned here. The presence of a RanGTP cloud around *mitotic* chromatin has been demonstrated *in vivo* using the fluorescent biosensor Rango, which increases its FRET signal when released from importin beta by RanGTP [99–101]. Increased RanGTP has also been observed using RanGTP biosensors around spindles assembled *in vitro* in mitotic *Xenopus* egg extracts [102]. Similarly, a RanGTP gradient has been observed *in vivo* in living mouse oocytes using FRET [64,103].

Excellent reviews of a number of seminal *in vivo* studies on the karyopherin/Ran dueling regulators by the Lavia group and others include [64,104]. Recently, Hasegawa *et al.* [105•] found a steep RanGTP gradient exists around the mitotic chromosomes of rapidly growing cells, while reduced RanGTP gradients are observed around the chromosomes in primary cells (HFF-1 cells). Interestingly, overexpression of the RanGEF RCC1 in these primary cells causes induction of a steep RanGTP gradient.

Further, cell-cell fusion studies lead the authors to propose that chromosome gain can also increase the RanGTP gradient [105•], a gain that might also be seen in cancer cells.

Strong *in vivo* evidence for importin beta's role in mitosis comes from microinjection of different importin beta protein fragments into cells at prophase or prometaphase: a fragment of importin beta (aa 71-876) lacking the RanGTP binding domain caused blockage of spindle assembly and/or proper chromosome segregation in a majority of cells [4,106]. Thus, the above *in vivo* findings reinforce the *in vitro* findings: RanGTP and karyopherins are dueling regulators and a correct balance helps coordinate correct mitotic assembly events.

New cellular arenas for karyopherin/RanGTP regulation

Karyopherin/Ran and the timing of anaphase

Rape and colleagues [107,108•] have discovered that – once anaphase begins and the Anaphase Promoting Complex (APC/C) is activated by successful chromosome attachment – a higher level of regulation takes over. They found that, unlike cyclin B1 which is ubiquitinated and degraded at the very onset of anaphase, SAFs such as TPX2, HURP, and NuSAP, remain stable through anaphase. These SAFs, presumably originally freed from importin beta by RanGTP, are now protected from ubiquitination and degradation through anaphase by their physical association with the spindle microtubules. Later, when released from microtubules, the SAFs are quickly modified by the APC/C and degraded. This protective mechanism ensures that these SAFs are maintained as long as the spindle needs them, and their subsequent degradation prepares conditions for the next phase of the cell cycle [107–110].

Spindle positioning along the cell axis

Often in cell division, the spindle in mitotic cells *orients* along the long axis of the cell. In a study designed to reveal why this positioning occurs, a new role for karyopherins and RanGTP was discovered. Proteins that generate pulling forces on the astral microtubules, LGN and NuMA, the latter of which was an early SAF target of importin α/β [9], were also found to be key for *spindle positioning* [111••]. When an inhibitor of the RanGTP/importin beta interaction, importazole, was added to human cells, a misoriented spindle resulted [111••]. It turned out that LGN and NuMA had become mislocalized. The addition of CLASP1, a protein that stabilizes aster microtubules, restored correct spindle orientation. It appears that the karyopherin/Ran system works with LGN and NuMA to define *proper spindle orientation* along the long axis of the cell [111••,112] (Figure 3E).

Actin cytoskeleton regulation

Up to this point the *cytoskeletal* elements influenced by the karyopherin/RanGTP team have been microtubule-related. Now Samwer *et al.* [113••] have found an actin connection. They identify a novel actin-bundling kinesin, NabKin (for **N**uclear and meiotic **a**ctin-**b**undling **Kin**esin), that binds to and stabilizes nuclear *actin* bundles in interphase and also stabilizes the actin-based cortical ring structure that divides cells during cytokinesis. *In vitro*, they find that importin beta blocks NabKin kinase interaction with filamentous actin, while RanGTP reverses this inhibition. They conclude that NabKin directly links microtubules to F-actin and does so in a classical karyopherin/Ran-regulated manner [113••].

These new arenas can be added to the previous most unexpected area for karyopherin regulation, that of synapse-to-nucleus communication, where importin beta mediates the retrotranslocation of damage signals from an injured nerve terminus to its neuronal cell nucleus [10•,114,115•].

Perspectives

The karyopherins, importin beta and transportin, and their RanGTP counterpart are in fact a unique way to impose a wide-reaching regulatory regime over disparate cellular events. While kinase/phosphatase and ubiquitinase/proteolysis pairs depend on enzymatic amplification, the karyopherin/Ran paradigm depends instead on: (a) the high and pervasive concentrations (micromolar) of these transport receptors throughout the cytoplasm during mitosis, (b) the ability of each karyopherin to bind to a very broad spectrum of motifs and molecules, and (c) importantly, the focused production of RanGTP in specified locales for targeted activation of assembly factors and pathways. Perhaps rivaled only by the hsp70 chaperone family for versatility of binding, the added element of localized RanGTP production renders the evolutionary power of the karyopherin/Ran regulation even greater. It now stands out as one to be watched by all.

Acknowledgments

The current studies concerning karyopherins and RanGTP number in the thousands with an increasing fraction concerning mitotic roles. The overall mitotic literature is even larger; we very much regret that only a fraction could be cited here. This work was supported by a National Institutes of Health grant, R01-GM033279, to D. J. F. The authors would like to thank Dennis Garland for helpful comments on the manuscript.

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Chapter 4

An Analysis of Exportin Regulation of Major Mitotic Assembly Events *In Vitro:*

Membrane Fusion, Nuclear Pore Formation, and Spindle Assembly.

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Abstract

Xenopus egg extracts provide a powerful tool for studying a wide range of biological processes, from DNA replication to nuclear pore complex (NPC) formation. They have been indispensable in the dissection of the roles of *Importins* during major mitotic assembly events, including spindle assembly, nuclear membrane fusion, and nuclear pore formation. In this study, *Xenopus* egg extracts were used to assess the regulatory roles that *Exportins* play during nuclear membrane fusion, nuclear pore formation, and spindle assembly *in vitro*. For this, recombinantly produced Exportins were added to nuclear and mitotic reconstitution reactions *in vitro*. Three Exportins were analyzed: Crm1 (Exportin-1), Exportin-t, and to a lesser extent, Exportin-5. It was found that Crm1 and Exportin-t were able to inhibit formation of the nuclear membranes by affecting vesicle fusion. This inhibition was counteracted by RanQ69L-GTP, a constitutively actively mutant of RanGTP. Crm1, Exportin-t, and Exportin-t were also found to inhibit nuclear pore formation in BAPTA nuclei, which are pore-free nuclear intermediates. The inhibitory effects on nuclear pore formation could be relieved by the simultaneous addition of RanQ69L-GTP, a constitutively active mutant of RanGTP. Looking at mitotic extracts, we found that both Crm1 and Exportin-t negatively regulate spindle assembly.

To probe for potential regulatory targets of exportins, GST pulldowns from interphase *Xenopus* egg extracts were performed using Crm1- or Exportin-t-bound glutathione beads with or without RanQ69L-GTP. It was found that Crm1 and Exportin-t pulled down a subset of nucleoporins known to be essential for both

spindle assembly and nuclear pore assembly. RanQ69L-GTP significantly decreased such interaction. The above study thus greatly expands the network of nuclear transport factors that are known to affect major mitotic structural assembly events.

Introduction

The nucleus is one of the most prominent and defining structures of the eukaryotic cell. The nuclear envelope, a double membraned structure provides a barrier between the contents of the nucleus and the cytoplasm. This barrier creates a highly-regulated environment that is crucial for safeguarding the genomic DNA. It is has become critical to many eukaryotic processes to maintain the separation between the nuclear and cytoplasmic contents without abolishing communication between the two compartments. Cells evolved specialized selective entries to regulate the communication and transport, termed nuclear pore complexes (NPCs). The NPC is a very large, macromolecular structure, 100-120 MDa in size in vertebrates, that perforates the double-membraned nuclear envelope (D'Angelo and Hetzer, 2008).

The nuclear pore complex is composed of ~30 different proteins, each found in 8-32 copies. These proteins are collectively known as nucleoporins, or Nups. There are distinct classes of Nups, including FG-Nups with phenylalanine-glycine (FG) repeats, central scaffold Nups, and integral membrane Nups. FG Nups, comprising one-third of the different Nups, are localized along the pathway of transport. The FG Nups of the central pore interact to form a hydrogel that acts as a selective barrier between the nucleus and cytoplasm (Labokha et al., 2013; Schmidt and Görlich, 2015). Furthermore, the FG repeats themselves serve as binding sites for the nuclear transport receptors that are responsible for transport of large cargoes between the cytoplasm and the nucleus (Cook et al., 2007; Fried and Kutay, 2003; Kapinos et al., 2017),.

Transport through the NPC is a tightly-regulated process. Molecules ranging in size from water, ions, and nucleotides to proteins less than 20-40 kDa can passively diffuse through the pore. However, proteins larger than this threshold must traverse the NPC with the aid of a nuclear transport receptor (Barry and Wente, 2000; Fried and Kutay, 2003; Kuersten et al., 2001a; Nigg, 1997; Weis, 2003). Multiple nuclear transport receptors exist and are collectively known as karyopherins.

Karyopherins encompass two distinct branches of proteins: importins and exportins. Importins transport cargo into the nucleus, while exportins transport cargo from the nucleus to the cytosol. All karyopherins possess HEAT repeats (Huntington's, eIF3, PP2A, and Tor1 kinase), each of which consists of two cylindrical coils positioned in an anti-parallel orientation (Cook et al., 2007; Yoshimura and Hirano, 2016). Karyopherins also commonly possess a region of homology at the N-terminal end of the protein, which is known as the CRIME domain (Crm1, Importin β, Etc.) The CRIME domain plays a large role in binding the regulatory factor, RanGTP, to the karyopherin (Kapinos et al., 2017; O'Reilly et al., 2011; Ström and Weis, 2001; Süel et al., 2006).

The directionality of transport across the nuclear envelope is in large part controlled by the small GTPase Ran. The GTP-exchange factor for Ran, termed RCC1, is a chromatin-bound enzyme, and activates Ran for transport by exchanging its GDP for GTP. This creates a large biochemical gradient where the concentration of RanGTP is high in the nucleus, and low in the cytosol (Bischoff and Ponstingl, 1991a, 1991b). RanGAP, which is bound to the cytoplasmic filament nucleoporin,

Nup358, (and is also present in the cytosol), enhances the steep gradient of RanGTP by ensuring Ran hydrolyzes its GTP to GDP at nuclear exit.

The karyopherin family of proteins harnesses the RanGTP gradient to produce directionality of transport (Hopper et al., 1990; Kalab et al., 1999, 2002). Importins in the cytoplasm bind cargo bearing a nuclear localization signal (NLS), then the import complex travels through the nuclear pore, and enters the nucleus. Inside the nucleus the importin binds RanGTP, which induces a conformational change that displaces the cargo, completing import. Conversely, inside the nucleus, exportins bind cargo with a nuclear export signal (NES) along *with* RanGTP to form a ternary complex that then exports through the NPC. RanGAP, bound to the Nup358 cytoplasmic filament, helps stimulate the RanGTP to hydrolyze its GTP to GDP, which causes the ternary export complex to disassemble, completing export (Barry and Wente, 2000; Izaurralde and Adam, 1998; Weis, 2003).

Past studies have shown that importins function as regulators not only of nuclear transport during interphase, but that they also critically regulate major mitotic assembly events. RanGTP was found to promote the assembly of mitotic structures, while the Importins α and β were found to be inhibitors of the same processes (Carazo-Salas et al., 2001; Du et al., 2002; Ems-McClung et al., 2003; Harel et al., 2003; Kalab et al., 1999; Nachury et al., 2001; Ohba et al., 1999; Schatz et al., 2003; Walther et al., 2003). Indeed, it was found that Importin β directly inhibited the fusion of nuclear membranes and the formation of nuclear pores *in vitro,* in direct opposition to RanGTP (Delmar et al., 2008; Harel et al., 2003; Hetzer et al., 2000;

Walther et al., 2003). Subsequent work with Importin β revealed a much wider array of regulatory targets regarding spindle assembly, of which reviews are available (Forbes et al., 2015; Kalab and Heald, 2008). A second importin, Transportin, was also found to directly regulate major mitotic assembly events in a pathway parallel to Importin β (Bernis et al., 2014; Lau et al., 2009a). Essentially, the two importins bind to and inhibit assembly factors in areas away from mitotic chromosomes and chromatin. However, the importins release their bound assembly factors upon binding to RanGTP, which is found in high concentration around the mitotic chromosomes and chromatin.

Much effort has been dedicated to *importin* regulation of mitotic events. However, to date, the other half of the karyopherin family, the exportins, have yet to be studied in *Xenopus* egg extracts for an effect on mitotic assembly events. The exportins have a distinct and interesting set of cargoes. Crm1 (Exportin-1, Xpo1) is the major nuclear protein export receptor for the cell. It is a relatively large protein, ~118 kDa, and consists of 21 HEAT repeats (Conti et al., 2006; Cook et al., 2007; Güttler and Görlich, 2011). Crm1 recognizes nuclear proteins that carry a leucine-rich NES, also termed a classical NES (Dong et al., 2009; Fornerod et al., 1997; Kutay and Güttinger, 2005). Of all the exportins, Crm1 has the largest and most diverse set of cargo, ranging from p53 to full ribosomal subunits (Kırlı et al., 2015; Thakar et al., 2013; Wühr et al., 2015; Xu et al., 2012). In certain cancer types, overexpression of Crm1 leads to improper nuclear export and cytoplasmic localization of anti-tumor proteins and apoptosis proteins, aiding in the proliferation

of the cancerous cells. This has led to the development of targeted inhibitors of Crm1, small molecules known as SINEs, or selective inhibitors of nuclear export (Boons et al., 2015; Dickmanns et al., 2015; Mendonca et al., 2014). While a large body of work detailing karyopherin regulation of mitotic events has been conducted *in vitro, in vivo* evidence for such regulation exists as well. For example, Importin β overexpression causes microtubule fragmentation, which can be rescued by the co-expression of TPX2 (Ciciarello et al., 2004). Crm1 has also previously been shown to impact *mitotic* processes in normal mammalian cultured cells. Specifically, Crm1 localizes to the mitotic spindle and kinetochores *in vivo* during metaphase where it contributes, for example, to the localization of RanBP2 and RanGAP to the kinetochore, both of which are proteins necessary for the stability of the microtubule kinetochore fibers (Arnaoutov et al., 2005a). Lastly, Importin β and Crm1 play dueling roles *in vivo* in RanBP2/RanGAP localization on the mitotic spindle and kinetochore (Gilistro et al., 2017; Roscioli et al., 2012).

During interphase Exportin-t, another exportin of interest, transports tRNAs from the nucleus to the cytoplasm (Arts et al., 1998; Kuersten et al., 2001b; Kutay et al., 1998). Exportin-t is ~135 kDa and is composed of 19 HEAT repeats. A third exportin, for example Exportin-5, is the primary transporter for pre-miRNAs and double stranded RNA- binding proteins (Bohnsack et al., 2004; Güttler and Görlich, 2011; Yi, 2003). Exportin-5 shares many structural elements with the other exportins, such as its RanGTP binding domain (CRIME domain) and multiple HEAT repeats. As opposed to Crm1, Exportin-t and Exportin-5 are less well understood and

their functions are unexplored in mitosis. To date, none of these key exportins have been tested for cell cycle affects in *in vitro* cell cycle extracts.

Understanding the full scope of karyopherin function in the cell cycle has proven to be challenging when working with intact cells. Any perturbations to import or export can lead to subsequent deficits in mitosis. For example, a dysfunction in interphase transport may lead to errors in mitosis due to the lack of import of factors needed to prepare the cell for mitosis. For importins, researchers have overcome these potential pitfalls by utilizing *Xenopus* egg extracts in specific cell cycle states. Many different cellular events can be studied in *Xenopus* egg extracts that biochemically mimic *in vivo* conditions (Desai et al., 1998; Macaulay and Forbes, 1996; Maresca and Heald, 2006; Smythe and Newport, 1991; Tutter and Walter, 2006). These extracts largely replicate the appropriate *in vivo* events such as the formation of nuclear membranes and nuclear pores as seen in telophase. Therefore, with the proper biochemical manipulations, *Xenopus* egg extracts allow analysis of specific and distinct biochemical steps in physiological processes including spindle formation, nuclear envelope fusion, and nuclear pore formation.

This study uses *Xenopus* egg extracts and recombinantly produced proteins to begin to dissect the role of Crm1 and Exportin-t in nuclear membrane fusion, with an analysis of Crm1, Exportin-t, and Exportin-5 in nuclear pore formation. We also preliminarily explore the potential roles of Crm1 and Exportin-t in spindle formation. Using *Xenopus* egg extracts to simulate different mitotic phases of the cell cycle, we explore the novel roles of export karyopherins in a role completely distinct from their

interphase transport duties. We propose a model where exportins selectively bind target assembly factors and release them in the presence of RanGTP, thus contributing to proper spatial regulation of the nascent-forming nucleus or spindle.

Results

Crm1 and Exportin-t inhibit nuclear membrane fusion with RanQ69L-GTP counteracting their inhibitory effects

To investigate whether exportins can regulate nuclear envelope fusion, we used an *in vitro* nuclear assembly assay derived from *Xenopus* interphase egg extracts and monitored nuclear membrane fusion. To this end, exogenous Crm1 or Exportin-t was added to interphase *Xenopus* egg extracts containing sperm chromatin and membrane vesicles at t=0 and allowed to incubate for 1 hour. The fusion of vesicles to form the nuclear membranes was determined by observing two different aspects of the reconstituting nuclei. First, the integrity of the nuclear membrane was assessed by the ability to exclude TRITC-labeled 70 kDa dextran. Second, the appearance of the membrane was assessed by addition of the fluorescent membrane dye, DHCC. DHCC stains membranes such as those surrounding the reconstituting nuclei, allowing for the visualization of either smooth contiguous membranes or, alternatively, bound membrane vesicles blocked in an unfused state. As a control, a known inhibitor of nuclear membrane fusion, Transportin, which is a RanGTP-sensitive import receptor (Bernis et al., 2014; Lau et al., 2009a), was used to compare to the phenotypes generated by the three exportins (Figure 4.1). Transportin, as expected, blocked the appearance of a smooth nuclear membrane (Figure 4.1C, green) and prevented nuclear exclusion of 70 kDa dextran (Figure 4.1C, red). The addition of either Crm1 (Figure 4.1E) or Exportin-t (Figure 4.1G) also resulted in the loss of TRITC 70 kDa dextran exclusion, and led to the appearance of unfused, non-smooth,

incomplete nuclear membranes. However, we found that the addition of exogenous RanQ69L-GTP simultaneously with the exportins was able to significantly restore the extract's ability to properly form fused nuclear membranes for both Crm1 (Figure 4.1F) and Exportin-t (Figure 4.1H) and these were capable of 70 kDa dextran exclusion. The quantification of the observed phenotypes is shown in Figure 4.2. We conclude that in the *in vitro* nuclear reconstitution system, exportins can negatively regulate the fusion of nuclear membrane precursor vesicles into intact double nuclear membranes.

Crm1, Exportin-t, and Exportin-5 inhibit nuclear pore formation, all of which are counteracted by RanQ69L-GTP.

We next asked whether one or more exportins were able to inhibit nuclear pore formation *in vitro.* Nuclei reconstituted in the presence of the Ca^{2+} chelator BAPTA are known to form nuclear intermediates with smooth double nuclear membranes that are devoid of nuclear pores, termed "BAPTA nuclei" (Figure 4.3A, red). However, when these pore-free intermediates are diluted into fresh interphase egg extract lacking BAPTA, nuclear pore assembly then occurs (Macaulay and Forbes, 1996). Nuclear pore assembly is assessed with a fluorescent antibody to FG nucleoporins (Figure 4.3B, red). BAPTA nuclei thus provide ideal pore-free intermediates that allow for the specific analysis of nuclear pore formation, separate from membrane fusion.

Using preformed BAPTA nuclear intermediates (Figure 4.3A), the effects of Crm1, Exportin-t, and Exportin-5, added with fresh BAPTA-free cytosol, on nuclear pore formation were analyzed. Transportin, a known nuclear pore inhibitor sensitive to RanGTP, was again used as a negative control. Inhibition of nuclear pore assembly was observed with Tranportin (Figure 4.3D, red), but not if RanQ69L-GTP was included (Figure 4.3E, red). Compared to the GST control (Figure 4.4B, red) or to RanQ69L-GTP addition alone (Figure 4.3C, red), the three exportins produced strong inhibitory effects on pore formation (Figure 4.3F, 4.3I, 4.3L). However, upon inclusion of RanQ69L-GTP, the inhibitory effects of these exportins were ameliorated, and nuclear pore formation was significantly rescued. For Crm1, Exportin-t, and Exportin-5, respectively, the observed phenotypes ranged from nuclear pore complex restoration at scattered sites on the nuclear rim (Figures 4.3G, 4.3J, 4.3M), to full nuclear pore complex restoration around the entire rim (Figures 4.3H, 4.3K, 4.3N). The quantification of this pore assembly is shown in Figure 4.4. Crm1 showed the most reversal by RanQ69L-GTP. In sum, the Exportins Crm1, Exportin-t, and Exportin-5 all are capable of inhibition of nuclear pore assembly, and this inhibition can be considerably counteracted by RanGTP.

Crm1 and Exportin-t inhibit spindle assembly in *Xenopus* egg extracts

Since we have shown that the three exportins tested can inhibit both nuclear membrane fusion and nuclear pore assembly, structures formed in telophase in the

cell, we asked if they could also affect an earlier mitotic event, spindle assembly, which is seen at metaphase. The import receptors Importin β and Transportin have both been shown to be negative regulators of spindle assembly when added to mitotic *Xenopus* egg extracts (Bernis et al., 2014; Du et al., 2002; Ems-McClung et al., 2003; Gilistro et al., 2017; Gruss et al., 2001; Lau et al., 2009a; Nachury et al., 2001; Ribbeck et al., 2006; Roscioli et al., 2012; Schatz et al., 2003; Wiese et al., 2001). Mechanistically, an excess of these importins has been shown to act by causing sequestration of spindle assembly factors (SAFs), even around mitotic chromosomes, leading to blocked spindle assembly.

To ask the simple question of whether an excess of the exportins Crm1 and Exportin-t would have an effect on spindle assembly in mitotic *Xenopus* egg extracts, spindle assembly reactions were performed (as in Bernis et al., 2014) by adding demembranated sperm chromatin packets and energy mix to *Xenopus* mitotic extracts. The reactions were supplemented at time 0 with either GST alone, 20 μM GST-Transportin as a negative control, or different concentrations of recombinant GST-Crm1. Rhodamine-labeled tubulin was added at the beginning of the reaction to allow for visualization of spindle assembly. After 60 minutes, the reactions were halted and analyzed for the presence of mitotic spindles around the chromatin packets. The latter were visualized with Hoechst DNA dye. Spindle assembly readily occurred in a control GST reaction: microtubules in a robust bipolar spindle assembled around mitotic chromosomes, as expected Figure (4.5A).

Addition of low concentrations of Crm1 (1-2 μM) had no significant effect on spindle formation (Figure 4.5C-D). Increasing Crm1 concentrations (8-10 μM) caused a visible lessening of spindle structure (Figure 4.5E-F). Addition of 15-20 μM exogenous Crm1 to the mitotic egg extract completely blocked spindle assembly, as shown by the absence of microtubules around the chromatin in 80 % of the cases (15 μM, Figure 4.5G; 20 μM, data not shown). This inhibition of spindle assembly was similar to that seen previously with addition of 20 μM Transportin (Figure 4.5B) (Bernis et al., 2014). The remaining 20% of the DNA packets at 15 μM Crm1 showed aberrant spindles or DNA with irregular and disorganized microtubules around it (data not shown).

Similar to Crm1, Exportin-t was tested in our *in vitro* spindle assembly assays. Exportin-t (15 μM) induced two distinct phenotypes: (a) full spindle inhibition (Figure 4.6D) and (b) mitotic chromatin (blue) flanked by very small aster-like microtubule nucleations (red) (Figure 4.6C). When RanQ69L-GTP was added simultaneously, there was a partial reversal of spindle inhibition. The main phenotypes observed in this case were: a very small bipolar spindle adjacent to the chromatin (Figure 4.6E, red), multipolar spindles (Figure 4.6F, red), groups of asters that are similar to the addition of RanQ69L-GTP to mitotic extract in the absence of DNA (Figure 4.6G), and condensed chromatin lacking any microtubule association (Figure 4.6H). Thus, exportin-t inhibits spindle assembly and aster assembly, both of which are partially restored by RanGTP.

Crm1 and Exportin-t interact with multiple nucleoporins, while RanQ69L-GTP decreases their interactions.

The exportins above, when tested, clearly inhibit nuclear membrane fusion, as well as nuclear pore formation, and spindle assembly (Figures 4.1, 4.3, 4.5,4.6). Nucleoporins are not known to play a role in nuclear membrane fusion, but do offer strong potential regulatory targets for nuclear pore assembly, as well as spindle assembly (Bernis et al., 2014; Cross and Powers, 2011; Lau et al., 2009a; Mackay et al., 2009; Orjalo et al., 2006; Rasala et al., 2006). Nucleoporins are also known to be required for proper bipolar spindle formation and kinetochore assembly (Chatel and Fahrenkrog, 2011; Cross and Powers, 2011; Forbes et al., 2015; Mackay et al., 2009; Mishra et al., 2010; Nakano et al., 2011; Orjalo et al., 2006; Rasala et al., 2006; Rotem et al., 2009; Wozniak et al., 2010).

To understand the mechanism by which exportins might regulate these processes, we performed GST pulldowns to look for protein-protein interactions between exportins and nucleoporins in our system. In mature *Xenopus* eggs, the disassembled subunits of the oocyte nuclear pores are stored in large quantity for later in development. These pore subunits, ~13 in number, have proven to be logical targets for binding in our past studies on importin regulation of nuclear pore assembly (Bernis et al., 2014; Delmar et al., 2008; Harel et al., 2003; Lau et al., 2009a; Shah et al., 1998). Thus, we asked which, if any, Nup complexes might be targets of exportin binding in our hands. To this end, GST pulldowns from interphase *Xenopus* egg extracts were conducted with GST-Crm1 or GST-Exportin-t bound to glutathione

beads. Extract was incubated with the two types of GST-exportin-beads, with or without the presence of RanQ69L-GTP. The bound proteins were eluted and probed by immunoblotting for the presence of nucleoporins from different nuclear pore subcomplexes, including Nup133 (Nup107-160 complex), Nup98 (Gle2), Nup358 (RanBP2/RanGAP), Nup214 (Nup88), Nup153 (Nup50), and Nup62 (Nup58, 54, 45). (The nucleoporins in parenthesis are Nups not probed for but known also to be present in the adjacent Nup subunit.) We found that Crm1 and Exportin-t were bound to all six tested, i.e., subunits containing Nup133, Nup98, Nup358, Nup214, Nup153, and Nup62 (Figure 4.7, lanes 4 and 6). This indicates that the exportin must have bound either the nucleoporin probed for, or, another nucleoporin in that subcomplex. Notably, the addition of RanQ69L-GTP significantly decreased interactions of the exportins (Figure 4.7, Lanes 5 and 7) with all the nucleoporins tested.

Discussion

This study shows that three exportins, Crm1, Exportin-t, and Exportin-5, can inhibit major mitotic assembly events. We predict that the binding and releasing activity of exportins with specific assembly target proteins could be used to ensure the proper spatial assembly of critical structures such as the nuclear membranes, nuclear pores, and the mitotic spindle. Exportins were shown to bind nucleoporin subcomplexes and release them in the presence of RanGTP. For nuclear pore formation, we believe the release of the nucleoporin subcomplexes would only occur near chromatin where RanGTP is produced and is therefore high. This localization is consistent with where the nascent forming nuclear envelope needs to be assembled. This model further suggests that while the three exportins have different export cargoes, ranging from Crm1's diverse array of NES-bearing proteins to the tRNA selectivity of Exportin-t, the three have a shared common mechanism of binding to nucleoporins, either directly or indirectly, and appear to release the Nups in a RanGTP-dependent manner (Figure 4.1, 4.3, 4.7).

The exportins Crm1 and Exportin-t are also able to inhibit spindle assembly *in vitro,* as shown in Figures 4.5 and 4.6. Could they be regulating spindle assembly in a by binding nucleoporins and releasing them upon encountering RanGTP? Several nucleoporins are indeed required for bipolar spindle assembly, including the Nup107-160 complex, ELYS, and Nup358 (Arnaoutov et al., 2005b; Chatel and Fahrenkrog, 2011; D'Angelo and Hetzer, 2008; Mishra et al., 2010; Nakano et al., 2011; Orjalo et al., 2006; Rasala et al., 2006; Wozniak et al., 2010). The Nup107-160 complex has

also been observed on the spindle itself (Orjalo et al., 2006). Exportin-t binding and inhibiting essential Nups in mitosis, and releasing them in areas of high RanGTP seems the most plausible mechanism/model, as tRNAs likely have little to do with spindle assembly, and we observed (Figure 4.7) that the addition of RanGTP significantly reversed Exportin-t inhibition of spindle assembly.

In sum, the evidence above provides new insight into novel regulatory mechanisms the cell could employ to ensure correct spatial arrangement of major mitotic structures for a faithful segregation of mitotic chromosomes, followed then by proper nuclear reassembly. This would greatly expand the current known roles of RanGTP and karyopherin regulation in major mitotic assembly events.

Materials and Methods

Expression and Purification of Recombinant Proteins and Antibodies

Recombinant proteins [GST, GST-hTransportin, GST-xCrm1, GST-hExportin-t, 6xHis-hExportin-5, and GST-RanQ69L] were expressed in BL21 (DE3) cells (New England BioLabs, C25271) by growing 1L at 37º for 3 hours or until the OD_{600} was ~0.500. Protein expression was induced by adding IPTG (0.5mL of 1M stock) to a final concentration of 0.5M and growing cells overnight at 16º. The cells were collected by centrifugation, resuspended in 25mL of bacterial lysis buffer (300 mM NaCl, 50 mM Tris, pH=7.5) plus 5 mg lysozyme (BioPioneer, C0021) and frozen at -80ºC. Cells were thawed on ice, then sonicated and centrifuged in an SS-34 rotor (Sorvall) to give cleared lysate (24k G, 45 minutes, 4ºC). The lysate was applied to Glutathione Agarose 4B beads (Prometheus Protein Biology Products, 20-542) or Super Ni-NTA Agarose Nickel beads (Lambda Biotech, G202) and the expressed proteins purified according to manufacturers' instructions. Proteins were stored in aliquots at -80ºC in Egg Lysis Buffer (250 mM Sucrose, 2.5 mM $MgCl_2$, 50 mM KCl, 10 mM HEPES, and the pH was adjusted to 7.8 with KOH (Harel et al., 2003). Antibodies used in this study were generated from rabbit serum against *Xenopus* anti-Nup98 (Lau et al., 2009) and *Xenopus* anti-Nup133 (Bernis et al., 2014; Harel et al., 2003).

Membrane Fusion Assays

Xenopus high speed mitotic or interphase cytosol and membranes were prepared as previously described (Bernis et al., 2014; Harel et al., 2003; Lau et al., 2009a). Membrane fusion assays were conducted as previously described (Bernis et al., 2014) (Figure 4.1) and were analyzed on an Axio Observer microscope with a 63 x 1.4 NA objective. Images were captured with an Axiocam 506 monochrome digital camera and analyzed using Zen 2.3 (all from Carl Zeiss MicroImaging). Alternatively, images were visualized using an Axioskop 2 microscope and a 63x objective (Carl Zeiss Microimaging). Membranes were visualized with 3,3'-Dihexyloxacarbocyanine iodide (DHCC; Sigma-Aldrich, 318426). Nuclear membrane exclusion capabilities were assessed with 70kd Rhodamine-labeled Dextran (Molecular Probes Life Technologies, D1819). DNA was stained with bis-Benzimide H 33258 dye (Hoeschst; Sigma-Aldrich, B1155). The reactions were set up as follows in a 1.5 mL tube: 20 μL high speed interphase extract, 1 μL 20x clarified membranes, 1.25 μL of ATP regenerative mix (200 mM phosphocreatine, 1.6 mg/mL creatine kinase, 20 mM ATP, 20 mM $MgCl_2$, 2 mM EGTA), 1 μL of ~50,000 sperm chromatin (~2,000 sperm chromatin packets/μL final concentration), and recombinant protein. The following recombinant proteins final concentrations were used in different controls: 25 μM GST, 25 μM GST-hTransportin, 37.5 μM GST-RanQ69L-GTP, and 25 μM GST-hTransportin plus 37.5 μM GST-RanQ69L-GTP. The experimental proteins added were as follows: 25μM GST-xCrm1, and 25μM GST-hExportin-t, plus or minus 37.5μM GST-RanQ69L-GTP. All the proteins were diluted to 200 μM and 3.3 μL of

each protein was added at t=0 to the appropriate reaction tube, which contained 23.25 µL before the addition of the recombinant protein. The reactions were allowed to progress for 90 minutes at room temperature, which was roughly 22ºC. For membrane fusion assays, after 90 minutes 1 µL (2.5µg) of 70 kDa Rhodamine-labeled Dextran was added to each reaction and then incubated on ice for 10 minutes. Next, the reactions were fixed by adding 9 µL of 16% paraformaldehyde (Sigma-Aldrich, P6148-500G) in PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4) to a final concentration of 4% paraformaldehyde and left on ice until microscopic analysis.

Nuclear Pore Assembly Assays

Nuclear pore assembly assays were conducted by first creating pore-free nuclear intermediates termed "BAPTA" nuclei (Figure 4.3), as previously described (Bernis et al., 2014; Harel et al., 2003; Lau et al., 2009a). To generate BAPTA nuclei, reactions were setup with the following in a 1.5mL tube: 20 µL high speed interphase extract, 1 µL 20x clarified membranes, 1.25 µL of ATP regenerative mix (200 mM phosphocreatine, 1.6 mg/mL creatine kinase, 20 mM ATP, 20 mM $MgCl_2$, 2 mM EGTA), ~1 µL containing 2000 sperm chromatin packets/µL, and 0.5 µL of 250 mM 1,2-bis(*o*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid in water (7.5 mM final concentration, BAPTA; Calbiochem, 196419), and allowed to progress for 1 hour at room temperature. To ensure nuclear pores had not formed in BAPTA nuclei, FG

Nups were probed for using 0.5μL of Alexa Fluor 594 anti-Nuclear Pore Complex Proteins (414; anti-FG Nups; BioLegend, 682202) to a 5 μL sample of BAPTA nuclei. After determining the BAPTA nuclei intermediates were in fact pore-free, the BAPTA nuclei were diluted 1:10 into fresh cytosol (5μL of BAPTA nuclei in 45μL of fresh cytosol and 2.5μL ATP regenerative mix). Before addition of the BAPTA nuclei, the following conditions (final concentration) were prepared as controls in the fresh cytosol: 7.5 mM BAPTA, 25 μM GST, 25 μM GST-hTransportin, 37.5 μM GST-RanQ69L-GTP, and 25 μM GST-hTransportin plus 37.5 μM GST-RanQ69L-GTP. The experimental conditions were as follows: 25 μM GST-xCrm1 plus or minus 37.5 μM GST-RanQ69L-GTP, 25 μM GST-hExportin-t, plus or minus 37.5 μM GST-RanQ69L-GTP, and 25 μM 6x-His-hExportin-5 plus or minus 37.5 μM GST-RanQ69L-GTP. All proteins were diluted to 423 μM in ELB, then 2.68 μL were added to the appropriate tube. One hour after dilution into fresh cytosol, 1μL of 414 ant-FG antibody was added to each reaction and incubated for 1 hour at room temperature. Then, 15 μL of 16% paraformaldehyde in PBS was added to a final concentration of 4%, and the reactions were left on ice until imaged. For all experiments, a minimum of 50 nuclei were counted, and each experiment was repeated at least three times. Error bars are standard error from the mean.

Spindle Assembly Assays

Spindle assembly assays were adapted from previously described protocols (Bernis et al., 2014; Lau et al., 2009b). In brief, *Xenopus* eggs were collected and were lysed in XB+EGTA (50 mM sucrose, 1 mM $MgCl_2$, 100 mM KCl, 10 mM HEPES, 7 mM EGTA, pH=7.7-7.8 with KOH) by centrifugation in a TOMY TX-160 centrifuge at 15K RPM for 20 minutes at 4ºC. The crude cytosol was removed and supplemented to 50 μg/mL Cytochalasin B (EMD Millipore Corp, 250233-5MG), 10 μg/Aprotinin and 10 μg/mL Leupeptin (USB, 11388 and 18413, respectively). The mitotic cytosol was then centrifuged again as described above, and the supernatant was removed and kept on ice until ready to use. For each spindle assembly reaction, 20 μL of mitotic extract, 1.6 μg of Rhodamine-labeled tubulin (Cytoskeleton, TL590M-A) and 1.5 μL ATP regenerative mix was added to each 1.5 mL tube. The recombinant protein was then added to the proper concertation. For the Crm1 spindle assembly, recombinant proteins were added to the following final concentrations: 15 μM GST, 15 μM Transportin, and 1, 2, 8, 10, 15, and 20 μM Crm1 (Figure 4.5). For Exportin-t analysis in spindle assembly analysis, recombinant proteins were added to the following final concentrations: 15 μM GST, 15 μM Transportin, 15 μM RanGTP-Q69L, 15 μM Exportin-t, and 15 μM RanGTP-Q69L plus 15 μM Exportin-t. Lastly, sperm chromatin was added to a final concentration of ~3000/μL. The reactions were allowed to progress for 1 hour at room temperature, then 2.5 μL of the reaction was plated and mixed with 1 μL spindle fixation buffer (48% glycerol, 11% formaldehyde, 10 mM HEPES, pH=7.5, 5 μg/mL Hoechst). All samples were

visualized with an Axioskope 2 microscope with a 63x objective (Carl Zeiss MicroImaging).

GST Pulldowns and Immunoblots

To prepare beads for pulldowns, 180 μL of Glutathione Agarose 4B beads (Prometheus Protein Biology Products, 20-542) were washed two times with PBS, and blocked with 2.5 μL of 20 mg/mL BSA for 1 hour at room temperature. Then 100 μg of GST (7 μL), GST-xCrm1 (3.3 μL), or GST-hExportin-t (3.3 μL) were bound to the beads for 1 hour at room temperature while rotating in a final volume of 0.5 mL of PBS. The tubes with GST-, Crm1-, and Exportin-t-bound beads were split evenly into two 1.5 mL tubes (yielding 50 μg of bound protein per tube) and 25 μL of interphase *Xenopus* egg extracts precleared of residual membranes by centrifugation in a TOMY TX-160 centrifuge at 15K RPM for 20 minutes at 4ºC was added to each tube, and the volume was then brought up to 500 μL with PBS. In half of the tubes, 100 μg of 6xHis-RanQ69L-GTP (3.8 μL) was added so that each condition (GST, GST-xCrm1, and GST-hExportin-t, respectively) was also tested alongside RanGTP. The tubes were rotated at 4º for 2 hours. The beads were centrifuged at 3k RPM in an Eppendorf Microcentrifuge 5415 C, and the supernatant carefully aspirated to avoid removing beads. The beads were washed three times with PBS+0.2% NP-40, then another three times with PBS. The PBS was removed and the samples were resuspended and boiled at 95ºC for 5 minutes in 30 μL of 2X gel sample buffer (31.5

mM Tris-HCl, pH=6.8, 10% glycerol, 1% SDS, 0.005% Bromophenol Blue) and run on a 10% SDS-PAGE gel until resolved. The proteins were transferred overnight in Transfer Buffer (3 g Tris Base, 14.4 g glycine, 1 g SDS and 20% ethanol per liter) to a nitrocellulose membrane at 30 V. The membrane was blocked in 5% milk (Apex BioResearch Products, 20-241) in TBS+0.1% Tween 20 for 1 hour at room temperature, then cut into sections for probing. Primary antibodies were prepared in the following dilutions in 5% milk in TBS+0.1% Tween 20, with 414 anti-FG antibody 1:1000, or rabbit anti-Nup133 1:200, or rabbit anti-Nup98 1:1000. The membranes were bound overnight with the antibodies at 4º, washed 3 times for 5 minutes at room temp in TBS+0.1% Tween 20. Then, HRP-conjugated Protein A for the rabbit antibodies (Invitrogen, 101023) or HRP-conjugated goat anti-mouse for the 414 anti-FG antibody (Invitrogen, 626520) were bound 1 hour at room temperature at 1:10,000 in 5% milk in TBS+0.1% Tween 20. After 3 washes, the membrane was treated with ECL Western Lighting Plus (Perkin Elmer, NEL103001EA), imaged on a ChemiDoc XRS Imaging System, and analyzed using Image Lab 4.1 (BioRad).

Figures

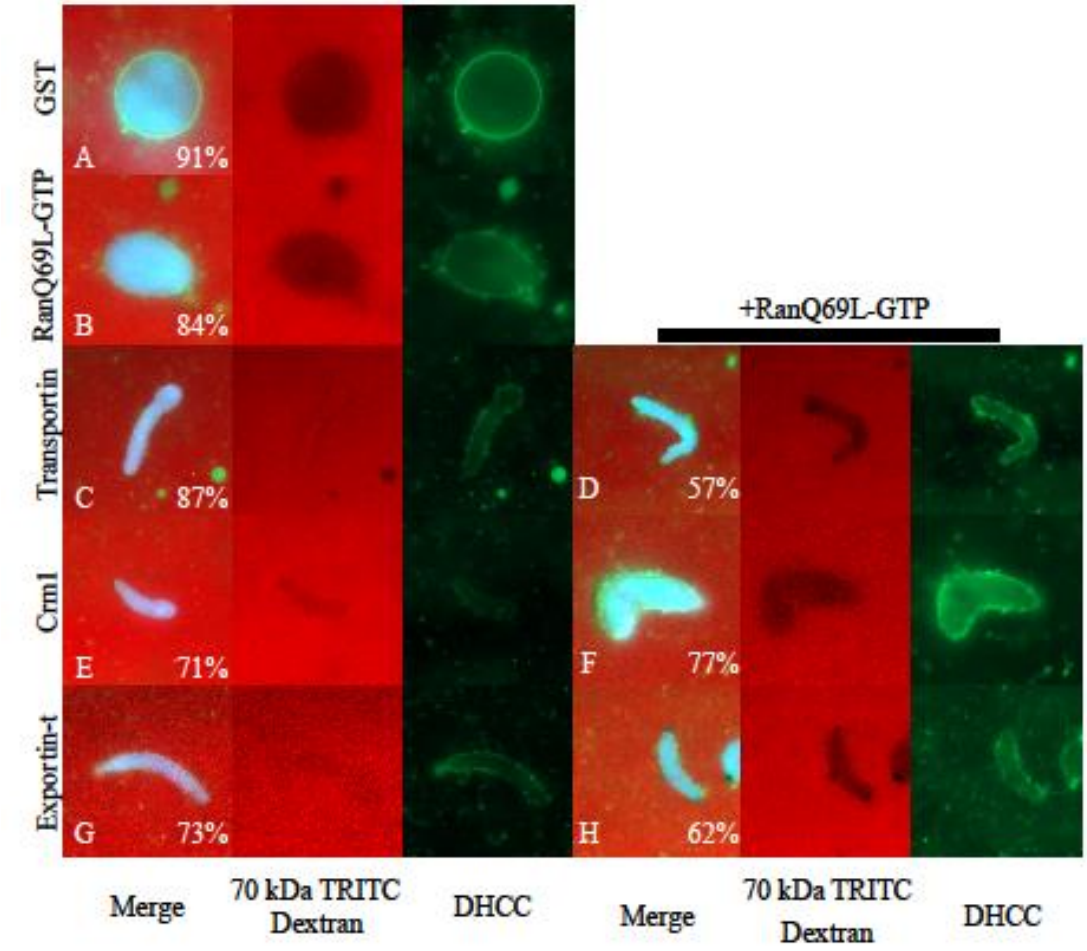


Figure 4.1: The Exportins Crm1 and Exportin-t inhibit nuclear membrane fusion and can be counteracted by RanQ69L-GTP. Crm1 and Exportin-t were added to extracts and membrane fusion was assayed. Nuclei were assembled for 1 hour at room temperature. Membranes are fused if there is the appearance of a smooth membrane around the DNA, and if the membranes are able to exclude a rhodamine-labeled 70kd Dextran. The conditions assayed included the following addition of recombinant protein. A: 25 μM GST, B: 37.5 μM RanQ69L-GTP, C: 25 μM Transportin, D: 25 μM Transportin+37.5 μM RanQ69L-GTP, E: 25 μM Crm1, F: 25 μM Crm1+37.5 μM RanQ69L-GTP, G: 25 μM Exportin-t, and H: 25 μM Exportin-t+37. 5μM RanQ69L-GTP. Note:RanGTP alone causes abundant membrane fusion, producing a nuclear membrane with many outfolds, which is less smooth than seen in GST alone nuclei (Harel et al., 2003).

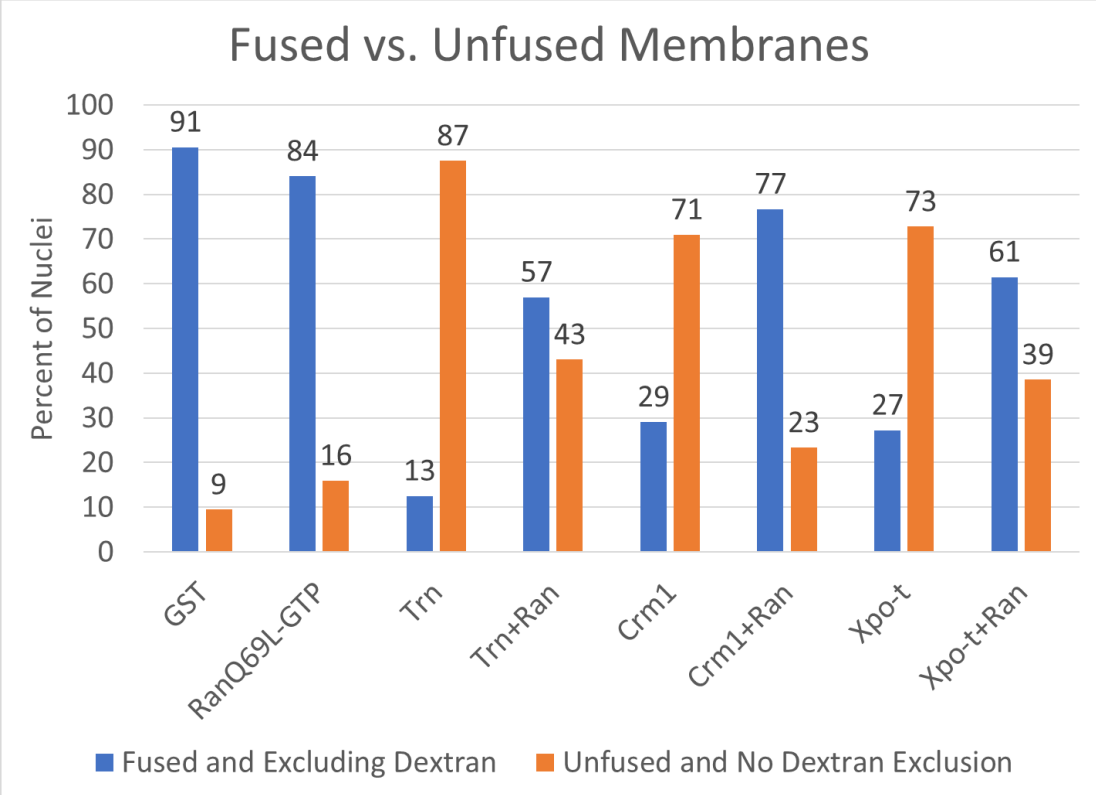
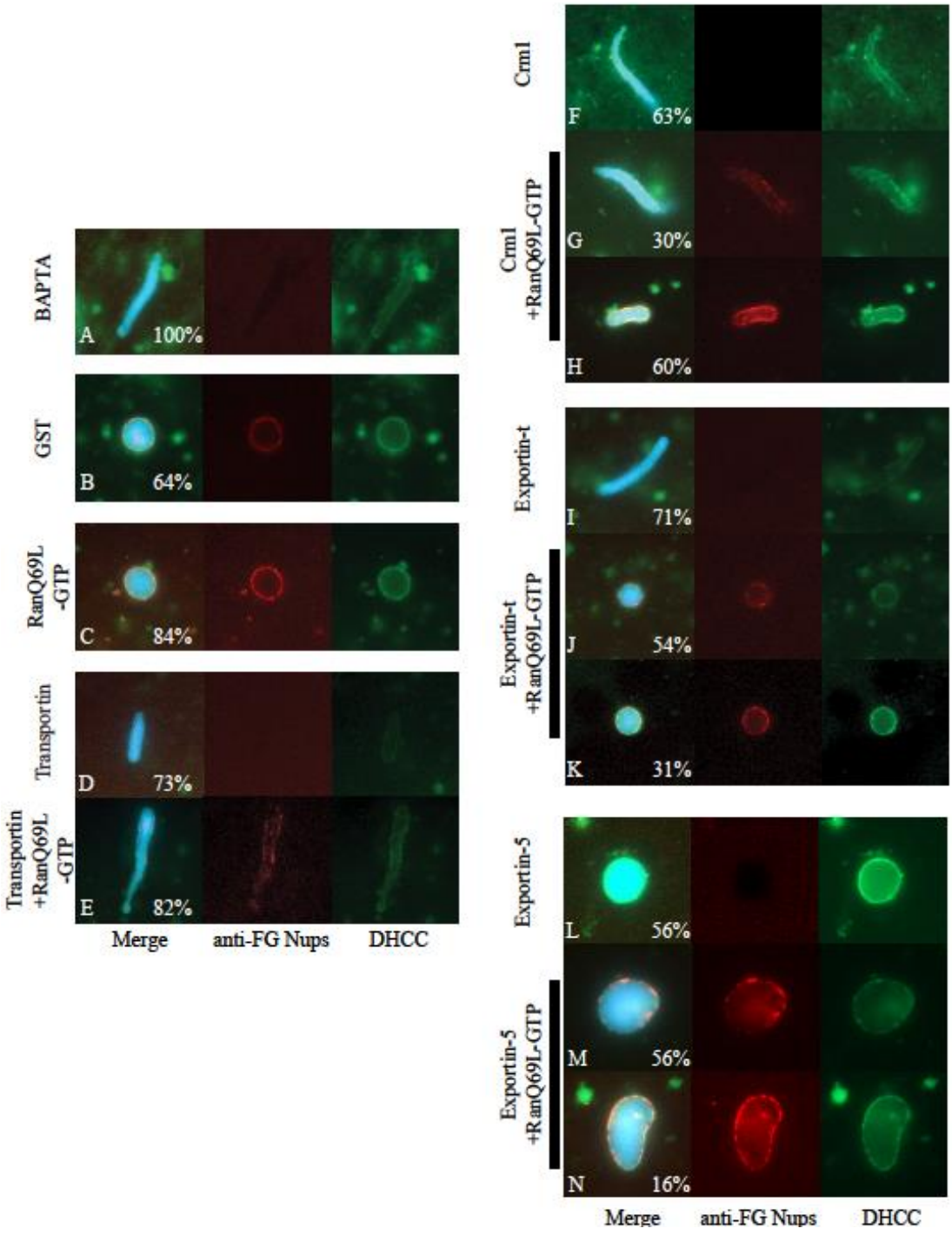


Figure 4.2: Quantification of membrane fusion assays. The percentages shown were determined by counting at least 50 nuclei from each condition. Each experiment was repeated three times. Error bars represent the Standard Error from the Mean. Trn=Transportin, Ran=RanQ69L-GTP, Xpo-t=Exportin-t.

Figure 4.3: Crm1, Exportin-t , and Exportin-5 inhibit nuclear pore formation in BAPTA nuclei, and all three are RanQ69L-GTP sensitive.

BAPTA nuclei were assembled for 1 hour at room temperature, then, upon checking that nuclear pores were indeed inhibited by staining with 414, diluted 1:10 into fresh cytosol to release from BAPTA inhibition. In advance of diluting the BAPTA nuclei into the fresh cytosol, the following was added to cytosol; A: 7.5 mM BAPTA. B: 25μM GST. C: 37.5μM RanQ69L-GTP. D: 25 μM Transportin. E: 25 μM Transportin+37.5 μM RanQ69L-GTP. F: 25 μM Crm1. G: 25 μM Crm1+37.5 μM RanQ69L-GTP (partial rim). H: 25 μM Crm1+37.5 μM RanQ69L-GTP (full rim). I: 25 μM Exportin-t. J: 25μ M Exportin-t+37.5μM RanQ69L-GTP (partial rim). K: 25 μM Exportin-t+37.5 μM RanQ69L-GTP (full rim). L: 25 μM Exportin-5, M: 25 μM Exportin-t + 37.5 μM RanQ69L-GTP (partial rim). N: 25 μM Exportin-t + 37.5 μM RanQ69L-GTP (full rim). Red is 414 anti-FG Nups, Green is DHCC, Blue is Hoechst.


BAPTA
A 100%
GST
B 64%
RanQ69L -GTP
C 84%
Transportin
D 73%
Transportin +RanQ69L -GTP
E 82%
Merge
anti-FG Nups
DHCC
Crm1
F 63%
Crm1 +RanQ69L-GTP
G 30%
H 60%
Exportin-t
I 71%
Exportin-t +RanQ69L-GTP
J 54%
K 31%
Exportin-5
L 56%
Exportin-5 +RanQ69L-GTP
M 56%
N 16%
Merge
anti-FG Nups
DHCC

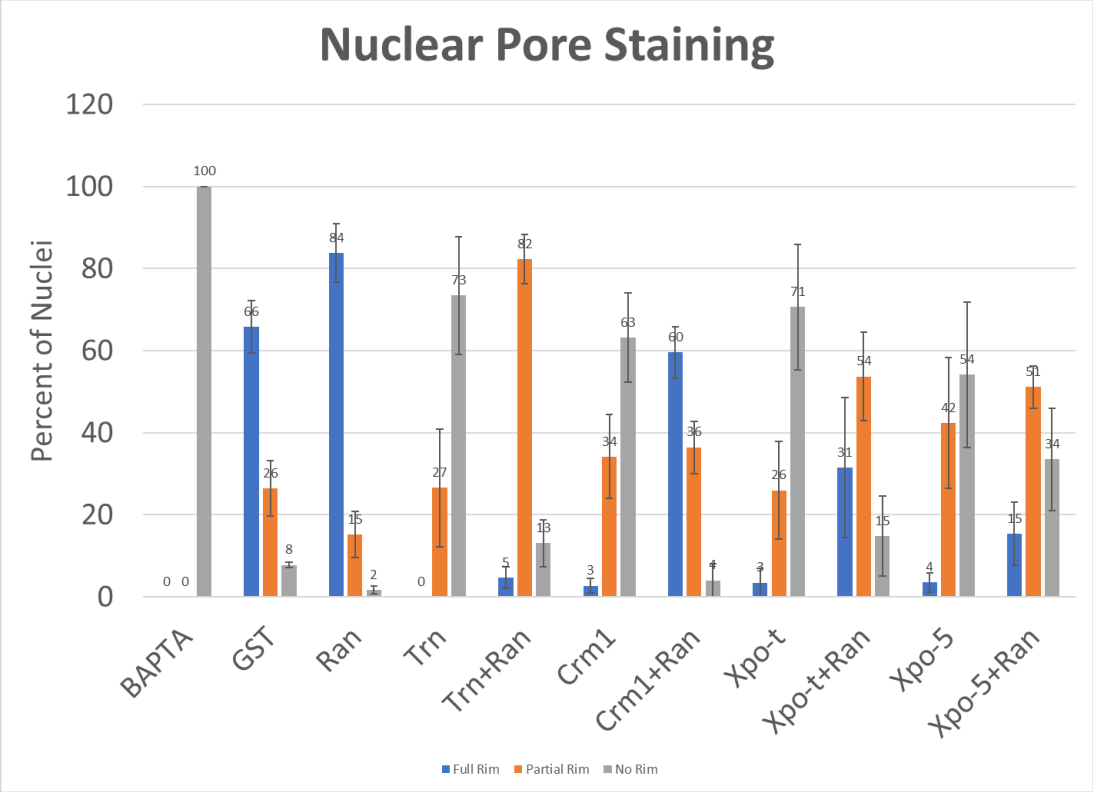


Figure 4.4: Quantification of nuclear pore formation assays. The percentages were determined by counting at least 50 nuclei from each condition. Each experiment was repeated three times. Error bars represent the Standard Error from the Mean. Trn=Transportin Ran=RanQ69L-GTP, Xpo-t=Exportin-t.

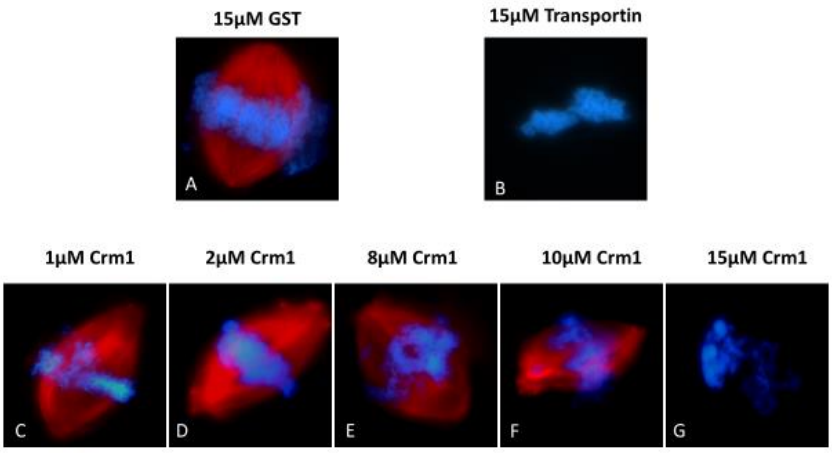


Figure 4.5: Crm1 inhibits spindle assembly. Recombinant Crm1 was added to mitotic extract and compared to both GST and Transportin controls (A and B, respectively). GST (A) produced strong bipolar spindles, while Transportin (B) inhibited bipolar spindle formation. Low concentrations (1-2 µM) of Crm1 (C and D) had little effect on bipolar spindle formation. However, increasing concentrations (8-10 µM had an increasingly deleterious effect on bipolar spindle formation (E and F) until finally bipolar spindle formation was completely inhibited (G, 15 µM).

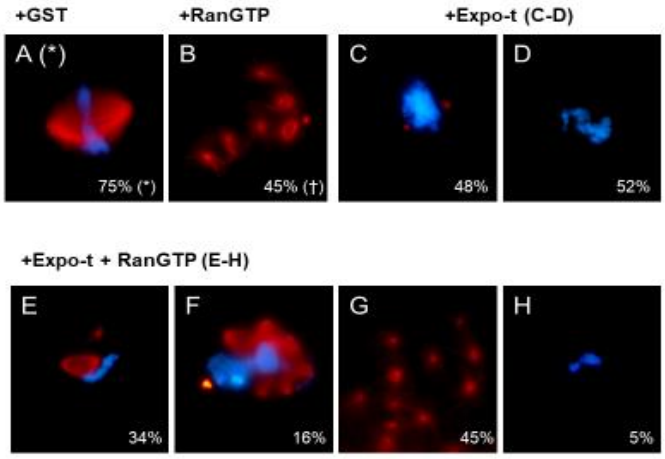


Figure 4.6. Spindle assembly with Exportin-t and RanGTP. Exportin-t was analyzed in mitotic extract to assess its effect on spindle assembly. A: In control 15 μM GST conditions, 75% of the structures observed were bipolar spindles (* indicates the remaining 25% of the structures were half spindles). B: RanQ69L-GTP addition created an abundance of microtubule nucleations called asters († indicates the remaining 55% of structures were bipolar spindles and multi-polar spindles). When 15 μM Exportin-t was added, two major phenotypes were observed shown in C: 48% of the structures were very small microtubule nucleations on opposite sides of the mitotic chromatin, and D: 52% were DNA structures completely inhibited for spindle assembly. When 15 μM Exportin-t and 15 μM RanQ69L-GTP were added simultaneously, several phenotypes were observed including, E: a small spindle directly adjacent to mitotic chromatin (35%), F: multipolar spindles (16%), G: groups of microtubule nucleations called asters (45%), and H: mitotic chromatin with no spindle associated (5%).

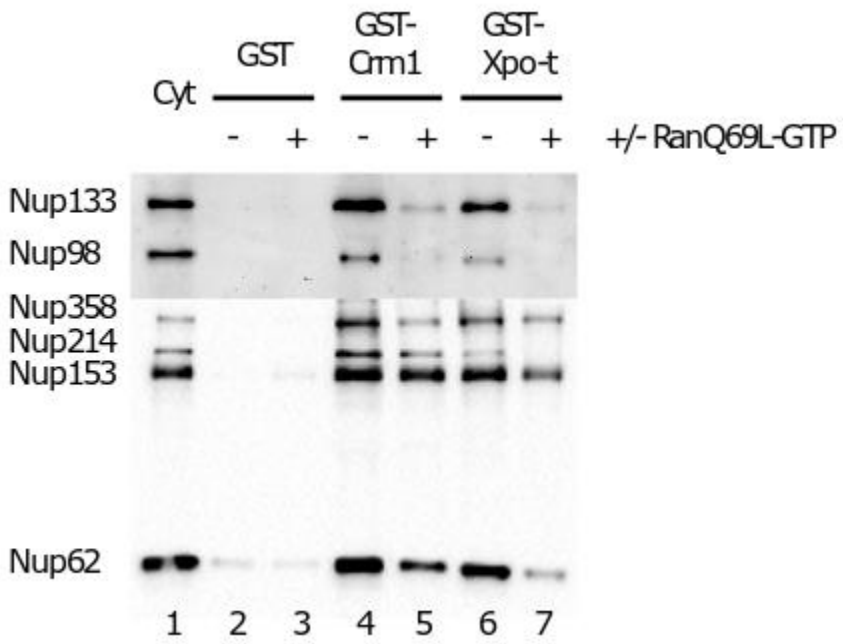


Figure 4.7: GST pulldowns and nucleoporin Interactions. GST, GST-Crm1 and GST-Exportin-t were bound to glutathione beads and incubated in cytosol with or without RanQ69L-GTP. The beads were washed in PBS+0.2% NP-40, the bound proteins were eluted from the beads, resolved on a gel (lanes 2-7), then transferred and probed with the following antibodies: 414 anti-FG Nups, anti-Nup133, and anti-Nup98. Lane 1: cytosol input. Lane 2: GST. Lane 3: GST+RanQ69L-GTP. Lane 4: GST-Crm1. Lane 5: GST-Crm1+ RanQ69L-GTP. Lane 6: GST-Exportin-t. Lane 7: GST-Exportin-t+ RanQ69L-GTP.

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Future Directions

Summary of Results

This dissertation focuses on regulation of mitotic assembly events by karyopherins. In the publication in Chapter 2, Transportin, a close relative of Importin β, was shown to inhibit assembly factors required for proper formation of the mitotic spindle, for nuclear membrane fusion, and for nuclear pore formation *in vitro* (Bernis et al., 2014). RanGTP relieved the inhibition by Transportin, as did the addition of the super NLS, M9M. In addition, the Transportin mutant TLB, which cannot release bound NLS-bearing proteins regardless of RanGTP binding, was shown to be counteracted by the addition of M9M, but not by RanGTP. The nucleoporin, ELYS, which is required to initiate nuclear pore formation, and is also an essential component of kinetochores needed for spindle assembly (Rasala et al., 2006), was identified in Chapter 2 as a target of Transportin that is M9M sensitive. Moreover, the addition of M9M alone to a mitotic *Xenopus* extract lacking induced the spontaneous polymerization of microtubule asters. These experiments indicated that Transportin does not act by titrating RanGTP and preventing Importin β from acting properly, but instead indicates that Transportin acts by binding and regulating key factors for all three mitotic assembly events.

With the above in mind, future directions include the search for additional protein spindle assembly factors as a logical step for moving forward. It was shown in early spindle assembly studies that the importin/assembly factor concentration in a

solution must be balanced for microtubule polymerization. Excess addition of purified assembly factors could overcome the inhibitory effects of the importin added (Gruss et al., 2001). A similar method could be used to identify assembly factors regulated by Transportin. Recombinant GST-Transportin and GST-Transportin TLB, bound to a glutathione-sepharose column, could be incubated with mitotic extract. After washing each column, a solution containing the super NLS, M9M, could be added to release bound proteins from both the Transportin and TLB columns. The eluates could then be collected, and the proteins separated by size using gel filtration chromatography. Each fraction could be tested for activity in fresh mitotic extracts supplemented with fluorescent tubulin to look for microtubule nucleation, i.e., aster assembly. A consideration one must take into account is, if nucleating activity is found in any of the eluates, is it generated from one individual protein or multiple distinct proteins that work together to promote aster formation?

Overview of Exportin Results

In Chapter 4 of this dissertation, we asked whether *exportins* might regulate or affect major mitotic assembly events. We did so by dissecting the effect of Crm1/Exportin-1, Exportin-t, and to a lesser extent, Exportin-5 on major mitotic assembly events. We found that for nuclear membrane fusion and nuclear pore formation, both Crm1 and Exportin-t did indeed strongly inhibit assembly. Moreover, both were able to be counteracted by the simultaneous addition of RanGTP. Studies with Exportin-5 showed a similar result, where Exportin-5 inhibited nuclear pore formation, and was also counteracted by RanGTP. Nuclear

membrane fusion remains to be tested for Exportin-5, but there is no logical reason that it would differ from either Crm1 or Exportin-t, although it is possible that Exportin-5 might differ if it binds no membrane fusion factors.

Using GST pulldowns, we found that GST-Crm1 and GST-Exportin-t pulled down multiple nucleoporin subcomplexes and this binding was reduced by the presence of RanGTP. While several previous studies have identified one or more Nups that interact with Crm1 or Exportin-t, the study here presents these interactions in a novel context: that of a post-mitotic and nascently forming nuclei, where membranes must fuse to form the double nuclear membranes and newly forming nuclear pores need to embed into the membranes. With these results in mind, the following describes a set of future experiments that will answer questions that our results have generated

This study is the first to show evidence that Crm1 can not only regulate aspects of major mitotic events, but that it can completely inhibit those assembly events. Previous studies *in vivo* have shown that Crm1plays required roles at kinetochores and at the centrosomes, both of which are involved in spindle assembly (Arnaoutov et al., 2005; Brodie and Henderson, 2012; Gilistro et al., 2017; Liu et al., 2009; Roscioli et al., 2012; Wang et al., 2005). However, our data show multiple roles for Crm1 in mitosis. Ours is the first study to show that Crm1 can regulate nuclear formation events such as nuclear membrane fusion and nuclear pore formation.

As Crm1 is the most widely studied exportin, there are more tools available to aid in future studies. These tools include a high affinity super NES, termed the MVM-NS2-NES, that can displace native cargo of Crm1 (Fu et al., 2018; Sun et al., 2013), Also, compounds such as Leptomycin B, SINEs and various other chemical inhibitors that covalently bind to and inactivate the NES-binding domain of Crm1 could be used to probe mitotic assembly events (Boons et al., 2015; Dickmanns et al., 2015; Mendonca et al., 2014). While the addition of Crm1 and either the MVM-NS2-NES or the SINEs did not prevent Crm1 from inhibiting membrane fusion (M. Nord, unpublished data), this could be that the inhibitors are being masked. There is precedent for small drugs being bound to/masked by the high lipid content or other unique aspects of *Xenopus* extracts (Mary Dasso, personal communication; Arshad Desai, personal communication). However, it should be noted that very recently several new peptide inhibitors of Crm1 have been developed. These Crm1 inhibitors are derived from naturally-occurring NES's that have been mutated further to have a picomolar K_d (Fu et al., 2018). As Crm1 is highly studied and of great medical interest, there will likely continue to be an expanding set of available tools to aid in the study of Crm1 in mitosis.

As described in the Discussion of Chapter 4, we believe that the exportins act by binding to and inhibiting nucleoporins needed for nuclear assembly and spindle assembly and have identified a set of Nup candidates by pulldown assays. However, we can also test if NES binding inhibition interferes with this. Chemical inhibitors could be pre-bound to the NES binding of Crm1 before its addition to the extracts. If

a function or open NES binding site is needed for the inhibitory action of Crm1, then pre-binding of a chemical inhibitor (SINEs) to Crm1 should negate its inhibitory action and allow membrane fusion and nuclear pore formation to occur. If the NES is not needed for inhibitory actions, other domains will be tested as below.

Probing Mechanism with Exportin Mutants

Another way to experimentally address mechanistic questions in *Xenopus* egg extracts would be with recombinant proteins containing mutations that disrupt different aspects of normal exportin function. For all three exportins, Crm1, Exportin-t, and Exportin-5, deletion of the N-terminal CRIME domain, largely responsible for RanGTP binding on all karyopherins (Güttler and Görlich, 2011; Ossareh-Nazari and Dargemont, 1999), would be useful to analyze in the extracts. What effect would the addition of these mutants produce on nuclear reconstitution? Also, would they change the nucleoporin binding dynamics in GST pulldowns? All are intriguing questions worth pursuing in the future. If this line of inquiry proves useful, further deletions or point mutations could be made, as there are several structural elements in karyopherins that modulate other processes, such as cargo selectivity and cargo dissociation (Dong et al., 2009; Fu et al., 2018; Güttler and Görlich, 2011; Leisegang et al., 2012; Monecke et al., 2009). The use of mutants will be especially valuable for answering whether potential assembly factor targets, such as the nucleoporins needed for nuclear pore and spindle assembly, bind to exportins

like Crm1 at particular sites on Crm1. For example, certain mutations in FG repeats exist in Nup214, which prevents Crm1 from binding to it (Roloff et al., 2013). Thus, mutations of specific functional domains in the exportins could be helpful in determining domains important to regulation.

RNA roles in major mitotic events

Previous work has shown that Pol II-transcribed RNAs play a structural role in mitotic spindle and kinetochore assembly (Blower et al., 2005, 2007; Gent and Dawe, 2012; Grenfell et al., 2016; Talbert and Henikoff, 2018). With this in mind, it is possible that the exportins, particularly Crm1 and Exportin-5, could regulate critical RNA-protein complexes needed in mitosis. Crm1 could do this: (a) by targeting a needed RNP to the kinetochore in a regulatory manner, or (b) by binding and preventing its release in incorrect areas of the cell during mitosis, much like Importin β and Transportin have been shown to do for other assembly factors.

For Exportin-t, although active protein translation is not required for spindle assembly in *Xenopus* egg extracts (Blower et al., 2007), perhaps there are other roles for tRNAs. It has recently been shown that tRNA-derived fragments can regulate gene expression by recruiting Argonaut proteins to silence targeted mRNAs via the RISC complex (Kuscu et al., 2018; Lee et al., 2009). The overexpression of such tRNA fragments in prostate cancer is correlated with increased cell proliferation and a poor prognosis (Lee et al., 2009; Sun et al., 2018). Could Exportin-t play a role in

this pathway? Overexpression of Exportin-t could lead to an excess of tRNA export, leading to an increase in the tRNA fragments and increased tumorigenic properties in the now cancerous cell. Further study of this new class of small tRNA fragments could prove fascinating.

Bioinformatics of Crm1

Several large scale screens for potential NES-bearing proteins that bind to Crm1 have been conducted, leading to over 1000 putative Crm1 NES-bearing cargoes; over 250 have been biochemically verified (Kırlı et al., 2015; Thakar et al., 2013; Wühr et al., 2015; Xu et al., 2012). However, there was relatively little overlap between the Crm1-binding proteins identified in the original studies. Separately, a program called LocNES was designed to identify possible NES's in proteins (Xu et al., 2015). Looking both at the proteins from the screens above and then those identified by the LocNES program, one could search specifically for cargoes associated with mitotic events. For example, Crm1 has been shown to localize to centrosomes, and indirectly to recruit γTURCs to the centrosome for the nucleation of microtubules (Liu et al., 2009). Recently, Crm1 in *S. pombe* has been shown to be repurposed as a docking protein for the microtubule nucleation protein Mto1, which causes unexpected microtubule nucleation at nuclear pores (Bao et al., 2018). Interestingly, Crm1 binds to Mto1 via an NES motif in Mto1 (even though it does not have an NLS). Extrapolating from the negative end stabilizing events involving Crm1 at both the nuclear pore in *S. pombe* and Crm1 at mitotic centrosomes, I speculate that a potential target of Crm1 might be the microtubule negative-end

stabilizing protein called Patronin in *Drosophila*, and the three related CAMSAPs in vertebrates (Goodwin and Vale, 2010; Hendershott and Vale, 2014; Jiang et al., 2014). When I analyzed these using the LocNES program, CAMSAP 2 and 3 score higher than many proteins with confirmed NESs (M. Nord, personal observation). Using such a targeted approach to identify Crm1-bidning mitotic targets, and then biochemically testing for a direct interaction between them, could yield new and interesting binding partners of Crm1 relevant to mitosis.

Karyopherins as global regulators of major mitotic events

In humans, there are 21 known karyopherins. In this study, we have added to the cast of karyopherins with the potential to regulate major mitotic events. What does this mean for the rest of the family? Might all 21 known karyopherins play a role? While logically all could have some type of impact on mitotic events, several question must be answered first. One major question is what is the concentration of an individual karyopherin in the cell? If it is comparatively low, perhaps it does not have the physical presence to dramatically impact the assembly of such massive structures in the cell. Another question is whether certain karyopherins might be post-translationally modified to be inactive and thus not play a role during mitosis. They could be phosphorylated and inactivated, ubiquitinated and degraded, or receive some other modification that inactivates them during mitosis. Alternatively, all karyopherins could be active in both interphase and mitosis.

As a final note to this dissertation, I would like to present a model of exportin regulation of nuclear pore formation *in vitro.* While the discussion of a model of both spindle assembly and nuclear membrane fusion would be relevant and interesting, a discussion of the formation of nuclear pores is most pertinent.

In this model, in a newly forming nucleus, where nuclear membranes are beginning to localize to decondensing chromatin and nuclear pores are beginning to form, RCC1 creates a high concentration of RanGTP inside the unfused nucleus, which then permeates out of the unfused membranes and into the future cytoplasm (Figure 5.1). Away from this local high concentration of RanGTP, exportins bind to and inhibit nucleoporin subcomplexes. However, as the exportin/nucleoporin complexes diffuse with time into higher RanGTP (near the unfused portions of the nuclear membranes), RanGTP binds to the exportins, which then release the nucleoporins so that nuclear pores can properly assemble into the nucleus (Figure 5.2). The nuclear envelope independently finishes forming over this time course also.

Overall, the study of karyopherins and their role in major mitotic assembly events has been a story that keeps growing. With their nuclear transport functions having such a large impact on the fate of the cell in interphase, continued study in mitosis will undoubtedly prove challenging. However, use of *Xenopus* egg extracts can continue to shed light on unforeseen roles of karyopherins in mitosis and, with many karyopherins playing a large role in both cancer and viral infections, the scientific yields of future study should prove fruitful.

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Figures

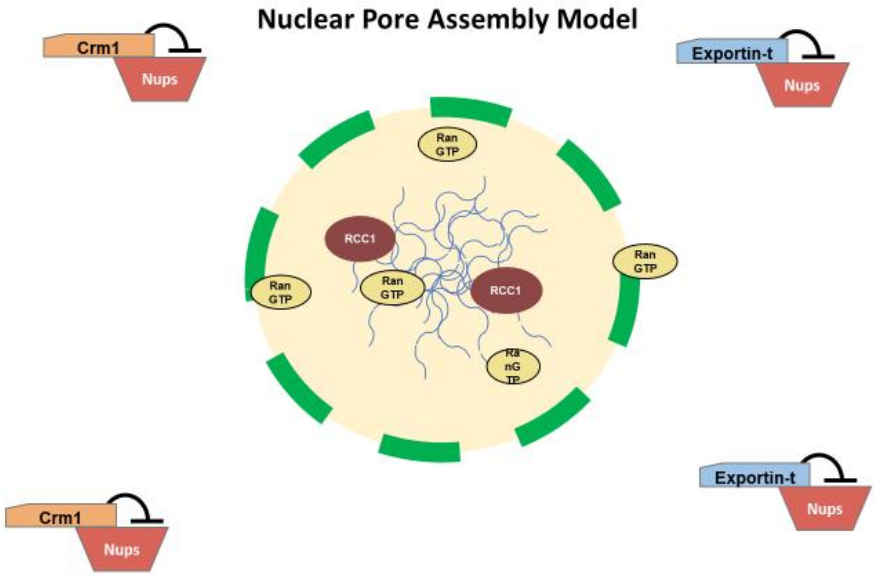

Figure 5.1: Exportins suppress nuclear pore formation away from the newly forming nucleus. Away from the newly forming nucleus, exportins bind to and inhibit nucleoporin subcomplexes so that they do no initiate nuclear pore formation in incorrect areas of the cell.

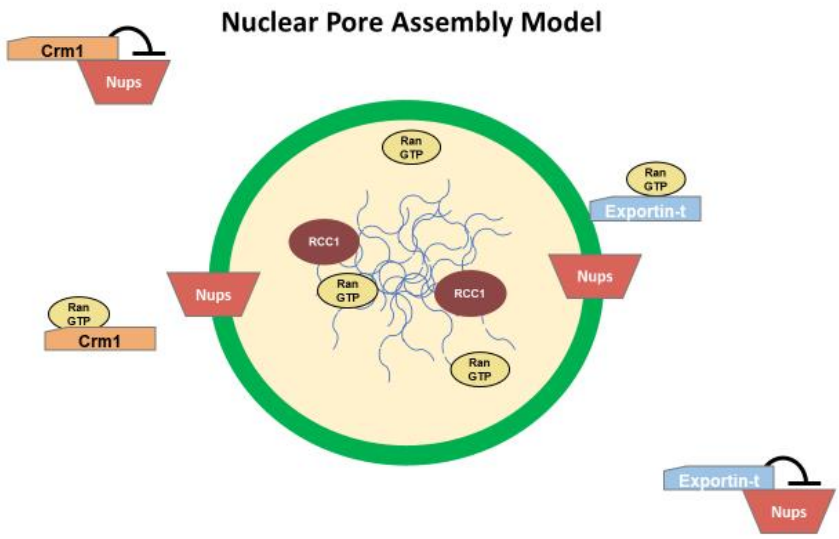

Figure 5.2: RanGTP stimulates the release of nucleoporins from exportins near the newly forming nucleus. As the exportin/nucleoporin complex diffuses closer to the newly forming nucleus, it encounters RanGTP. RanGTP binds to the exportin, which causes the release of the nucleoporins only near the newly forming nucleus. This results in a fully functional nuclear pore. Note that over the same time course the nuclear membranes independently continue to form, ultimately giving a closed nuclear envelope.

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