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The Nucleoporin Nup98 Regulates Microtubule Dynamics During Spindle Assembly

By

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The Nucleoporin Nup98 Regulates Microtubule Dynamics During Spindle Assembly

By
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B.S., Middle Tennessee State University, 2003

Advisor: Maureen A. Powers, Ph.D.

An Abstract of
a dissertation submitted to the Faculty of the Graduate School of Emory University in partial fulfillment
of the requirements for the degree of
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Graduate Division of Biological and Biomedical Sciences
Program in Biochemistry, Cell, and Developmental Biology

2009

Abstract

The Nucleoporin Nup98 Regulates Microtubule Dynamics
During Spindle Assembly

By Marie Cross

During interphase, the nuclear pore complex regulates trafficking between the nucleus and the cytoplasm. However, during mitosis, the nuclear pore complex is disassembled and increasingly, nucleoporins have been found to have alternate mitotic functions in mitotic checkpoints and in assembly of the mitotic spindle. Using Xenopus extract spindle assembly assays, we found that the nucleoporin Nup98 functions in mitotic spindle assembly through regulation of microtubule dynamics. When added to spindle assembly assays, the C-terminal domain of Nup98 stimulates uncontrolled growth of microtubules. Addition of inhibitory antibodies or depletion of Nup98 from extract leads to formation of stable monopolar spindles. Importantly, addition of purified Nup98 C-terminus to depleted extract rescued bipolar spindle formation. This activity does not require the known interaction between Nup98 and Nup96, one member of a nucleoporin complex associated with both the kinetochore and spindle. We have mapped the relevant region of Nup98 to a portion of the C-terminal domain lacking a previously characterized function. Both interphase and mitotic phosphorylations occur within this region of Nup98, and mutation of selected mitotic phosphorylation sites significantly alters the excess microtubule phenotype. Furthermore, we have identified a molecular interaction between the C-terminal domain of Nup98 and the microtubule depolymerizing kinesin MCAK. These data support a model in which Nup98 acts through MCAK to regulate the microtubule dynamics required for spindle bipolarity.

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Chapter 1

Introduction

Nearly all cells in the human body contain a full molecular blueprint for their own assembly and function. These important instructions are encoded by deoxyribonucleic acid (DNA), which is divided into individual chromosomes and sequestered into a distinct compartment by the nuclear envelope. The nuclear envelope protects the DNA from free accessibility to proteins in the cytoplasm. However, essential communication is maintained between these compartments through the nuclear pore complexes. These are large proteinaceous structures that span the nuclear envelope and form channels connecting the nuclear interior and the cytoplasm. Nuclear pore complexes (NPCs) both facilitate and regulate the movement of molecules between these compartments, and consist of multiple proteins termed nucleoporins.

When a human cell divides, the nuclear envelope is disassembled to allow for the formation of the bipolar spindle, which then segregates the replicated genome into daughter cells. During this time, when the cytoplasmic and nucleoplasmic compartments intermingle, the transport function of the nuclear pore complex is unnecessary. Along with the nuclear envelope, the nuclear pore complex disassembles. This results in soluble nucleoporin subcomplexes, and, with the exception of the transmembrane nucleoporins, these subcomplexes are dispersed throughout the cell. It was presumed for many years that nucleoporins were dormant during cellular division, simply loitering in the cytoplasm until the nuclear pore complex reassembled at telophase. However, in recent years, it has

developed that multiple nucleoporins have additional roles during cell division, and such nucleoporins are proving to be important contributors to bipolar spindle assembly and function.

The focus of this dissertation is the discovery of a mitotic role for one such nucleoporin, Nup98. During interphase, when the NPC is intact, Nup98 functions in both RNA export and protein import. Studies from our lab and that of Jan Ellenberg demonstrated that Nup98 is a dynamic nucleoporin, shuttling between the pore, the nucleoplasm, and intra-nuclear bodies termed GLFG bodies. New evidence presented in this dissertation establishes for the first time that, in mitosis, Nup98 contributes to regulation of microtubule dynamics during spindle assembly through the microtubule depolymerizing kinesin, MCAK. This represents a novel function of Nup98, which is carried out through a previously uncharacterized region within the C-terminus.

In order to understand how this finding fits into the budding field of mitotic nucleoporin function, a variety of topics will be considered in this introduction. First I will cover the basics of nuclear pore complex structure, as well as the soluble components of the transport machinery that operate to move molecules through the pore. I will then move on to discuss bipolar spindle structure and what is currently known about contributions made by nucleoporins and soluble transport factors to spindle assembly during cell division. Finally, I will review the varied functions of Nup98, and will end with a brief overview of the experimental evidence to be presented on Nup98 function in mitotic spindle assembly.

The Nuclear Pore Complex

Structure

The nuclear pore is an immense multiprotein complex that spans the double lipid bilayer of the nuclear envelope. In vertebrates, this structure is thought to have a mass of ~100 MDa, although remarkably, the NPC only contains ~30 unique proteins (Cronshaw et al., 2002; Rout et al., 2000). Models of nuclear pore structure have come into increasingly sharper focus over the years, as imaging technology has improved. In the 1950s, the use of electron microscopy generated a first, very primitive picture of the pore as a structure possessing 8-fold symmetry around a central axis perpendicular to the nuclear envelope (Callan and Tomlin, 1950; Gall, 1954). In recent years, imaging technologies such as field emission in lens scanning electron microscopy (FEISEM), atomic force microscopy (AFM), 4Pi, and cryoelectron tomography have improved immensely our understanding of nuclear pore complex structure (Figure 1A) (Beck et al., 2004; Beck et al., 2007; Goldberg and Allen, 1993; Goldberg et al., 1992; Huve et al., 2008; Maco et al., 2006; Stoffler et al., 2003). On the cytoplasmic side of the pore, eight fibers extend into the cytoplasm. These filaments are attached at their base to a large cytoplasmic structural ring, which is connected by eight radial spokes to another large structural ring on the nucleoplasmic side of the pore. The radial spokes surround a central channel through which transport of macromolecules occurs. Fibers extend out from the nucleoplasmic ring towards the nuclus and are joined together by a small distal ring structure to create a basket-like structure.

Recent cryoelectron tomography studies of the nuclear pore complex indicate that the central channel is ~75 nm in diameter (Beck et al., 2007). However, the effective limit for diffusion through the channel is ~9 nm. Subsets of proteins within the nuclear pore complex contain domains of interspersed hydrophobic amino acid repeats, which are though to be naturally unstructured (Denning et al., 2003). It is presumed that these unstructured, hydrophobic domains line the central channel (Patel et al., 2007). Although we have a much clearer picture today of the pore complex, especially in comparison to structures described in the 1950s, research efforts continue to refine our model of the pore and to investigate the environment of the central channel.

Nucleoporins

The proteins that make up the nuclear pore complex are referred to as nucleoporins (Nup), and are designated by their molecular weight (Cronshaw et al., 2002; Rout et al., 2000). For example, Nup98 is a nucleoporin of ~98 kDa. Nucleoporins join together into subcomplexes, which in turn assemble to generate the structure of the nuclear pore complex (Figure 1B). For example, Nup358 and the Nup214-Nup88 subcomplex associate to form the cytoplasmic filaments (Walther et al., 2002). The Nup107-160 complex, a large assemblage of nine nucleoporins, with its binding partner ELYS form the basis for the cytoplasmic and nucleoplasmic structural rings of the pore (Belgareh et al., 2001; Rasala et al., 2006). On the nucleoplasmic face, Nup153, Nup50, and TPR together comprise the nuclear basket structure (Bodoor et al., 1999). Immuno-

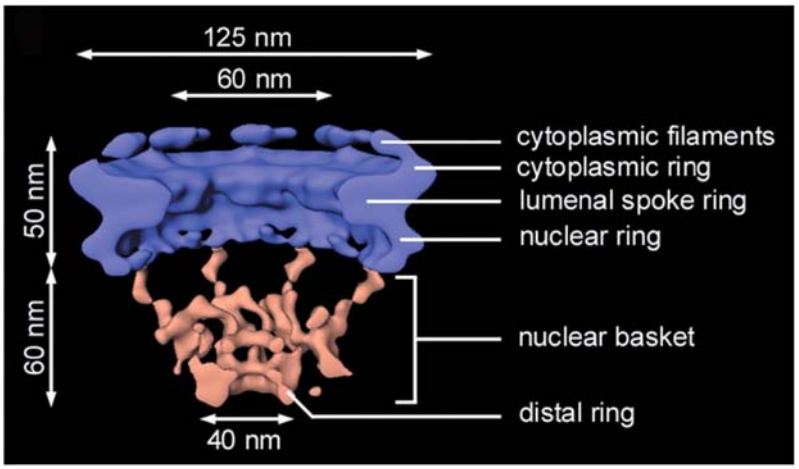
gold labeling experiments indicate that some nucleoporins, such as the Nup107 complex and Nup98, are found symmetrically on both the cytoplasmic and nucleoplasmic faces of the pore, while others, such as Nup358 and TPR are found exclusively on the cytoplasmic or nucleoplasmic face respectively (Griffis et al., 2003; Krull et al., 2004).

Individual nucleoporins can be divided into three different classifications depending on their structure and function at the pore (Table 1). The transmembrane nucleoporins, Pom121, gp210, and Ndc1, reside with their transmembrane domains at the curve of the nuclear envelope where the inner nuclear membrane and the outer nuclear membrane fuse at the nuclear pore complex (Schwartz, 2005). These nucleoporins serve to anchor the other components of the pore complex within the nuclear envelope. Scaffold nucleoporins, such as the Nup107 complex, are responsible for the major structural elements of the pore (Belgareh et al., 2001; Devos et al., 2006). Peripherally associated nucleoporins consistute a third class, including Nup98, Nup153 and Nup214, and these often contain phenylalanine-glycine (FG) repeat domains (Rout and Wente, 1994). Nucleoporin FG-repeat domains contain repeating motifs of FG, or variants of this sequence such as glycine-leucine-phenylalanine-glycine (GLFG) or FxFG repeats, where x is any amino acid. These FG domains are unstructured elements that face the central channel of the pore, where they provide interaction sites for soluble transport factors (Denning et al., 2003). Furthermore, homo- and hetero-typic interactions of FG-repeats within the central channel of the pore are thought to generate the

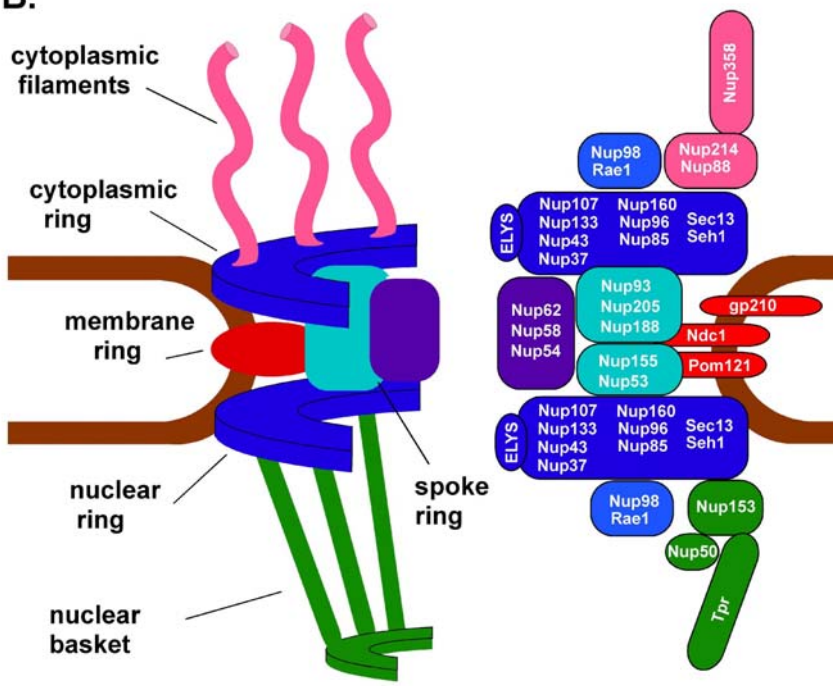
Figure 1. Structure and subcomplexes of the nuclear pore complex. **A)** Cryoelectron tomography image of a nuclear pore complex from Dictyostelium with major structural features highlighted (Beck et al., 2004). Reprinted with permission from Science. **B)** Diagram of nuclear pore complex subcomplexes (Xu, 2009).

Figure 1.

A.


125 nm
60 nm
50 nm
60 nm
40 nm
cytoplasmic filaments
cytoplasmic ring
lumenal spoke ring
nuclear ring
nuclear basket
distal ring

B.


cytoplasmic filaments
cytoplasmic ring
membrane ring
nuclear ring
nuclear basket
spoke ring
Nup358
Nup98 Rae1
Nup214 Nup88
ELYS
Nup107 Nup133 Nup43 Nup37
Nup160 Nup96 Nup85
Sec13 Seh1
Nup62 Nup58 Nup54
Nup93 Nup205 Nup188
gp210
Ndc1
Nup155 Nup53
Pom121
Nup153
Nup50
Tpr

permeability barrier of the pore, and thereby influence the size selectivity of the pore channel (Patel et al., 2007).

Dynamics of the pore

While descriptions of the nuclear pore complex structure may invoke a static picture of the pore, this is far from the full picture. Elegant experiments combining GFP-tagged protein expression constructs with live cell imaging and inverse fluorescence recovery after photobleaching (iFRAP) have deduced dynamic association of nucleoporins with the nuclear pore complex (Table 1) (Rabut et al., 2004). In the process of iFRAP, the majority of the cell is bleached, and dynamics are measured by monitoring the loss of fluorescent signal from the unbleached region (Dundr and Misteli, 2003). Highly dynamic nucleoporins include Nup50, Nup153 and gp210, with residence times lasting seconds to less than 15 minutes (Rabut et al., 2004). Nucleoporins classified in an intermediate range of dynamics, with residence times of 2.9 hours to 20 hours, are Nup98, Nup35, Nup58, Nup62, and Pom121. Finally, nucleoporins that have a residence time at the pore of 35 hours or greater include the members of the Nup107 complex, Nup214, Aladin and Nup93. This long residence time correlates with the proposed structural role of these nucleoporins. Note that gp210, one of the transmembrane nucleoporins, is surprisingly one of the most dynamic nucleoporins. Nup50 and Nup153, two highly dynamic nucleoporins, were each found to have two seperable populations at the pore with distinct residence

Table 1. Nucleoporin location, dynamics and function

Location	Vertebrate Nups	Dynamics	Predicted Function
Cytoplasmic face	Nup358	nd	structural, transport
	Nup214 complex	stable	structural
	CG1	intermediate	structural, transport
Symmetrically distributed	Nup62 complex	intermediate	structural, transport
	Nup107 complex	stable	structural
	Nup93 complex	intermediate	structural, transport
	Rae1/Gle2	dynamic	transport
	Nup98	dynamic	transport
Transmembrane	Pom121	stable	structural
	gp210	dynamic	transport
	NDC1	nd	structural
Nuclear Face	Nup50	dynamic	transport
	Nup153	dynamic	structural, transport
	Tpr	nd	structural, transport

Nup62 complex = Nup62, Nup58, Nup54, Nup45

Nup107 complex = Nup160, Nup133, Nup107, Nup96, Nup85, Nup43, Nup37, Sec13, Seh1

Nup93 complex = Nup93, Nup205, Nup188

Nup214 complex = Nup214, Nup88, Nup62

times, suggesting potential differences in binding and/or function between the two populations.

There have been several hypotheses as to why nucleoporins might move on and off the pore. One theory is that this movement represents a mechanistic contribution to nuclear transport wherein nucleoporins contribute to delivery of cargo to the pore. Indeed, previous work found that the dynamics of both Nup98 and Nup153 are much reduced in the presence of transcription inhibitors such at Actinomycin D and DRB (Griffis et al., 2002; Griffis et al., 2004). Thus, in the absence of new RNA cargo, these nucleoporins do not traffic on and off the pore. Alternatively, nucleoporin dynamics could also be a means of global regulation of nuclear pore complex function. Changing the composition of the pore through the redistribution of nucleoporins may differentially affect the ability of individual classes of cargo to be transported through the pore (Fahrenkrog et al., 2002; Paulillo et al., 2005). Finally, this dynamic association could be, at least in part, a mechanism to replace nucleoporins that have been damaged through oxidative stress or other disruptive modifications accumulated over time. A recent study on age-dependent deterioration of nuclear pore complexes showed that, over time in post-mitotic cells, a subset of exposed nucleoporins such as Nup93 and Nup153 collected carbonyl groups, a sign of oxidative damage (D'Angelo et al., 2009). Perhaps observed dynamics represents replacement of a “used” nucleoporin with a “new” nucleoporin.

In addition to the dynamic nature of nucleoporins, the pore complex structure is flexible. Studies utilizing both transmission electron microscopy

(TEM) and field emission in-lens scanning electron microscopy (FEISEM) showed that the basket structure on the nucleoplasmic face changes during export of large ribonucleoprotien (RNP) cargo (Kiseleva et al., 1996). Furthermore, studies using domain-specific antibodies directed towards Nup153, a component of the nuclear basket, revealed that the C-terminus of Nup153 is highly flexible and can extend through the central channel to the cytoplasmic face of the pore (Fahrenkrog et al., 2002). Views of the pore generated through cryoelectron tomography reveal that the cytoplasmic filaments are dynamic entities that interact with incoming cargo, although they are not required for cargo import (Beck et al., 2004; Walther et al., 2002). As well, cryoelectron tomography has highlighted that the central channel of the pore can also dilate and constrict during transport, which has been suggested to result from intermolecular sliding of members of the Nup62 complex (Melcak et al., 2007). Thus, the pore is far from being passive, but rather plays a very active role to ensure passage of molecules.

Nuclear Transport

Transport Receptors

While small molecules and ions passively diffuse through the pore, larger proteins and RNA complexes over ~40 kDa must be actively transported (Fried and Kutay, 2003). Transport of these large cargos generally is facilitated by families of transport receptor proteins, collectively termed karyopherins (Kap) (Pemberton and Paschal, 2005). The largest family of transport receptors, the

Importin β superfamily, is comprised of at least 21 members in humans, including Importin β, exportin1/Crm1, transportin, and Cas/Cse1 (Mosammaparast and Pemberton, 2004). Karyopherins are classified into this family based on similarities in sequence and structure, and are often separated into either Importins or Exportins depending on the direction they typically carry cargo through the pore. Transport receptors often require adaptor proteins which bind to the cargo and the transport receptor simultaneously. For classical nuclear localization signals (see below), Importin α acts as an adaptor for Importin β (Görlich et al., 1995; Moroianu et al., 1995). Other transport factors include NTF2, which carries RanGDP (see below) from the cytoplasm to the nucleus, and the mRNA export receptor TAP/NXF1, which functions as a heterodimer together with p15/NXT (Katahira et al., 1999; Paschal and Gerace, 1995). There is also evidence that some proteins, such as β-catenin, can interact with the pore directly due to their structural resemblance to karyopherins, and thus have no need for a transport receptor (Fagotto et al., 1998).

Transport Signals

Transport receptors function by recognizing specific localization signals within cargo needing to be moved across the nuclear pore complex. The classical protein nuclear localization signals (cNLS) were first discovered within simian virus 40 (SV40) large T antigen and nucleoplasmin, and are characterized by stretches of basic amino acids (Dingwall et al., 1982; Kalderon et al., 1984; Robbins et al., 1991). SV40 large T antigen contains a monopartite signal

(PKKKRKV), while the NLS signal of nucleoplasmin is described as bipartite (KRPAATKKAGQKKKK) because the signal is bisected by a linker sequence. The Importin α/Importin β receptor heterodimer recognizes these signals and carries cargo through the pore into the nucleus. Importin β can function in the absence of Importin α by binding directly to cargo containing a non-classical NLS through region distinct from the Importin α binding site (Cingolani et al., 2002). The classical signal for a protein to be exported from the nucleus, the nuclear export signal (NES), is a short sequence enriched in leucine or other hydrophobic resides, such as the original NES identified in HIV Rev (LWLPPLERLTL) (Fischer et al., 1995). This signal is present in many cellular proteins and is recognized by Crm1/Exportin1, which carries molecules from the nucleus to the cytoplasm.

Most RNA species employ import and export mechanisms similar to those of proteins. Import and export signals are found within adaptor proteins that bind the RNA, and these signals are then recognized by karyopherin family members. For example, small nuclear RNA (snRNA) and 5s ribosomal RNA (rRNA) bind NES-containing proteins, which in turn are recognized by Crm1/Exportin1, and the complex is exported out of the nucleus (Hamm et al., 1990; Moy and Silver, 1999). An exception to the use of adaptor proteins is the export of transfer RNA (tRNA), which is bound directly by exportin-t, a β karyopherin that recognizes specific RNA structural motifs as export signals. In contrast, messenger RNA (mRNA) is transported by a complex, multi-component system (Arts et al., 1998; Kutay et al., 1998). Its interface with the nuclear pore complex is mediated by the TAP/NXT heterodimer, a transporter unrelated to the karyopherin β or α families

(Gruter et al., 1998). mRNA export is quite distinct from other types of export, due to extensive maturation and rigorous quality control mechanisms linked to its export, and will be discussed in more detail below.

The RanGTPase system

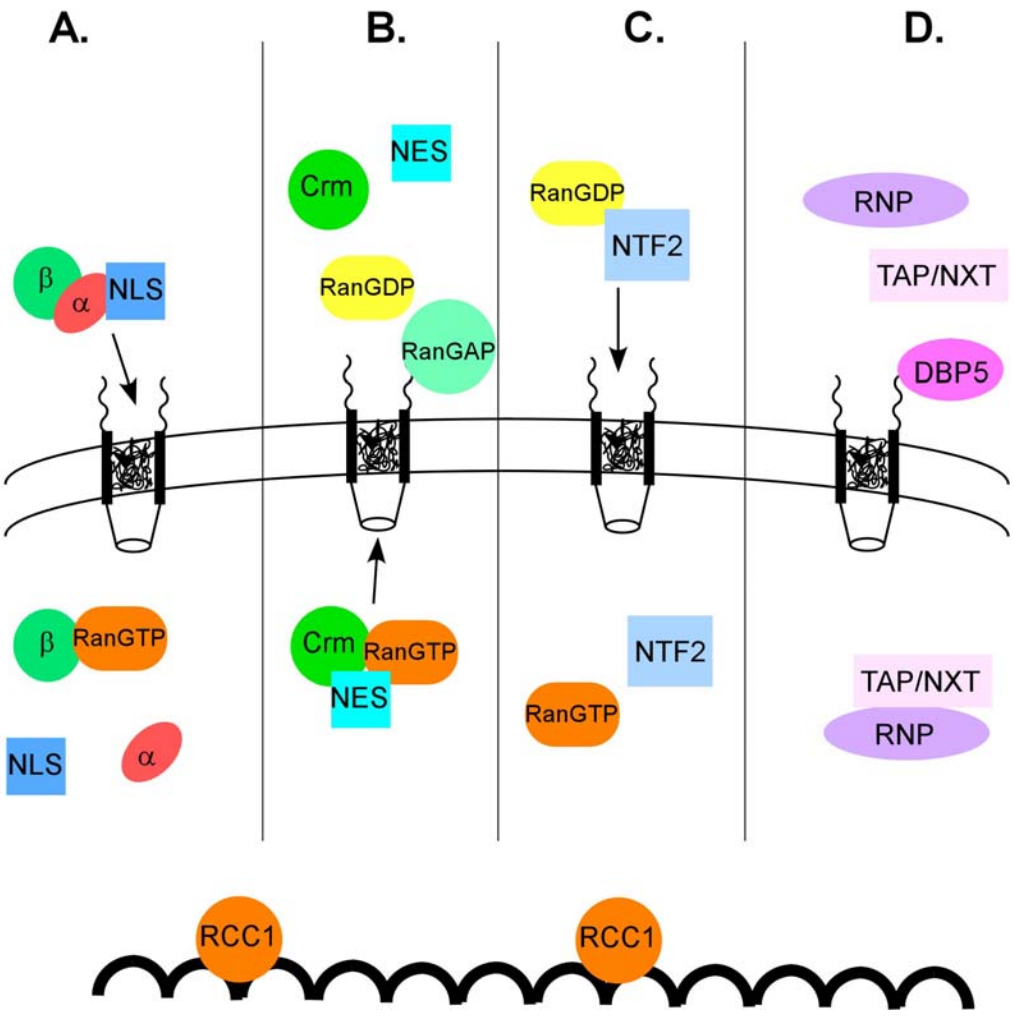
The master regulator of nucleocytoplasmic transport is Ran, a low molecular weight guanosine triphosphatase (GTPase) of the Ras superfamily (Bischoff and Ponstingl, 1991b). Ran controls the directionality of transport through regulation of interactions between cargo and receptors. As is typical of members of the Ras family, the nucleotide binding state of Ran is regulated by a guanosine exchange factor (RanGEF) and a GTPase activating protein (RanGAP). The RanGEF (Regulator of Chromosome Condensation 1; RCC1), binds to chromatin and is thus sequestered in the nucleus (Bischoff and Ponstingl, 1991a). Complimentary to RCC1 is the RanGAP, which is found in the cytoplasm and associates in part with the cytoplasmic fibers of the nuclear pore complex (Bischoff et al., 1994). RanGEF stimulates the formation of RanGTP by facilitating release of GDP from Ran, while RanGAP, with the help of its binding partner Ran-binding-partner1 (RanBP1), increases the rate of GTP hydrolysis by Ran (Bischoff and Gorlich, 1997). The compartmentalized manner in which the RanGAP and RanGEF are localized results in a high concentration of RanGTP in the nucleus and a high concentration of RanGDP in the cytoplasm of the cell. This is typically referred to as the “Ran gradient” (Görlich et al., 1996; Izaurralde et al., 1997).

The Ran gradient dictates directionality of cargo movement across the pore through controlled binding and release of cargo from transport receptors. Import receptors bind their cargo in the presence of RanGDP, which is found at high concentration in the cytoplasm. However, they are dissociated on the nuclear side of the pore when the importin/cargo complexes encounter and bind RanGTP (Figure 2A) (Rexach and Blobel, 1995). In the case of classical NLS protein import, RanGTP binds directly to Importin β, resulting in a conformational change of Importin β that releases both cargo and Importin α into the nucleoplasm (Cingolani et al., 2000; Vetter et al., 1999). Conversely, the exportins form a complex with their nuclear export cargo in the presence of RanGTP (Fornerod et al., 1997; Kutay et al., 1997b). Binding of the exportin to the cargo blocks the minimal rate of nucleotide hydrolysis by Ran, however following translocation through the nuclear pore complex, the exportin/cargo complex encounters the RanGAP/RanBP1 complex. RanBP1 binds the complex and facilitates interaction between RanGAP and RanGTP thus relieving the block to hydrolysis (Bischoff and Gorlich, 1997). GTP hydrolysis and the resulting conformational change drive disassembly of the exportin/cargo complex (Figure 2B). Thus, the high concentrations of RanGTP in the nucleus support both import cargo delivery and formation of export cargo/receptor complexes, while the localization of the RanGAP allows for release of cargo into the cytoplasm.

In order to perpetuate cycles of transport, import receptors must be recycled to their original cytoplasmic compartment and the export receptors must be relocated back to the nucleus. Importin β can directly facilitate its own

Figure 2. Nuclear Transport. Cartoon diagram of different nucleocytoplasmic transport events. **A)** Classical nuclear import – cNLS-containing cargo binds to the Importin β/Importin α heterodimer in the cytoplasm and is disassociated in the nucleus upon the binding of RanGTP to Importin β. **B)** Classical nuclear export – NES-containing cargo forms a trimeric complex with Crm and RanGTP in the nucleus, and disassembles in the cytoplasm when RanGAP stimulates RanGTP to hydrolyze. **C)** The transport receptor NTF2 recycles RanGDP back into the nucleus after export, which is then converted back to RanGTP by the chromatin-associated RanGEF RCC1. **D)** mRNA complexes with multiple proteins during processing in the nucleus, which generates an mRNP that can the recruit the export receptor heterodimer TAP/NXT. The RNA helicase DBP5 associates with the cytoplasmic side of the nuclear pore complex and aids in release of the mRNP to the cytoplasm.

Figure 2


A.
B.
C.
D.
β
α
NLS
Crm
NES
RanGDP
RanGAP
NTF2
RNP
TAP/NXT
DBP5
β
RanGTP
NLS
α
Crm
RanGTP
NES
RanGTP
NTF2
TAP/NXT
RNP
RCC1
RCC1

passage through the nuclear pore complex (Hieda et al., 1999; Izaurralde et al., 1997). However, Importin α requires a specific β family member, Cas, which is specialized for export of importin α from the nucleus (Kutay et al., 1997a). In order to maintain RanGTP levels in the nucleus, NTF2 binds to RanGDP and facilitates its movement back through the pore, where it is exchanged to RanGTP by RCC1 (Figure 2C) (Moore and Blobel, 1995; Paschal and Gerace, 1995).

mRNA Export

mRNA export from the nucleus is unique in multiple ways but chief among them is that it does not rely on the Ran gradient for directionality. Once an mRNA molecule is synthesized in the nucleus, it goes through extensive processing before it can be exported from the nucleus, including being capped at the 5' end, trimmed and polyadenlyated at the 3' end, and spliced to remove introns. During these processes, a host of different proteins are sequentially recruited to the mRNA, leading to formation of a messenger-ribonucleoprotein complex (mRNP) (Hieronymus and Silver, 2003). The TAP/NXT mRNA export receptor complex is targeted to mRNPs by export adaptor proteins, including SR proteins, ALY/REF, and the TREX complex (Caceres et al., 1997; Gruter et al., 1998; Reed and Hurt, 2002). These export adaptor proteins are recruited to the mRNA during transcription, splicing and quality control steps of maturation. This sequential recruitment mechanism prevents TAP/NXT from prematurely binding and exporting an immature message to the cytoplasm. TAP/NXT is able to interact with FG nucleoporins through two separate binding sites as well as with Rae1

(Blevins et al., 2003). These interactions facilitate the movement of RNPs from the nucleus to the cytoplasm.

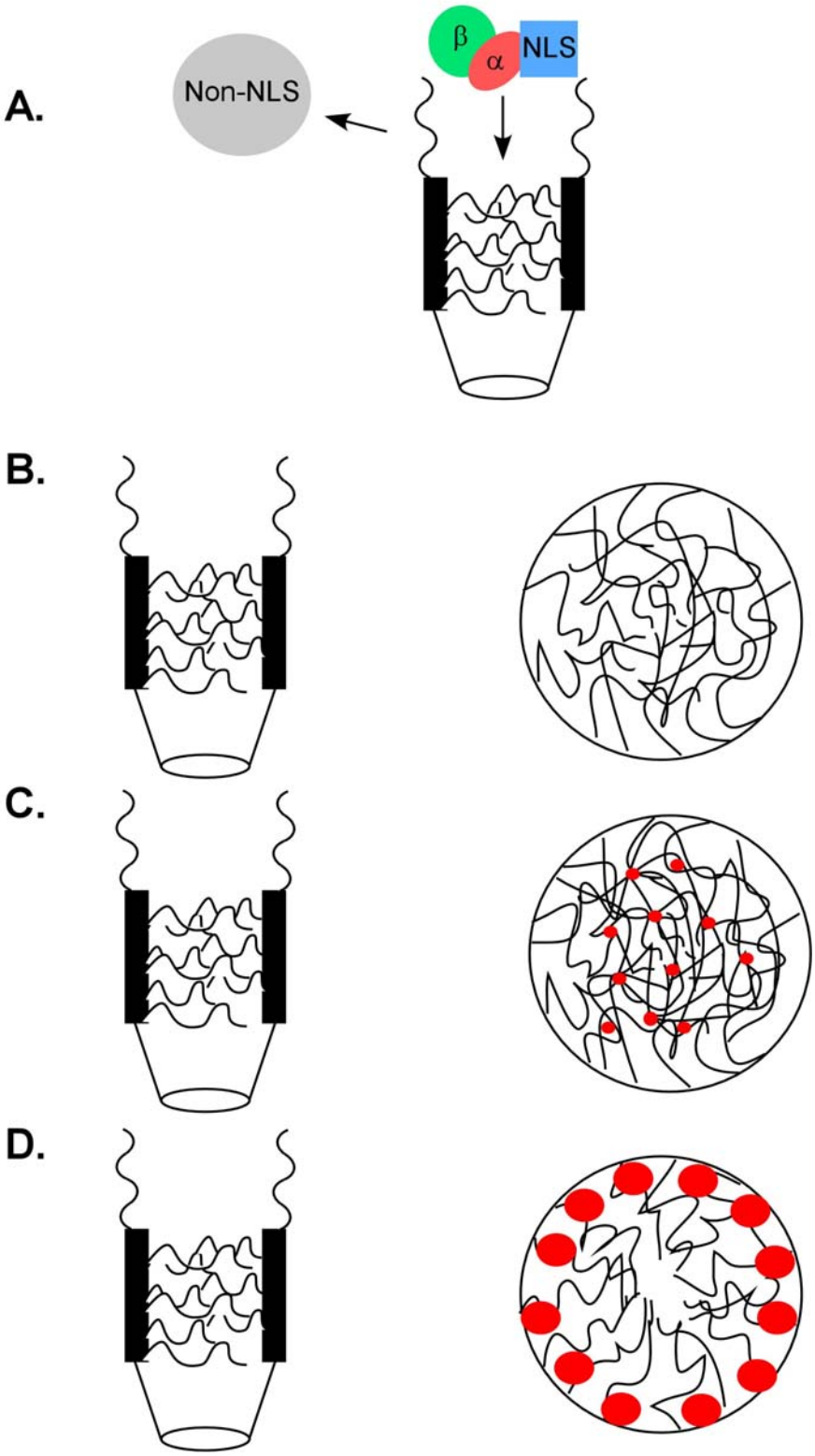
Since mRNP export is not dependent on Ran, another mechanism is needed to dissociate mRNP cargo from its receptor in the cytoplasm. The ATP-dependant, DEAD-box RNA helicase protein, Dbp5, is thought to provide such a mechanism (Tseng et al., 1998). Dbp5 is found on the cytoplasmic side of the pore, where it binds to Nup214, a component of the cytoplasmic fibers (Weirich et al., 2004). The intrinsic RNA helicase activity of Dbp5 is low, but can be stimulated by the dynamic nucleoporin/export factor Gle1 and the signaling molecule inositol hexakisphosphate ($InsP_6$) (Alcazar-Roman et al., 2006). Local activation of the Dbp5 helicase on the cytoplasmic face of the pore leads to rearrangements that force the mRNP out into the cytoplasm while simultaneously releasing the transport receptor (Figure 2D) (Stewart, 2007).

Models of Translocation

Both single-molecule imaging experiments and *in vitro* data suggest that there are ~1000 molecules traversing each nuclear pore complex every second (Ribbeck and Gorlich, 2001). Although much information about the players in this process has been gleaned from experimentation, there is still considerable debate about how exactly proteins and RNA, bound by their appropriate transport receptors, actually move through the nuclear pore complex. Multiple models have been proposed to explain translocation through the pore; all of these build on the

Figure 3. Nuclear pore translocation models. Cartoon diagram of different translocation models. In figure 3B, 3C, and 3D, the large circle on the right represents a view of the central channel of the pore looking down from the top. **A)** The “virtual gate model” proposes that the cytoplasmic fibers act as entropic bristles – preventing non-transporting proteins from entering the pore. Cargo bound to transport receptors can translocate through the pore by interacting with the FG-repeat domains. **B)** The “oily spaghetti model” advocates that the hydrophobic FG-repeat domains form a meshwork in the center of the pore that transport receptors transiently bind to. **C)** The “selective phase model” proposes that the FG-repeat domains interact with each other (red dots) to form a meshwork or phase. Transport receptors locally dissolve these FG-FG interactions to move through the pore. **D)** The “reduction of dimensionality” model states that the FG-repeats line the nuclear pore (red circles), and transport receptors glide along these repeats to translocate through the pore. Molecules too large to diffuse through the central channel that cannot interact with FG-repeats are unable to enter the pore.

Figure 3



well established binding between transport receptors and the FG-repeat domain of nucleoporins.

The “virtual gate model”, also termed the “entropic bristle model”, proposes that the selectivity of the nuclear pore complex is based on changes in entropy (Figure 3A) (Rout et al., 2003). In order for a molecule to move from a freely diffusing state in the cytoplasm to a much less mobile state within the central channel of the nuclear pore complex, there would be a rapid and substantial drop in its entropy. However, when a molecule is bound to a transport receptor that is capable of interacting with the FG-repeat domains found in the central channel, the chance is increased that, even with the drop in entropy, the molecule will translocate through the pore. Additionally, in this model the cytoplasmic filaments are though to extend out towards the cytoplasm like bristles. Movement of these “entropic bristles” serves to exclude non-transporting proteins from entering the pore.

Alternative models suggest that selectivity of the pore is generated via the physical barrier created by interactions between FG-repeat domains. One such model nicknamed the “oily-spaghetti model” advocates that the many copies of flexible FG-repeat domains form a kind of meshwork that fills the central channel of the pore (Figure 3B) (Macara, 2001). Transport-cargo complexes navigate through the pore by transiently binding to the meshwork. The “selective phase model” is slightly different in that it suggests that the FG-repeat domains interact with each other through hydrophobic interactions in the central channel, thus generating a connected meshwork or phase (Figure 3C) (Ribbeck and Gorlich,

2001). The transport receptors locally dissolve the meshwork by competing for and disrupting FG-FG interactions to allow for passage of cargo through the pore. In support of this model, it was recently shown that high concentrations of purified FG-repeat domain from the yeast nucleoporin, Nsp1p, could form a hydrogel that displayed similar permeability properties as intact nuclear pores (Frey and Gorlich, 2007). However, rather extreme conditions were required to generate this hydro-gel and this result remains controversial.

Most recently, the “reduction of dimensionality model” proposed that transporters can simply slide along the FG-repeat lined corridor of the nuclear pore complex, and that those molecules too large to diffuse through the central channel that are unable to bind the FG-repeat domains are excluded from the nucleus (Figure 3D) (Peters, 2005). In all probability, the “correct” model for how molecules move through the nuclear pore complex probably includes aspects from each of the proposed models.

The Mitotic Spindle

When a cell undergoes division, it is critical that each pair of replicated chromosomes is separated correctly into the daughter cells. The apparatus responsible for this process is the bipolar mitotic spindle, which is a complex microtubule-based machine accountable for aligning the chromosomes together on the metaphase plate at the center of the cell and then dividing the sets of chromosomes correctly into individual daughter cells. The typical spindle structure consists of two centrosomes that form the poles of the spindle, and

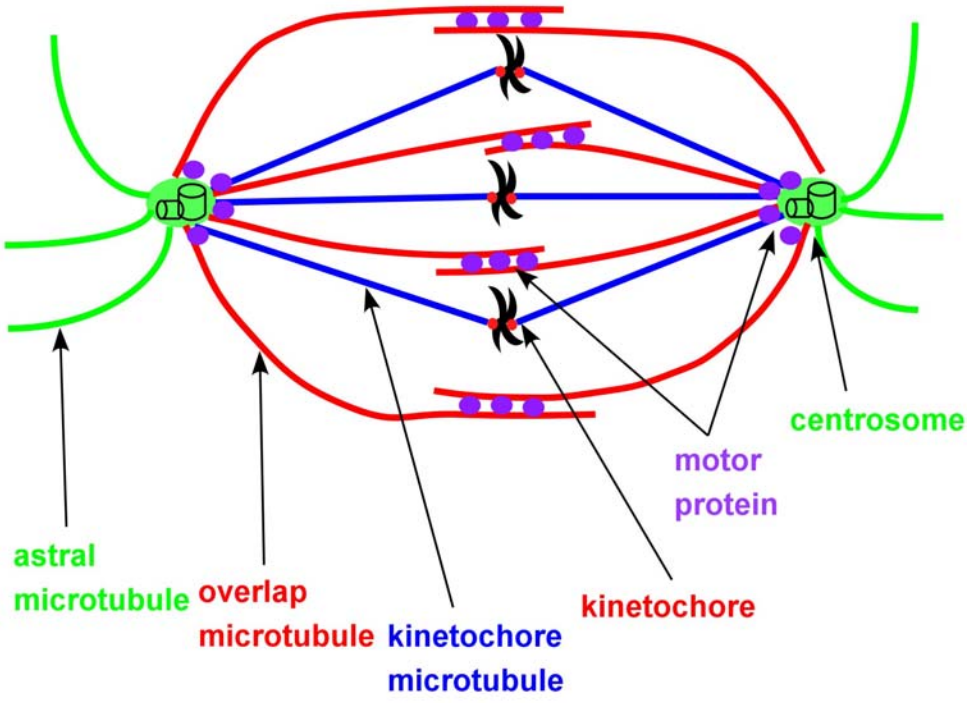
microtubules, which emanate from the centrosomes and form a scaffold between the poles and the chromosomes (Figure 4). Components of the soluble transport machinery, introduced above, have critical roles during spindle assembly. Recently, it has been established that a subset of nucleoporins are also instrumental in formation and maintenance of the spindle structure. In this section, I will discuss spindle structure and formation, and review what is currently known about functions of nucleoporins in spindle assembly during mitosis.

Microtubules

Microtubules are the major structural component of the bipolar spindle, and are built from the ~50 kDa GTP-binding protein, tubulin (Burns, 1991). Heterodimers of α- and β-tubulin bind in a head-to-tail fashion to form a polarized protofilament (Amos and Klug, 1974). In turn, ~13 αβ protofilaments in the same orientation bind laterally to form a cylindrical microtubule (Evans et al., 1985). One end of this polarized microtubule, the plus end, is the site for rapid microtubule growth, whereas the opposite, minus end, is much less efficient at addition of αβ subunits and grows slowly. These differences in dynamics result from the polarized manner in which the microtubule is built with the β subunit always facing the plus end (Allen and Borisy, 1974). Once the αβ heterodimer incorporates into a polymer, the GTP bound to the β subunit is hydrolyzed to GDP, while the GTP associated with the α subunit remains stable (David-Pfeuty et al., 1977; Spiegelman et al., 1977).

Figure 4. The mitotic spindle and spindle assembly factors. Cartoon diagram of a bipolar spindle.

Figure 4


centrosome
motor
protein
astral
microtubule
overlap
microtubule
kinetochore
microtubule
kinetochore

The dynamics of microtubules are a direct consequence of the hydrolysis of GTP in the β subunit after polymerization. GTP hydrolysis by β-tubulin is not required for microtubule polymerization, but hydrolysis does affect the stability of the microtubule polymer once it is formed. When non-hydrolyzable analogs of GTP are incorporated into microtubules, the microtubule polymers are much more stable and structurally rigid than natural microtubules (Kirschner, 1978; Mejillano et al., 1990; Mickey and Howard, 1995; Vale et al., 1994). Conversely, GTP hydrolysis lessens the stability of the microtubule, and can lead to depolymerization from the plus end (Caplow et al., 1994). When growth proceeds more rapidly than hydrolysis, the result is a region of GTP-bound β-tubulin subunits at the plus end, referred to as the GTP cap, which protects the microtubule filament from depolymerization (Mitchison and Kirschner, 1984). Under conditions of slowed polymerization, hydrolysis “catches up” to the incorporating tubulin subunits, leading to a loss of the stabilizing GTP cap. Such a microtubule is prone to sudden, rapid depolymerization or “catastrophe”. CryoEM studies detected differences in microtubule structure when GTP is hydrolyzed to GDP, resulting in destabilizing curvature of the tubulin polymer (Hyman et al., 1995). The combination of growth and depolymerization results in “dynamic instability”, first described by Mitchison and Kirschner in 1984 (Mitchison and Kirschner, 1984). Dynamic instability is an important feature in the “search and capture” model of spindle assembly, described in more detail below.

Microtubule polymers also undergo a process called “treadmilling”, in which addition of tubulin heterodimers on the plus end is balanced by removal of

tubulin dimers at the minus end (Kirschner, 1980; Margolis, 1981). Within the mitotic spindle, a similar process termed poleward flux occurs, in which tubulin subunits are added onto microtubules at the kinetochore and move down the polymer towards the centrosome (Maddox et al., 2003; Mitchison, 1989). However, in a spindle, an additional force provided by molecular motors, is required to drive poleward flux. Real-time microscopy of live microtubules confirmed that both dynamic instability and poleward flux do occur *in vivo* (Cassimeris et al., 1988; Mitchison et al., 1986; Mitchison, 1989). As discussed below, microtubule dynamics have proved essential for correct spindle assembly and function.

There is a dramatic change in microtubule dynamics during the cell cycle (Zhai et al., 1996). During interphase, microtubule polymers extend far out into the cytoplasm, away from their point of origin at the centrosome, and very rarely undergo catastrophes. However, during mitosis, experiments in *Xenopus* egg extracts (see below) and imaging in cultured cells demonstrated a dramatic increase in catastrophe frequency causing mitotic microtubules to be highly dynamic arrays of relatively short microtubules (Belmont et al., 1990; McNally, 1996; Verde et al., 1992). This change in microtubule dynamics in response to the cell cycle is strongly suggestive of regulatory factors in addition to GTP hydrolysis that influence the stability of the microtubule. Such regulators include both microtubule stablilizing and destabilizing factors.

A class of proteins called microtubule associated proteins or MAPs are responsible for much of the stabilizing activity. Classical MAPs bind to

microtubules, stabilize the polymer, and, in some cases, can prevent catastrophe while promoting assembly (Pryer et al., 1992; Trinczek et al., 1995). Other, non-classical MAPs have more complex activities that contribute to microtubule stability. For example, XMAP215, first identified in *Xenopus* egg extract, associates with the plus end tips of polymerizing microtubules and increases the rate of αβ subunit addition, thus promoting growth (Gard and Kirschner, 1987). Alternatively, XMAP215 can also accelerate microtubule depolymerization when conditions are not favorable for microtubule growth through mechanisms that are poorly understood. Phosphorylation of MAPs during mitosis in most cases inhibits their ability to stabilize microtubules (Trinczek et al., 1995).

Opposing the action of MAPs are the microtubule destabilizers, which promote catastrophe of microtubule fibers by a variety of means. Op18/stathamin sequesters αβ tubulin subunits, thereby altering the balance between polymerized and free tubulin and promoting depolymerization of microtubules (Belmont and Mitchison, 1996). A second microtubule destabilizer is the Mitotic Centromere-Associated Kinesin (MCAK). Abnormally long microtubules were observed in egg extracts depleted of MCAK, suggesting that MCAK acts to promote microtubule depolymerization (Walczak et al., 1996). Initial characterization in cells revealed a population of MCAK at the centromeric region of chromosomes during mitosis (Wordeman and Mitchison, 1995). MCAK was later characterized as a member of the Kin I family of kinesins, named for their internal as opposed to N- or C-terminal motor domains (Desai et al., 1999b). Kinesins of this family capture the energy of ATP-hydrolysis to depolymerize

microtubules (Hunter et al., 2003; Maney et al., 2001). The microtubule depolymerizing activity of MCAK may in part serve to correct missattachments of microtubules to kinetochores (Kline-Smith et al., 2004). MCAK has additional roles beyond correction of kinetochore-microtubule misattachments, as spatial regulation of MCAK activity by the chromosomal passenger complex is required for bipolar spindle assembly in *Xenopus* egg extract (Kelly et al., 2007; Ohi et al., 2004; Sampath et al., 2004). In egg extract, the microtubule depolymerizer MCAK and the microtubule stabilizer XMAP215 are the main opposing regulators of microtubule dynamics in both interphase and mitosis (Tournebize et al., 2000).

Centrosomes and Kinetochores

The centrosome functions as the primary microtubule organizing center of the cell. As the cell cycle progresses towards M phase, the centrosome is duplicated during S phase in a process initiated by Cyclin A or Cyclin E complexed with CDK2 (Lacey et al., 1999). During prophase, before nuclear envelope breakdown, the duplicated centrosomes spearate from one another and position themselves on either side of the nucleus, where they are situated to become the opposite poles of the bipolar spindle. The metazoan centrosome is made up of a pair of centrioles surrounded by a pericentriolar matix which consists of a lattice of pericentrin and other large proteins (Dictenberg et al., 1998). This lattice serves as a scaffold for multiple protein complexes needed for correct function of the centrosome. One such complex is the γ-tubulin ring complex (γ-TuRC), a ring shaped protein complex that functions to nucleate

microtubule polymerization (Zheng et al., 1995). The protein composition of the centrosome is altered in a cell cycle dependent manner, regulated by the kinases Aurora A and Polo-like Kinase 1 (Plk 1) (Donaldson et al., 2001; Giet et al., 2002). During mitosis, the concentration of the γ-TuRC at the centrosome is increased, resulting in greater microtubule nucleation capacity (Khodjakov and Rieder, 1999; Piehl et al., 2004).

During mitosis, γ-TuRC-nucleated microtubules connect the centrosomes to the chromosomes via the kinetochore, a protein complex built on the centromeric region of the chromosome. In human cells, electron microscopy has shown that each kinetochore binds the plus ends of 15-20 microtubules, which bundle together to form the “K-fiber” (McEwen et al., 2001; Wendell et al., 1993). Mass spectrometric analysis of isolated kinetochores and mitotic spindles, as well as co-immunoprecipitations of kinetochore components have identified ~80 proteins that associate to form the massive kinetochore structure (Cheeseman et al., 2004; Sauer et al., 2005; Wigge and Kilmartin, 2001). The kinetochore complex is not only responsible for the physical microtubule-kinetochore attachment, but also for correction of mis-attached microtubules, and for ensuring that these attachements are complete and correct before signaling for anaphase. As will be discussed below, several nucleoporins are functional members of the kinetochore.

Centrosome-driven spindle assembly

An early model of spindle assembly was proposed by Mitchison and Kirschner in 1986, and focused solely on the centrosome (Kirschner and Mitchison, 1986; Mitchison and Kirschner, 1984). This “search and capture” model hypothesized that, through dynamic instability, microtubules randomly search cytoplasmic space for chromosomes. When a microtubule makes contact with the kinetochore on a chromosome, its dynamics rapidly change to a stable state, and the chromosome is captured by that microtubule. Over time, multiple connections are made this way, with many microtubules becoming stabilized at the kinetochore. Differential interference-contrast (DIC) images of live newt lung cells and serial section electron microscopy analysis support the “search and capture” model (Hayden et al., 1990; Rieder and Alexander, 1990). Centrosome-driven spindle assembly is thought to be the major pathway by which bipolar spindles are built in most cells.

Nonetheless, there have been indications that the “search and capture” mechanism may not be sufficient for building a mitotic spindle. Mathematical modeling predicted that it would take hours for microtubules to find kinetochores without the introduction of a bias towards the chromosome (Wollman et al., 2005). Furthermore, bipolar spindles can form in cells lacking a centrosome, such as meiotic eggs, some types of plant cells. Thus, one or more alternative spindle assembly pathways must exist.

Such a pathway has now been identified and is driven by chromatin. As will be discussed in detail below, chromatin can direct microtubule nucleation and

spindle assembly. The chromatin-driven spindle assembly pathway is the major pathway in cells naturally lacking centrosomes and in meiotic *Xenopus* egg extracts. However, this pathway also exists and is functional in cells containing centrosomes. Remarkably, cells can still assemble spindles when their centrosomes have been ablated with lasers (Basto et al., 2006; Karsenti et al., 1984a; Khodjakov et al., 2000).

Chromatin-driven Spindle Assembly

Karsenti and colleages reported 25 years ago that prokaryotic DNA injected into *Xenopus* eggs could induce formation of a bipolar spindle-like structure (Karsenti et al., 1984a). Prokaryotic DNA lacks the centromeric DNA sequence upon which the kinetochore is built, and *Xenopus* eggs do not contain centrosomes. Therefore, this most unexpected finding established that kinetochores and centrosomes themselves are not absolutely required for spindle assembly and suggested that other unidentified factors make important contributions to the bipolar spindle. Subsequent experiments in which magnetic beads were coated with plasmid DNA and incubated in mitotic *Xenous* egg extract demonstrated that microtubules are able to organize into an anti-parallel spindle structure around the beads. The molecular motor dynein is critical to formation of this spindle as it is involved in generating spindle poles by focusing microtubule minus ends (Heald et al., 1996). Even more unexpectedly, multiple groups later reported that addition of non-hydrolyzable RanGTP or the RanGEF, RCC1, to *Xenopus* egg extracts was sufficient to induce microtubule

polymerization and formation of spindle-like structures in the absence of centrosomes or DNA; these structures are typically termed Ran asters (Carazo-Salas et al., 1999; Kalab et al., 1999; Ohba et al., 1999; Wilde and Zheng, 1999; Zhang et al., 1999). Conversely, depletion of RCC1 from egg extracts led to an inhibition of spindle assembly even in the presence of chromatin (Ohba et al., 1999). Since RCC1 binds to chromatin, this prompted a model in which RCC1 generates a gradient of RanGTP around the chromatin, and this, in some manner, activates microtubule polymerization and organization into a bipolar spindle.

During interphase, a gradient of RanGTP across the nuclear envelope serves to direct soluble transport factors in the Importin β superfamily. Strikingly, the Ran gradient works in a related fashion during chromatin-driven spindle assembly. Depletion of Ran from *Xenopus* egg extracts severely inhibited spindle assembly, which led to a search for Ran targets in microtubule polymerization and spindle organization (Nachury et al., 2001). The soluble transport receptor Importin β was found to be a potent spindle assembly inhibitor. Depletion of Importin β using a RanGTP column led to spontaneous Ran aster formation, which could be reversed with addition of purified recombinant Importin β (Nachury et al., 2001). Specific spindle assembly factors were found to be sequestered by either Importin β or the Importin α/Importin β heterodimer in a RanGTP-sensitive manner (Gruss et al., 2001; Nachury et al., 2001; Wiese et al., 2001). Furthermore, the β family member Crm, which binds cargo in the presence of RanGTP, aids in localizing specific spindle factors to the kinetochore

(Arnaoutov and Dasso, 2005). Thus, the prevailing model of chromatin driven spindle assembly is that spindle assembly factors are sequestered by soluble transport receptors in mitotic cytosol, and these factors are released the vicinity of the chromatin or regions of the spindle when the karyopherins reach specific thresholds of RanGTP concentrations within the mitotic Ran gradient (Figure 5). Many such spindle assembly factors have now been identified, and will be discussed in the following section. Additionally, the chromatin-based pathway of spindle assembly has been visualized in both meiotic *Xenpus* egg extracts and mitotic HeLa cells using fluorescent sensors that can detect the molecular interactions in that pathway (Kalab et al., 2006).

Spindle Assembly Factors

Numerous spindle assembly factors interact in a cooperative manner to build the bipolar spindle structure (Table 2). Many of these factors are regulated directly by the Ran pathway of spindle assembly, while others are downstream effectors. The first critical step in Ran-mediated spindle assembly is nucleation of microtubules. Both γ-tubulin and the microtubule associated protein TPX2 are essential for this first step (Gruss et al., 2001; Haren et al., 2006; Luders et al., 2006; Wilde et al., 2001). TPX2 can nucleate microtubules *in vitro,* although it requires other factors to aid in this function in *Xenopus* extracts. The cyclin-dependent kinase, CDK11 is essential for stabilizing these nucleated microtubules, although substrates of CDK11 in this process have not yet been identified (Yokoyama et al., 2008).

Figure 5. Chromatin-driven spindle assembly. Cartoon diagram of chromatin-driven spindle assembly. The RanGEF, RCC1, localizes to chromatin (black) and generates a gradient of RanGTP (blue). Importin β sequesters spindle assembly factors (SAF) in the cytoplasm until it comes in contact with the RanGTP gradient. Importin β undergoes a conformational change when bound by RanGTP, thus dropping the SAFs in the vicinity of the chromatin. Multiple SAFs are responsible for microtubule (red) nucleation, stabilization and organization into a spindle structure (adapted from (Nachury et al., 2001)).

Figure 5

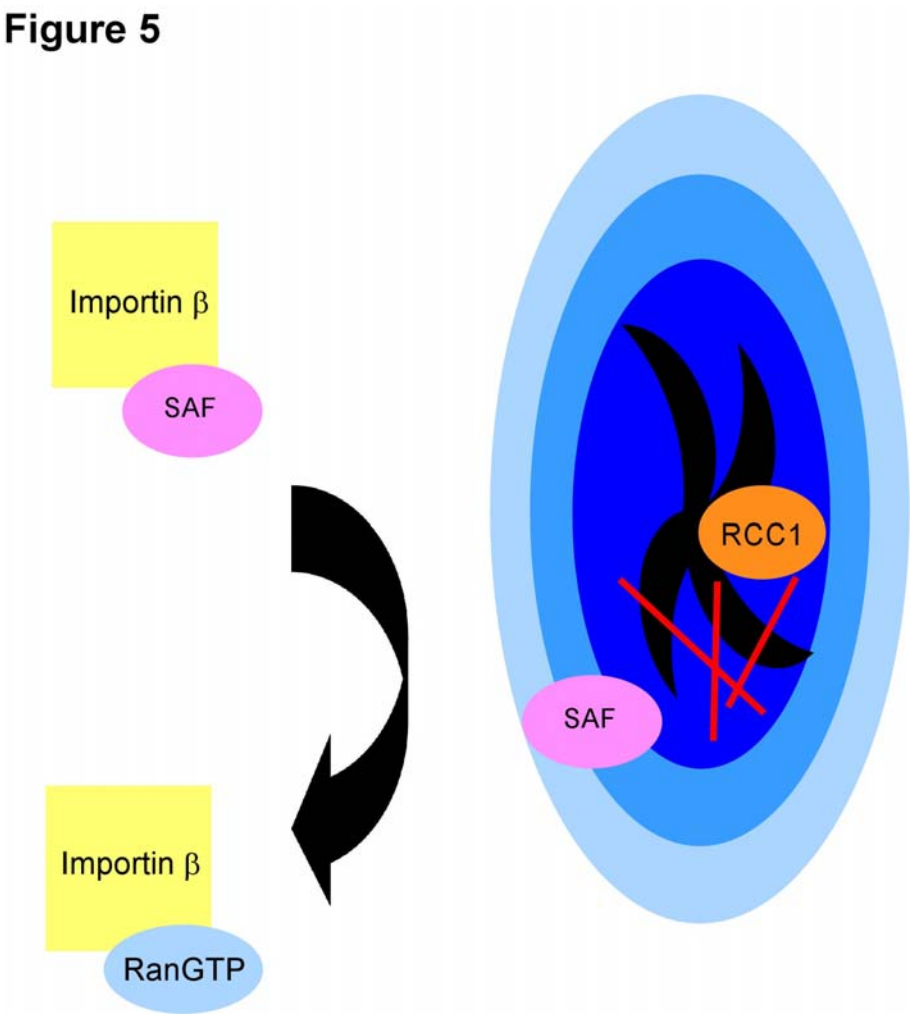


Table 2. Spindle Assembly Factors

Spindle Factor	Protein Class	Function/Activity
TPX2	MAP, spindle pole	MT nucleation, Aurora A localization, spindle pole formation
Maskin	MAP, spindle pole	recruits MT stabilizer XMAP 215 to pole, anchors MT at centrosome
NuMA	MAP, spindle pole	spindle pole focusing
NuSAP	MAP, spindle midsection	MT bundling, MT-chromatin attachment
Xnf7	MAP	MT bundling, APC/C inhibitor
HURP	MAP	MT bundling, chromosome alignment
Lamin B	intermediate filament	Spindle matrix formation
Rae1	mRNA export factor nucleoporin	MT polymerization
Mast/Orbit/ CLASP	MAP, MT plus end	MT polymerization
Eg5	Kinesin-5	spindle bipolarity, MT flux
XCTK2	Kinesin 14, spindle pole	spindle pole focusing, spindle bipolarity
MCAK	Kinesin 13, centromere, spindle pole	error correction of MT-kinetochore attachment, chromosome segregation
XMAP215	MAP	MT stabilizer

Once microtubules are nucleated, numerous MAPs, molecular motors and other components are recruited to organize the microtubules into the spindle structure. Following initiation of microtubule polymerization, TPX2 localizes to microtubule minus ends, where it serves to correctly position the mitotic kinase Aurora A at the forming spindle pole. Aurora A, in turn, phosphorylates numerous substrates involved in spindle assembly, including Maskin, HURP, BRCA1, and the kinesin Eg5 (Brunet et al., 2004; Groen et al., 2004; Gruss et al., 2001; Kufer et al., 2002; Tsai et al., 2003; Wittmann et al., 2000). Maskin is a spindle pole MAP, and a member of the transforming acidic coiled coil (TACC) family of proteins (O'Brien et al., 2005). Phosphorylated maskin recruits the microtubule stabilizer XMAP215 to spindle poles (Kinoshita et al., 2005). Recently, Maskin has also been implicated in anchoring microtubule polymers to centrosomes (Albee et al., 2006; Albee and Wiese, 2008). HURP is a MAP required for correct chromosome alignment, and phosphorylation of HURP by Aurora A promotes its microtubule binding activity (Koffa et al., 2006; Sillje and Nigg, 2006; Wong and Fang, 2006; Wong et al., 2008). The kinesin Eg5 is responsible for poleward flux of tubulin subunits within the spindle and its activity is required for bipolarity of the spindle; in the absence of Eg5 activity, spindles are monopolar (Giet et al., 1999; Mayer et al., 1999; Miyamoto et al., 2004; Sawin et al., 1992; Shirasu-Hiza et al., 2004). Monastral, a small molecule that inhibits Eg5, reduces Eg5 affinity for microtubules and promotes reversal of ATP hydrolysis (Cochran et al., 2005; Maliga et al., 2002; Mayer et al., 1999). Finally, Aurora A phosphorylation of the tumor suppressor BRACA1, an E3 ubiquitin ligase, inhibits its ubiquitinating

activity, which promotes spindle pole formation by releasing BRACA1 inhibition of centrosome-dependent microtubule nucleation (Sankaran et al., 2007). However, some level of ubiquitinating activity of the BRACA1/BARD1 complex is required to correctly localize TPX2 to spindle poles (Joukov et al., 2006).

Other Importin β-regulated factors involved in spindle pole formation include the MAP NuMA and the kinesin XCTK2. NuMA is responsible for spindle pole focusing, and it is trafficked to the poles through an interaction with the molecular motor dynein/dynactin complex (Fant et al., 2004; Merdes et al., 1996; Nachury et al., 2001; Wiese et al., 2001). XCTK2 is a kinesin motor that promotes formation and stability of spindle poles (Ems-McClung et al., 2004; Walczak et al., 1997; Walczak et al., 1998). Importin β regulated spindle assembly factors are not confined to function at spindle poles; NuSAP is located in the midsection of the spindle, where it has roles in both bundling of microtubules and in attachments between microtubules and chromatin (Ribbeck and Mitchison, 2006; Ribbeck et al., 2007). Xnf7 functions as a microtubule crosslinker and also contributes to inhibition of the anaphase-promoting complex/cyclosome (APC/C) to prevent premature transition to anaphase (Casaletto et al., 2005; Maresca et al., 2005).

Finally, there are some spindle assembly factors that have more nebulous roles in the spindle assembly process. One such factor is Lamin B, which is proposed to tether spindle assembly factors and form a scaffold for spindle assembly termed the “spindle matrix”. While Eg5, XMAP215 and dynein have been described to associate with this matrix, the existence of this scaffold is

controversial and still heavily debated in the spindle field (Ma et al., 2009; Tsai et al., 2006). Rae1, a nuclear pore complex protein, has also been identified as a component of an interesting RNA-containing complex that is required for correct spindle assembly, and will be discussed in more detail below.

Factors involved in microtubule-kinetochore interactions

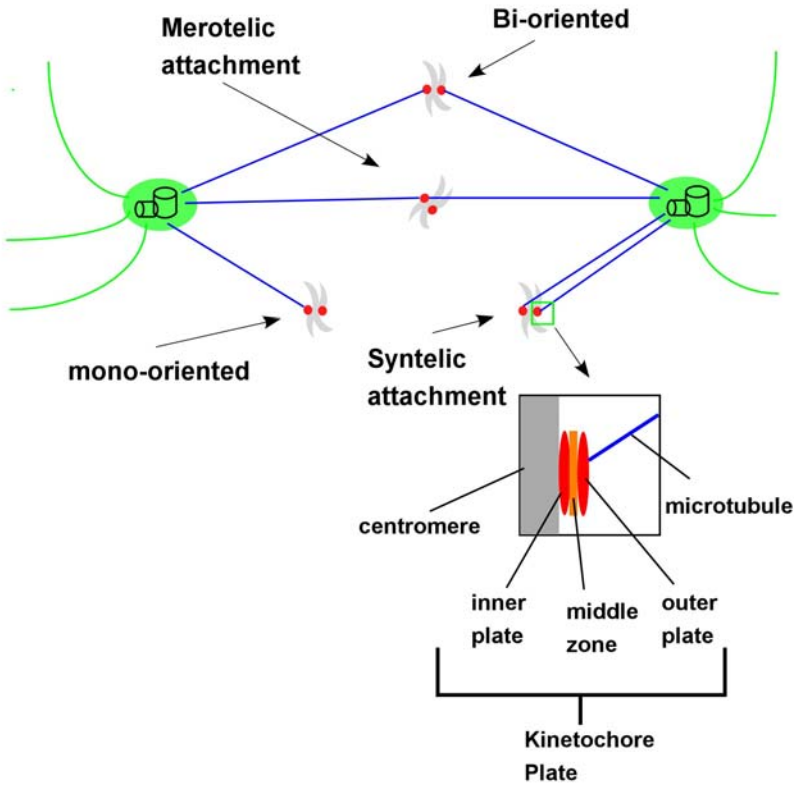
One of the most critical steps in spindle assembly is generation of correct attachments between microtubules and kinetochores. Establishment of proper attachments is essential for correct chromosome alignment, checkpoint signaling, and ultimate segregation. The highly conserved NDC80 kinetochore complex, which consists of NDC80 itself (also referred to as Hec1), Nuf2, Spc24 and Spc25, is responsible for the direct interaction with microtubules (McCleland et al., 2003; Miller et al., 2008). The NDC80 complex is found in the outer kinetochore region, and knockdown of this complex reduces the extent of microtubule binding to the kinetochore, while leaving kinetochore structure intact (DeLuca et al., 2005; DeLuca et al., 2002; McCleland et al., 2003; McCleland et al., 2004). Biochemical studies in the nematode *C. elegans* established that the NDC80 complex binds microtubules directly *in vitro* and that additional components, including Mis12 and KNL-1, increased the strength of this interaction (Cheeseman et al., 2006; Cheeseman et al., 2008; Cheeseman et al., 2004). Members of the +TIPs family of plus end binding proteins also function in microtubule-kinetochore interactions. MAST/Orbit (also referred to as CLASP) for example, associates with the plus ends, drives tubulin polymerization, and is

required for end-on binding of microtubules to kinetochores (Maiato et al., 2003; Maiato et al., 2005; Maiato et al., 2002). Studies in yeast identified the Dam1 complex, which forms a ring-like structure around the microtubule polymer and is essential for stabilizing kinetochore-microtubule attachments (Jones et al., 1999; Westermann et al., 2005). The Dam1 complex has lacked a definitive ortholog in vertebrates. Recently, the Ska I complex was proposed as a potential vertebrate ortholog of the Dam I complex (Welburn et al., 2009). Alternatively, another recent study suggests that the NDC80 complex itself may fulfill the function of the Dam1 complex in vertebrates (Powers et al., 2009).

Sometimes, connections between microtubules and the kinetochore are made incorrectly (Figure 6). Kinetochores from sister chromatids can accidentally be attached to only one pole (syntelic attachment) or single kinetochores may misadvertantly attach to microtubules from both poles (merotelic attachment) (Peters, 1985; Salmon et al., 2005). The mitotic kinase Aurora B is responsible for adjusting these faulty connections (Ditchfield et al., 2003; Hauf et al., 2003; Kallio et al., 2002; Murata-Hori and Wang, 2002). Aurora B is a member of a protein complex called the chromosomal passenger complex (CPC), which consists of the kinase Aurora B, the scaffold protein INCENP, and Survivin and Borealin/Dasra (Gassmann et al., 2004; Sampath et al., 2004). The CPC is targeted to chromatin via interaction of Survivin with the kinetochore localized export factor Crm1 (Knauer et al., 2006). Two Aurora B substrates are essential for correcting misattachments of microtubules to the kinetochore: NDC80 and the mitotic centromere associated kinesin (MCAK). Phosphorylation of MCAK by

Figure 6. Models of kinetochore-microtubule attachments. Cartoon diagram of different microtubule-kinetochore attachments. When microtubules are correctly attached to the sister kinetochores, and the chromosomes are aligned on the metaphase plate, the chromosomes are referred to as bi-oriented. Prior to bi-orientation, when only one sister kinetochore is attached to microtubules, the chromosome is referred to as mono-oriented. Incorrect attachements can arise, when either microtubules from both poles are attached to one kinetochore (merotelic attachment) or microtubules from one pole are attached to both sister kinetochores (syntelic attachment). The inset shows a magnified diagram of the centromere/kinetochore region of the chromosome (adapted from (Moore and Wordeman, 2004)).

Figure 6


Merotelic
attachment
Bi-oriented
mono-oriented
Syntelic
attachment
centromere
microtubule
inner
plate
middle
zone
outer
plate
Kinetochore
Plate

Aurora B inhibits its depolymerizing activity and regulates its localization at the inner centromeric region (Andrews et al., 2004; Lan et al., 2004). Without Aurora B phosphorylation, MCAK is unable to localize to the centromere, although pockets of both phosphorylated and unphosphorylated MCAK were found at the centromere, which suggests high turnover (Lan et al., 2004) Furthermore, a study using phosphomutant forms of MCAK showed the S→E phosphomimic localized to the inner centromere, whereas the S→A mutant associated with the kinetochore (Andrews et al., 2004). NDC80, which is the protein responsible for actually making the connection between the microtubule and the kinetochore complex is also regulated by Aurora B (Cheeseman et al., 2006; DeLuca et al., 2005; Pinsky et al., 2006). Addition of a mutant form of Ndc80 unable to be phosphorylated by Aurora B resulted in erroneous microtubule-kinetochore attachments.

Nucleoporins in Mitosis

Just as the RanGTPase system and soluble transport factors such as Importin β have functions during mitosis, nucleoporins have also been identified as critical players in spindle assembly. To date, the multimeric Nup107 complex, its binding partner ELYS, Nup358/RanBP2, Rae1, Nup153 and TPR are all nucleoporins with described roles in mitosis. However, nucleoporin function in mitosis is a relatively new branch of nuclear pore biology, and thus, we are far from a full understanding of these alternative roles of nucleoporins.

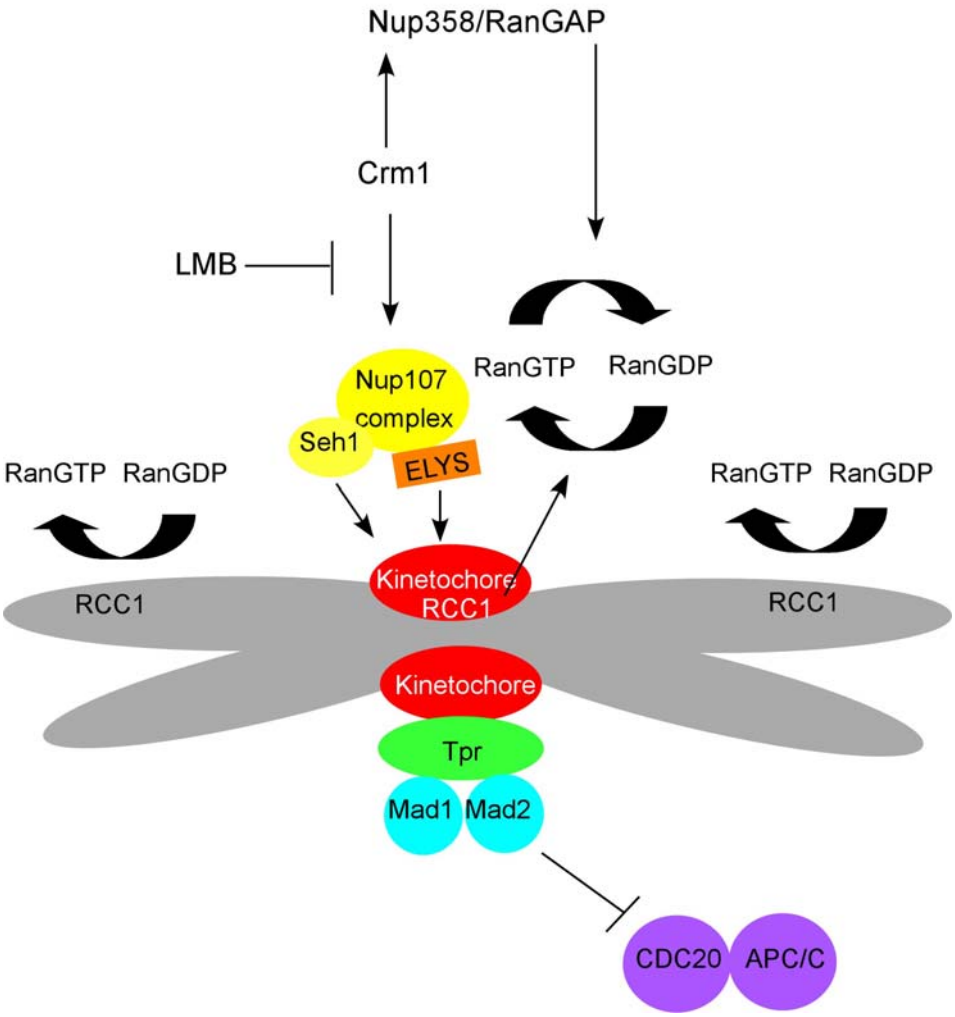
Nup107 complex and ELYS

The Nup107 complex is comprised of Nup160, Nup133, Nup107, Nup96, Nup85, Nup43, Nup37, Seh1, and Sec13. This large NPC subcomplex, which is the main component of the structural nuclear and cytoplasmic rings of the pore, remains together during mitosis. Although the majority is dispersed throughout the cell during mitosis, a subfraction (10-15%) of the complex localizes to the kinetochore (Figure 7) (Belgareh et al., 2001; Loiodice et al., 2004). The Nup107 complex is also found transiently on spindle poles during prometaphase in mammalian cells, and in Xenopus egg extract, the Nup107 complex is seen throughout the entire spindle structure (Orjalo et al., 2006). Interestingly, the Nup107 complex is found at higher levels on unattached kinetochores, where it forms a crescent-like structure akin to other outer kinetochore components such as Mad 2, dynein and CenpE (Hoffman et al., 2001; Maiato et al., 2004; Orjalo et al., 2006; Zuccolo et al., 2007).

Functional studies of the Nup107 complex in mitotic *Xenopus* egg extracts indicate an important role in spindle assembly. Spindles assembled in Nup107-immunodepleted egg extract are unstable (Orjalo et al., 2006). Time course experiments establish that while early stages of spindle assembly such as nucleation from centrosomes occur normally, later stages in the process fail. Bipolar spindles formed in immunodepleted extract, but proved unstable and consequently disassembled, leaving beind chromatin structures without associated microtubules. The phenotype of spindles formed in the presence of

Figure 7. Nucleoporins at the kinetochore during mitosis. Cartoon diagram of multiple nucleoporins found at the kinetochore during mitosis. The Nup107 complex is targeted to the kinetochore by Seh1 and ELYS. The Nup358/RanGAP complex localizes to the kinetochore via the transport receptor CRM, although this localization requires the kinetochore subfraction of Nup107 complex and is sensitive to treatment with Leptomycin B (LMB). The proximity of the RanGAP and the RanGEF at the kinetochore results in a highly localized functional Ran cycle. TPR is responsible for targeting the mitotic checkpoint proteins Mad1 and Mad2 to the kinetochore.

Figure 7


Nup358/RanGAP
Crm1
LMB
RanGTP
RanGDP
Nup107
complex
Seh1
ELYS
RanGTP
RanGDP
RanGTP
RanGDP
Kinetochore
RCC1
RCC1
RCC1
Kinetochore
Tpr
Mad1
Mad2
CDC20
APC/C

inhibitory antibodies directed towards the Nup107 complex was much less severe, consisting of bipolar spindles with reduced microtubule density.

The NDC80 complex, which connects microtubules to the kinetochore, is largely responsible for recruitment of the Nup107 complex (Zuccolo et al., 2007). siRNA knockdown of the NDC80 complex led to a massive reduction in the Nup107 complex at the kinetochore, although no direct molecular interactions between the Nup107 complex and the NDC80 complex have yet been identified. The Nup107 complex member Seh1 is critical for kinetochore localization of the complex, as siRNA knockdown of Seh1 alone can abolish the entire Nup107 kinetochore subfraction.

Investigations in somatic cells indicate that the kinetochore fraction of the Nup107 complex is required for correct microtubule-kinetochore attachment. Phenotypes that result from loss of the Nup107 complex at the kinetochore through depletion of Seh1 include a checkpoint-dependent delay of mitotic progression from ~45 minutes to ~90 minutes. In the absence of the Nup107 complex, there were no microtubules attached to kinetochores of unaligned chromosomes, and there were far fewer kinetochore microtubules attached to aligned chromosomes. Furthermore, the tension between sister kinetochores of aligned chromosomes was reduced and the distance between sister-kinetochores dropped by half from ~1.68 μm to ~0.84 μm. These data firmly establish that the Nup107 complex plays a role in correct microtubule-kinetochore attachment, although the precise mechanism remains uncertain.

Several years ago, a novel binding partner of the Nup107 complex was identified. Embryonic Large Molecule derived from Yolk Sac (ELYS) is a conserved AT-hook DNA binding protein that was previously identified as a putative transcription factor. ELYS associates with the Nup107 complex at the nuclear pore complex both during interphase and at the kinetochore during mitosis (Franz et al., 2007; Rasala et al., 2006). Knockdown of ELYS in mammalian cells resulted in a decrease in kinetochore-localized Nup107 complex, suggesting that ELYS supplements the major contribution of Seh1 in targeting the Nup107 complex to the kinetochore. Additionally, knockdown of ELYS resulted in a delay in or failure to complete cytokinesis at the end of mitosis, a function of ELYS that appears to be independent of the Nup107 complex.

Nup358/RanBP2

Nup358/RanBP2, a major constituent of the cytoplasmic filaments of the pore, is found at both spindle poles and kinetochores during mitosis, along with its binding partner RanGAP (Figure 7) (Joseph et al., 2004; Joseph et al., 2002; Salina et al., 2003). Interactions between Nup358 and RanGAP during both interphase and mitosis requires modification of RanGAP by the Small Ubiquitin-like MOdifier (SUMO) The Importin β superfamily member Crm1, which complexes with cargo in the presence of RanGTP, is involved in recruiting Nup358/RanGAP to the kinetochore. Treatment of mitotic mammalian cells with leptomycin B, a potent inhibitor of Crm-NES binding, results in a loss of the

Nup358 complex from kinetochores (Arnaoutov and Dasso, 2005). Furthermore, kinetochore-associated Nup107 complex is also required for Nup358/RanGAP kinetochore localization, as siRNA knockdown of the Nup107 complex resulted in a loss of both Crm1 and Nup358/RanGAP at the kinetochore (Zuccolo et al., 2007).

Similar to the Nup107 complex, the Nup358/RanGAP complex largely contributes to the formation of stable microtubule-kinetochore interactions. Nup358/RanGAP associates with the kinetochore shortly after stable microtubule attachment (Joseph et al., 2004; Joseph et al., 2002). siRNA knockdown of Nup358 in cells results in a striking relocalization of the checkpoint proteins Mad1, Mad2, CENP-E, CENP-F, ZW10 and dynein off of the kinetochore in prometaphase cells (Joseph et al., 2004; Salina et al., 2003). In contrast to Nup358/RanGAP, these proteins typically associate with the kinetochore before microtubule attachments are made. In the absence of Nup358/RanGAP, electron microscopy confirms the formation of aberrant kinetochore structures that are unable to capture microtubules (Salina et al., 2003). Thus, without Nup358/RanGAP, the kinetochore structure is unable to fully mature and capture microtubules. This in turn leads to the inability of chromosomes to congress to the metaphase plate, and therefore faulty chromatid segregation and aneuploidy (Askjaer et al., 2002; Salina et al., 2003). In contrast to RCC1, which is found along the length of the chromatin, the localization of Nup358/RanGAP to the kinetochore completes a highly-localized, RanGTPase cycle at the kinetochore.

This RanGTP cycling most likely contributes to molecular function at the kinetochore, but the mechanism is unknown.

Nup358 appears to have an additional, even less well-defined mitotic role at the spindle pole. Nup358 knockdown in mammalian cells results in an increase in multi-polar spindles (Joseph et al., 2004). Immunofluorescent staining of centrin revealed disruption of centriole distribution; some cells contained extra microtubule organizing centers (MTOC) lacking centrioles completely, some MTOCs contained single, unpaired centrioles, and other cells had more than two pairs of centrioles, some of which were not associated with obvious MTOCs. This alternate mitotic function of Nup358 remains poorly understood.

Rae1

Rae1 has been implicated in multiple mitotic roles, one of which was shown to require Nup98. Much, but not all of Rae1/Gle2 is found in complex with its binding partner Nup98, and this complex appears to persist during mitosis (Blevins et al., 2003; Macaulay et al., 1995; Matsuoka et al., 1999; Pritchard et al., 1999). Although knockout of Rae1 is lethal, cells from Rae1 heterozygous mice were defective in a mitotic checkpoint, and karyotyping revealed an elevated level of aneuploidy (9%). Strikingly, although cells from Nup98 +/- mice were not aneuploid, in Nup98 +/- Rae1 +/- double heterozygotes aneuploidy was elevated to 32%. There was evidence of premature separation of sister chromatids in cells from these mice. Coimmunoprecipitation experiments indicated an interaction between the Nup98-Rae1 complex and the cdh1 form of

the Anaphase Promoting Complex/Cyclosome (APC/C). The APC/C is an E3 ubiquitin ligase that is critical for degradation of specific targets at the metaphase to anaphase transition. One of these targets is Securin, an inhibitor of sister chromatid separation that is typically a substrate of the CDC20 form of the APC/C. Nonetheless, a decreased level of Securin was observed in the Nup98-Rae1 double heterozygote cells, in keeping with the premature separation of sister chromatids observed in these mice (Jeganathan et al., 2006; Jeganathan et al., 2005). Thus, the Nup98-Rae1 complex is a regulator of Securin degradation and the metaphase to anaphase transition.

A novel, Rae1-containing complex required for spindle assembly was identified in Xenopus egg extract (Blower et al., 2005). This complex binds directly to Importin β during mitosis and is released in the vicinity of chromatin following interaction with RanGTP. When Rae1 was immunodepleted from *Xenopus* egg extracts, both Ran aster formation and spindle assembly were inhibited, and only sparse, highly bundled microtubules were formed. Furthermore, siRNA knockdown of Rae1 in somatic cells, led to inhibition of chromosome congression and an increase in multipolar spindles. Importantly, the Rae1 complex proved to contain multiple RNA-binding proteins such as Maskin, Fubp2, Rap55, VERA, and CBTF, as well as a wide range of mRNAs. mRNAs encoding proteins that function in mitosis, DNA metabolism, and early development were found to be enriched on the spindle (Blower et al., 2007). Thus, the Rae1 complex may serve as a delivery system to ensure that essential

mRNAs are partitioned between daughter cells. The presence of Nup98 in this complex was not directly demonstrated and remains uncertain.

Rae1 was also identified as a binding partner of NuMA, a MAP responsible for organization of spindle poles through crosslinking of microtubules (Wong et al., 2006). Rae1 and NuMA form a transient complex during mitosis, and colocalize at spindle poles from prophase to anaphase. The multipolar spindle phenotype that arises from siRNA knockdown of Rae1 in cells is rescued by co-siRNA depletion of NuMA (Blower et al., 2005; Wong et al., 2006). Conversely, overexpression of NuMA also results in multipolar spindles, which can be rescued by overexpression of Rae1 (Quintyne et al., 2005; Wong et al., 2006).

Nup153 and TPR

During interphase, the spindle checkpoint proteins Mad1 and Mad2 are sequestered at the nuclear pore complex (Campbell et al., 2001; Iouk et al., 2002; Scott et al., 2005). TPR is the main constituent of the nuclear basket structure of the pore, and it was recently shown that Mad1 and Mad2 bind TPR directly (Figure 7) (Lee et al., 2008). Depletion of TPR using siRNA resulted in loss of Mad1/Mad2 nuclear pore localization during interphase. During mitosis, the Mad1/Mad2 complex relocalizes to the kinetochore (Li and Benezra, 1996; Luo et al., 2002). Interestingly, the kinetochore localization of Mad1 is also dependent upon TPR (Lee et al., 2008). When TPR is knocked down, ~45% of cells proceeded to anaphase with misaligned chromosomes, suggesting that the

spindle assembly checkpoint had been disarmed. Thus, TPR has an important role in maintaining a functional spindle assembly checkpoint.

Nup153, another nucleoporin that localizes to the nuclear face of the pore, has at least two distinct mitotic functions in both early and late mitosis (Mackay et al., 2009). Partial Nup153 knockdown by siRNA increased the number of unresolved midbodies, suggesting a cytokinesis defect. More complete knockdown of Nup153 protein resulted in many multi-lobed nuclei. While the excess midbody phenotype was rescued by expression of the C-terminal FG-domain of Nup153, the multi-lobed nuclei phenotype was rescued by the zinc-finger domain of Nup153. Thus, Nup153 appears to have different mitotic functions controlled by distinct domains within the protein. However, the targets of these domains are unknown.

Nup98

Nup98 domains and post-translational modifications

The 920 amino acid Nup98 protein can be divided into distinct domains: the N-terminal half of the protein contains GLFG (Glycine-Leucine-Phenylalanine-Glycine), as well as FG and two FXFG (where X is any amino acid) nucleoporin repeat motifs (Figure 8 A). The repeat domain is bisected by a small alpha-helical binding site for Rae1/Gle2 (aa 181-224) (Pritchard et al., 1999). The very C-terminus of Nup98 (aa 711-870) forms a unique structural fold which is required both for autoproteolyis following amino acid 863, and for targeting to the NPC via interaction with the Nup107 subcomplex (Hodel et al.,

2002; Vasu et al., 2001). The region between the repeat and autoproteolyic domains assumes a natively disordered structure (unpublished data, Cross M.K., Powers M.A., Schwartz T.) and as this dissertation will establish, this region plays a functional role in spindle assembly.

Nup98 was first characterized in Xenopus egg extract, where it was identified as a GLFG repeat-containing nucleoporin (Powers et al., 1995a). Characterization and cloning of rat Nup98 followed closely and confirmed the presence of GLFG repeats, as well as FG and FXFG repeats (Radu et al., 1995b). Nup98 is the only metazoan nucleoporin to contain a substantial number of GLFG motifs, and is homologous to a family of *S. cerevisae* GLFG repeat nucleoporins: Nup100, Nup116 and Nup145 (Wente et al., 1992). However, *S. cerevisae* may be an evolutionary aberration since, like human, all other eukaryotic species sequenced contain only one Nup98 homolog that combines the functions of all the *S. cerevisae* GLFG repeat nucleoporins.

Nup98 is post-translationally modified by O-linked N-acetylglucosamine at two or more sites (Finlay et al., 1987; Holt et al., 1987). Additionally, Nup98 is phosphorylated during interphase and hyperphosphorylated during mitosis (Macaulay et al., 1995). One facet of this dissertation is investigation of mitosis-specific phosphorylation sites within the C-terminal domain of Nup98.

Nup98 function at the pore

Nuclei formed in Nup98-depleted Xenopus egg extract provided the first indication that this nucleoporin had an important function within the nuclear pore

complex. Depleted nuclei were competent for import of cNLS-containing proteins, which are recognized by the Importin α/Importin β heterodimer. However, the nuclei were much reduced in size compared to control nuclei, and were unable to replicate their chromosomal DNA (Powers et al., 1995b). Although Nup98 was not essential for the import of cNLS-containing proteins, subsequent *in vitro* studies demonstrated that multiple human karyopherins, including importin β and transportin, bound *in vitro* to the repeat region of Nup98, suggesting a possible contribution to protein import pathways (Bonifaci et al., 1997; Fontoura et al., 2000; Radu et al., 1995a; Radu et al., 1995b; Yaseen and Blobel, 1997). Strong evidence implicated Nup98 in RNA export pathways as well; Nup98 antibodies injected into Xenopus oocyte nuclei blocked export of multiple classes of RNAs, including snRNA, 5S RNA, large ribosomal RNA, and mRNA, while having no effect on export of tRNA, or on the import of protein and snRNPs (Powers et al., 1997). Further evidence, from both *in vitro* and *in vivo* experiments, confirmed that Nup98 functions in RNA export mediated by TAP which Nup98 binds through its GLFG repeats (Bachi et al., 2000; Blevins et al., 2003; Levesque et al., 2006). The Nup98 binding partner Rae1/Gle2 also binds directly to TAP, associates with the NPC along with exporting RNA and may serve to deliver TAP and cargo to Nup98 (Blevins et al., 2003; Sabri and Visa, 2000).

Subcellular localization

Extensive research has been done to investigate the subcellular localization of Nup98 via immunofluorescence and immunoelectron microscopy.

Nup98 is a component of the nuclear pore complex, and thus localizes to the nuclear envelope in cells (Figure 8B) (Powers et al., 1995b). Nup98 was originally proposed to assemble specifically into the nuclear basket of the NPC (Frosst et al., 2002; Radu et al., 1995b) but was subsequently shown by immunofluorescence and confirmed by immunoelectron microscopy to be present on both nuclear and cytoplasmic faces of the pore (Griffis et al., 2003). Post-embedding immunoelectron microscopy analysis suggests that Nup98 may have a slight bias towards the nuclear side (Krull et al., 2004). The C-terminal domain of Nup98, and to a lesser extent the GLFG domain, both aid in targeting Nup98 to the pore complex (Griffis et al., 2003). Nup98 is also found in the nucleoplasm and in nucleoplasmic bodies. The GLFG domain of Nup98 is required for targeting to these bodies, which were consequently termed GLFG bodies. It remains unknown whether additional proteins are associated with these structures. Nup98 moves dynamically between the GLFG bodies, the nucleoplasm, and the nuclear pore complex and shuttles between the nucleus and cytoplasm as shown by FRAP analysis. Within the nucleus, this mobility requires, in part, ongoing transcription. Treatment of cells with transcription inhibitors such as Actinomycin D and DRB, which target RNA pol I and II, halt Nup98 shuttling. During cellular division, when the nuclear pore complex is disassembled, the majority of Nup98 is found diffusely throughout the cell.

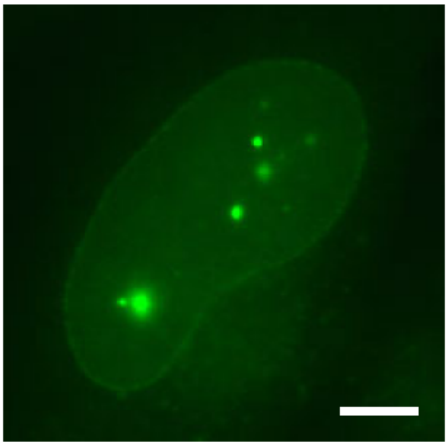
Figure 8. Domains and cellular localization of Nup98. A) Cartoon diagram of the domains of Nup98. **B)** Fluorescent microscopy images of the subcellular localization of Nup98. HeLa cells were transfected with GFP-Nup98, and demonstrates Nup98 localization to the nuclear pore complex and intranuclear GLFG bodies. Error bar is 10 μm.

Figure 8.

A.


FG repeats
FG & GLFG repeats
Autocatalytic Cleavage
Transportin
Rae1
Tap/NXF1
Karyopherins
NPC

B.



Nup98 and viral infection

The cellular importance of Nup98 function is underscored by the fact that Nup98 is a target during many different types of viral infection. One strategy of numerous viruses is to inhibit host cell activities, such as mRNA export, while promoting the corresponding viral function, such as translation of viral RNAs. Vesicular Stomatitis virus (VSV) accomplishes this in part through the viral Matrix (M) protein which binds to and inhibits a component of the mRNA export pathway. M protein was originally thought to bind Nup98, but was subsequently shown to interact directly with the Nup98 partner protein Rae1/Gle2, thus targeting Nup98 indirectly (Enninga et al., 2002; Faria et al., 2005; von Kobbe et al., 2000). Influenza and Polio viruses both interfere with mRNA export and specifically target Nup98 for degradation soon after infection, while apparently leaving other nucleoporins untouched. Poliovirus is less discriminating when choosing nuclear pore proteins for degradation, targeting Nup153 and Nup62 in addition to Nup98. However, Nup98 is a very early Poliovirus target, and unlike other nucleoporins, Nup98 degradation does not require viral replication (Park et al., 2008). Thus, Nup98 may be targeted by a protease delivered with the infecting viral particle.

Interestingly, cellular immune responses can combat viral interference with cellular mRNA export by increasing levels of Nup98 and Rae1/Gle2 proteins. Treatment of human monocytic cells (U937) with interferon-gamma (IFN-γ), led to increased Nup98 mRNA and protein and reversal of the M protein-mediated block to mRNA export (Enninga et al., 2002). The promoters of both the NUP98

and RAE1 genes contain IFN-γ response elements. Presumably, increased IFN-γ produced in response to viral infection leads to upregulation of both these proteins to counteract viral pathogenesis.

Nup98 knockout mouse

When Nup98 heterozygous mice were intercrossed, no homozygous progeny survived. Nup98-/- cells taken from day 8.5 embryos, although greatly impaired, could be propagated *in vitro* which suggests that Nup98 is not absolutely required for cell viability (Wu et al., 2001). Similarly, we observed that even when the majority of Nup98 is removed by siRNA, cells are surprisingly able to continue growth in culture (unpublished data, Hilbert B., Pierce A.K., Powers M.A.). In Nup98 knockout cells, several nucleoporins from the cytoplasmic face of the pore, including Nup214, Nup358, Nup88, and Nup62, were released into the cytoplasm (Wu et al., 2001). Thus, Nup98 may also play a role in targeting and maintaining other nucleoporins at the pore. The mechanism underlying this is unclear as in temporal studies Nup98 is one of the first nucleoporins to leave the NPC during mitotic disassembly and one of the last to return when the NPC reassembles in telophase (Dultz et al., 2008).

Nup98 in Leukemia

A substantial and growing area of research has focused on the function of Nup98 in leukemia. This link between Nup98 and leukemia first arose when the NUP98 gene was reported as part of a rare but recurring chromosomal

translocation in leukemia patients (Borrow et al., 1996; Nakamura et al., 1996). Nup98 chromosomal translocations lead to expression of oncogenic fusion proteins comprised of the N-terminal half of Nup98, typically aa 1-469, joined to a variety of C-terminal partners (reviewed in (Moore et al., 2007)). These Nup98 translocations result most often in acute myelogenous leukemia, but have more rarely been identified in chronic myelogenous leukemia and T-cell acute lymphoblastic leukemia. Thus far, Nup98 has been found fused to the homeodomains for 11 different transcription factors, and 15 different non-homeodomain proteins. Many, but not all, of the latter group have DNA binding or chromatin modifying activites. In the context of several fusion proteins, the GLFG domain of Nup98 has been shown to recruit the CBP/p300 histone acetyltransferase or HDAC1 histone deacetylase (Bai et al., 2006; Kasper et al., 1999). Thus, the current model suggests that the Nup98 fusions become rogue transcription factors that can aberrantly activate or repress target genes. However, other fusion partners have unidentified functions, which leaves the potential for additional leukemogenic mechanisms.

Xenopus egg extract

The data contained in this dissertation was largely collected through studies using *Xenopus* egg extract. The egg extract is a powerful system that can be utilized to study cell cycle events *in vitro* (Desai et al., 1999a; Powers et al., 2001). *Xenopus laevis* frogs are injected sequentially first with pregnant mare serum gonadotropin and two or more days later with human chorionic

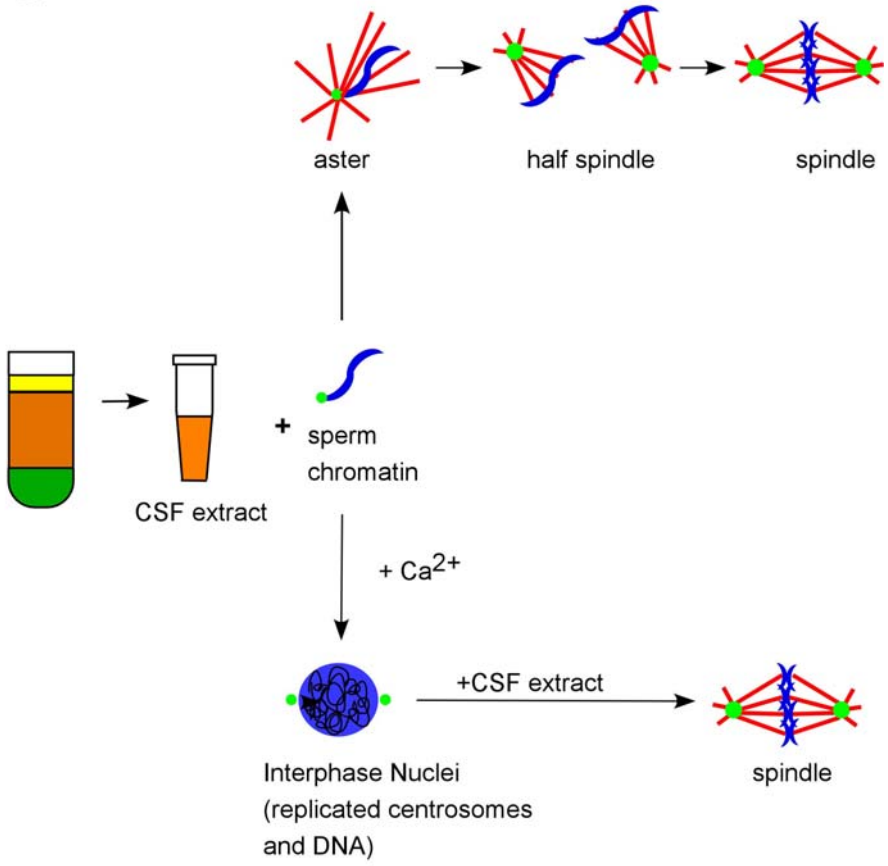
gonadotropin. This hormone treatment induces the frogs to ovulate and lay eggs. When the eggs are laid by the frog, they are arrested in metaphase of meiosis II through the action of a multiprotein complex termed Cytostatic Factor (CSF) which stabilizes the cyclinB/cdk1 complex (Tunquist and Maller, 2003). CSF is subject to calcium-stimulated inactivation; however, when lysed in the presence of EGTA, eggs produce an extract that is biochemically arrested in mitosis (CSF extract).

If *Xenopus* sperm chromatin is added to CSF extract, a bipolar spindle will form around the chromatin (Figure 9) (Heald et al., 1996; Karsenti et al., 1984b). The centrosome associated with the sperm chromatin forms one pole of the spindle, and the other pole of the spindle is formed through the chromatin-driven spindle assembly pathway discussed previously. Addition of calcium disrupts the CSF activity and results in degredation of cyclins responsible for maintaining mitotic activity of the extract. This treatment thus shifts extracts into interphase, where a nucleus forms around chromain and both the DNA and centrosomes are replicated. If an equal volume of CSF extract is added, the extract shifts back into mitosis, and now forms a bipolar spindle with centrosomes at either pole, otherwise referred to as a cycled spindle.

The extract can easily be manipulated in order to study the function of a particular protein in spindle assembly (Desai et al., 1999a). Common experimental manipulations include immunodepletion, antibody addition, and protein fragment additions. Importantly, extracts are powerful tools for

Figure 9. Spindle assembly assays in Xenopus egg extract. CSF extract is made from a lysate of Xenopus eggs. When sperm chromatin (blue) is added directly to CSF extract, microtubules are nucleated from the centrosome (green) and forms an aster. These asters are classified as half spindles when the microtubules become focused towards the chromatin, and bipolar spindles form from these half spindles through as yet undefined mechanisms. If calcium is added to the extract with the sperm chromatin, the extract will shift into interphase. During interphase, the centrosomes and the chromatin are replicated, and if an equal volume of unshifted CSF extract is added to the interphase extract, it will shift back into mitosis and form a bipolar spindle (adapted from (Desai et al., 1999a)).

Figure 9


aster
half spindle
spindle
+
sperm
chromatin
CSF extract
+ Ca^{2+}
+CSF extract
Interphase Nuclei
(replicated centrosomes
and DNA)
spindle

investigation of proteins with multiple functions within the cell, because function in one discrete phase of the cell cycle can be studied in isolation.

Scope of the Dissertation

Less than a decade ago, the first localization of a nucleoporin to the mitotic apparatus was discovered (Belgareh et al., 2001). Subsequently, one important facet of research in the area of nucleoporin function in mitosis has been simply discovering which, if not all, nucleoporins have an alternate function at this stage of the cell cycle when the nuclear pore complex is disassembled. Indeed, the list of nucleoporins contributing to mitosis continues to grow, as Nup153 was identified as having mitotic functions only earlier this year (Mackay et al., 2009).

Even more important than identification of the nucleoporins with a mitotic role, is actually establishing the molecular mechanism behind the mitotic functions and fitting that into the complex, still-emerging model of spindle assembly. Mitotic nucleoporin function is still poorly understood. At most, it is clear that both the Nup107 complex and Nup358 have important roles at the kinetochore in stabilizing the microtubule-kinetochcore attachment (Joseph et al., 2004; Orjalo et al., 2006; Salina et al., 2003; Zuccolo et al., 2007). However, molecular and mechanistic details behind nucleoporin function in spindle assembly have been scarce.

The work presented here began with an initial hypothesis that the nucleoporin Nup98 might play a role during mitosis. I tested this through addition

of different fragments of purified Nup98 C-terminal domain to spindle assembly assays in Xenopus egg extract, which resulted in disrupted spindle formation. This result led to the hypothesis that the C-terminus of Nup98 contains an activity that promotes microtubule polymerization. Inhibition/depletion of Nup98 in the egg extract demonstrated that the C-terminal region of Nup98 does indeed contain a functional domain required for microtubule polymerization and correct bipolar spindle assembly.

Prior published studies had established Nup98 as a nucleoporin that is phosphorylated during mitosis (Macaulay et al., 1995). Initial studies I performed indicated that the relevant region within the C-terminus is phosphorylated in a mitosis-specific manner. From this data, I hypothesized that phosphorylation regulates the mitotic activity of Nup98. I tested this hypothesis by mapping and mutating mitotic phosphorylation sites within the minimal region of the C-terminus required for the mitotic activity. These phosphomutants revealed that the spindle assembly function of Nup98 is regulated, in part, by phosphorylation.

When the microtubule depolymerizing kinesin MCAK is depleted from egg extract, long microtubules result, similar to microtubules formed in the presence of Nup98 C-terminal domain. Conversly, when MCAK is overactive in the extract, the number of monopolar spindles increases; this is reminiscent of the increased formation of monopolar spindles when Nup98 activity is decreased. My data was in keeping with a model in which Nup98 functions by inhibiting MCAK activity in the egg extract to promote microtubule polymerization and bipolar spindle formation. To test this hypothesis, I investigated the possibility of an interaction

between Nup98 and MCAK. Pulldown experiments indicated that MCAK binds the C-terminus of Nup98 in the same region that is required for the mitotic function of Nup98.

Data summarized above is presented in Chapter Two, which establishes a functional domain within the C-terminal domain of Nup98 that interacts with MCAK to regulate bipolar spindle assembly. Chapter Three of this dissertation contains a brief overview of the application of fluorescent-speckle microscopy to microtubule dynamics, and results I obtained using this technique, which establish that Nup98 does not influence microtubule flux. Finally, in Chapter Four, I discuss how these findings have advanced the mitotic nucleoporin field and consider future directions for this project.

Chapter Two

Nup98 regulates bipolar spindle assembly through the depolymerizing kinesin, MCAK.

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Running Title: Nup98 regulates microtubule dynamics in the mitotic spindle.

ABSTRACT

During mitosis, the nuclear pore complex is disassembled and increasingly, solubilized nucleoporins are proving to have mitotic functions. We find that the nucleoporin Nup98 contributes to mitotic spindle assembly through regulation of microtubule dynamics. When added to Xenopus extract spindle assembly assays, the C-terminal domain of Nup98 stimulates uncontrolled growth of microtubules. Conversely, inhibition or depletion of Nup98 leads to formation of stable monopolar spindles. Spindle bipolarity is restored by addition of the purified Nup98 C-terminus. The minimal required region of Nup98 corresponds to a portion of the C-terminal domain lacking a previously characterized function. Multiple phosphorylations occur within this region and mutation of mitotic phosphorylation sites influences the excess microtubule phenotype. Lastly, we show interaction between this region of the C-terminus of Nup98 and the microtubule depolymerizing kinesin, MCAK. These data support a model in which Nup98 acts through MCAK to regulate the microtubule dynamics required for spindle bipolarity.

INTRODUCTION

The nuclear pore complex (NPC) is a large, multiprotein structure that functions as a gateway for regulated movement of macromolecules between the nucleus and cytoplasm (reviewed in (D'Angelo and Hetzer, 2008; Tran and Wente, 2006). The NPC is made up of roughly thirty different proteins termed nucleoporins or Nups, one third of which contain a domain with multiple interspersed copies of the peptide repeat phenylalanine-glycine (FG)(Devos et al., 2006; Schwartz, 2005). The FG-repeat domains of nucleoporins are thought to be relatively unstructured regions that associate with one another largely through hydrophobic interactions. Nuclear transport receptors such as Importin β and other karyopherin family members are capable of regulated interaction with FG domains in order to selectively import and export proteins and RNA (reviewed in (Terry et al., 2007). The meshwork of FG domains also provides a barrier to non-specific diffusion of macromolecules while allowing free passage of smaller proteins and molecules. Thus, NPCs play a critical role in the maintenance of correct cellular compartmentalization.

During mitosis, metazoan cells break down their nuclear envelopes and disassemble their nuclear pores into conserved nucleoporin subcomplexes (Rabut et al., 2004). With the exception of transmembrane nucleoporins, these complexes are soluble and are generally dispersed throughout the dividing cell. Until recently, such mitotic nucleoporin subcomplexes were thought to remain dormant until the nuclear envelope and NPC began to reform in telophase. Unexpectedly, it has developed that, in addition to the established mitotic role of

nuclear transport receptors, nucleoporins also make functional contributions to mitosis although in most cases the underlying mechanisms remain unclear (Kutay and Hetzer, 2008; Roux and Burke, 2006).

Transport factors such as Importin β, Importin α, and Crm1/Exportin-1 are responsible for sequestering various spindle components during mitosis and releasing them appropriately in the vicinity of mitotic chromatin (reviewed in (Clarke and Zhang, 2008; Kalab and Heald, 2008). The GTPase Ran directs the localized release of assembly factors from transport receptors. This is accomplished by RCC1, the Ran GEF, which is bound to chromatin and generates a high, localized Ran-GTP concentration. As in nuclear import, interaction with Ran-GTP induces release of bound cargo from transport receptors. This ensures that potent mediators of spindle assembly function only within the specific area around chromatin. The Importin β/Importin α complex is responsible for correct localization of a variety of microtubule associated, spindle assembly factors (SAFs) including TPX2, NuMA, Xnf7 and NuSAP (Gruss et al., 2001; Maresca et al., 2005; Nachury et al., 2001; Raemaekers et al., 2003; Wiese et al., 2001). Importin β can directly bind and regulate additional factors, such as HURP, Maskin, and Rae1 (Blower et al., 2005; Koffa et al., 2006; O'Brien et al., 2005). The export factor Crm1 is responsible for targeting both the Nup358/RanGAP complex and the Chromosomal Passenger Complex (CPC) to the centromere/kinetochore region of the spindle (Arnaoutov and Dasso, 2005; Knauer et al., 2006).

A still growing list of nucleoporins play roles in mitotic spindle assembly or in mitotic checkpoints. A small percentage of both the Nup107/ELYS and Nup358/RanGAP subcomplexes are localized to mitotic kinetochores where they contribute to formation and/or stabilization of microtubule-kinetochore attachments (Arnaoutov and Dasso, 2005; Belgareh et al., 2001; Franz et al., 2007; Joseph et al., 2004; Joseph et al., 2002; Loiodice et al., 2004; Orjalo et al., 2006; Rasala et al., 2006; Salina et al., 2003; Zuccolo et al., 2007). Independently, ELYS, as well as Nup153, function in cytokinesis through poorly defined activities (Mackay et al., 2009; Rasala et al., 2006). Rae1 has multiple contributions through roles in spindle assembly and regulation of the Anaphase Promoting Complex/Cyclosome (APC/C), and is required for a fully functional mitotic checkpoint (Blower et al., 2005; Jeganathan et al., 2005; Whalen et al., 1997; Wong et al., 2006). Tpr is responsible for targeting of Mad1/2 to the kinetochore and is also involved in the mitotic checkpoint (Lee et al., 2008). Although this list of mitotically active nucleoporins continues to grow, the mechanisms underlying these roles are often incompletely understood.

Here we show that the nucleoporin Nup98 functions in mitotic spindle assembly by regulating microtubule dynamics. Nup98 is a GLFG-repeat containing nucleoporin dynamically associated with both nuclear and cytoplasmic faces of the NPC (Griffis et al., 2002; Griffis et al., 2003; Radu et al., 1995b). During interphase, Nup98 participates in RNA export and protein import (Fontoura et al., 2000; Powers et al., 1997). During mitosis, Nup98 is soluble and largely dispersed throughout the cell. We found that addition of the Nup98

C-terminal domain to Xenopus spindle assembly assays caused excess tubulin polymerization and highly disordered spindles. In contrast, inhibition of Nup98 in the egg extract led to accumulation of monopolar spindles. Importantly, addition of recombinant Nup98 C-terminus restored spindle bipolarity in depleted extracts. We have mapped the specific region of Nup98 involved and established that its function is independent of the Nup107 complex, a Nup98 binding partner in the NPC. This Nup98 activity is regulated, at least in part, through mitotic phosphorylation. We propose a model in which Nup98 regulates plus end microtubule dynamics, and in support of this model, we show that the relevant region of Nup98 interacts with the depolymerizing kinesin, MCAK, a major regulator of microtubule dynamics.

RESULTS

Addition of purified Nup98 C-terminal domain to CSF extract disrupts spindle assembly.

In order to investigate the potential mitotic function of Nup98, we added a bacterially expressed, Nup98 C-terminal fragment (his-tagged aa 506-920; Figure 10) to *Xenopus laevis* spindle assembly assays in vitro. The purified fragment was added to Xenopus CSF egg extracts to a final concentration of 6 μM along with sperm chromatin, and at different time points during spindle assembly samples were fixed and analyzed by fluorescence microscopy. As expected, by 15 minutes microtubule asters had formed in control samples (Figure 11A, panel a). Surprisingly, in samples to which the Nup98 C-terminal fragment had been added, we observed many long microtubules emanating from centrosomes (Figure 11A, panel b). This excess microtubule phenotype persisted throughout the time course of spindle formation and resulted in highly perturbed bipolar spindle structures. In contrast, spindles formed in the presence of the same concentration of a control protein (BSA) displayed normal bipolar spindle morphology (Figure 11A, compare panels i and m to panels j and n). Protein obtained by purification of control bacterial lysate over a nickel chromatography column had no effect when added to the spindle assembly assay (data not shown).

To independently demonstrate through biochemical means that this Nup98-induced phenotype represented excess microtubule growth, we employed a tubulin spindown assay. Following formation of spindles in the presence or

Figure 10. Mapping of the minimal region of the Nup98 C-terminus required for excess microtubule polymerization. A series of deletion or mutant constructs were generated as indicated and fragments were expressed in bacteria and purified using an N-terminal 6xHis tag. Nup98 fragments were added to egg extract and tested for induction of excess microtubules as described in Experimental Procedures.

Figure 10

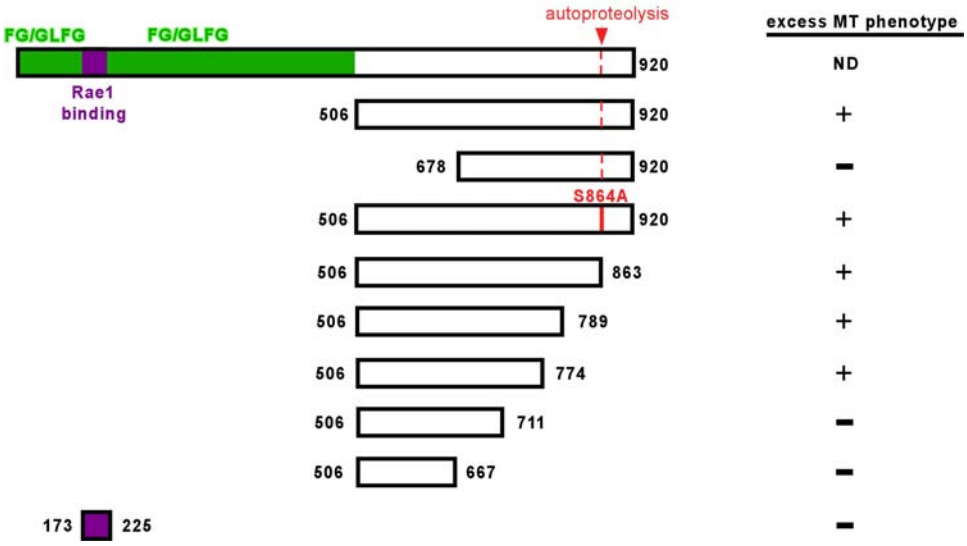
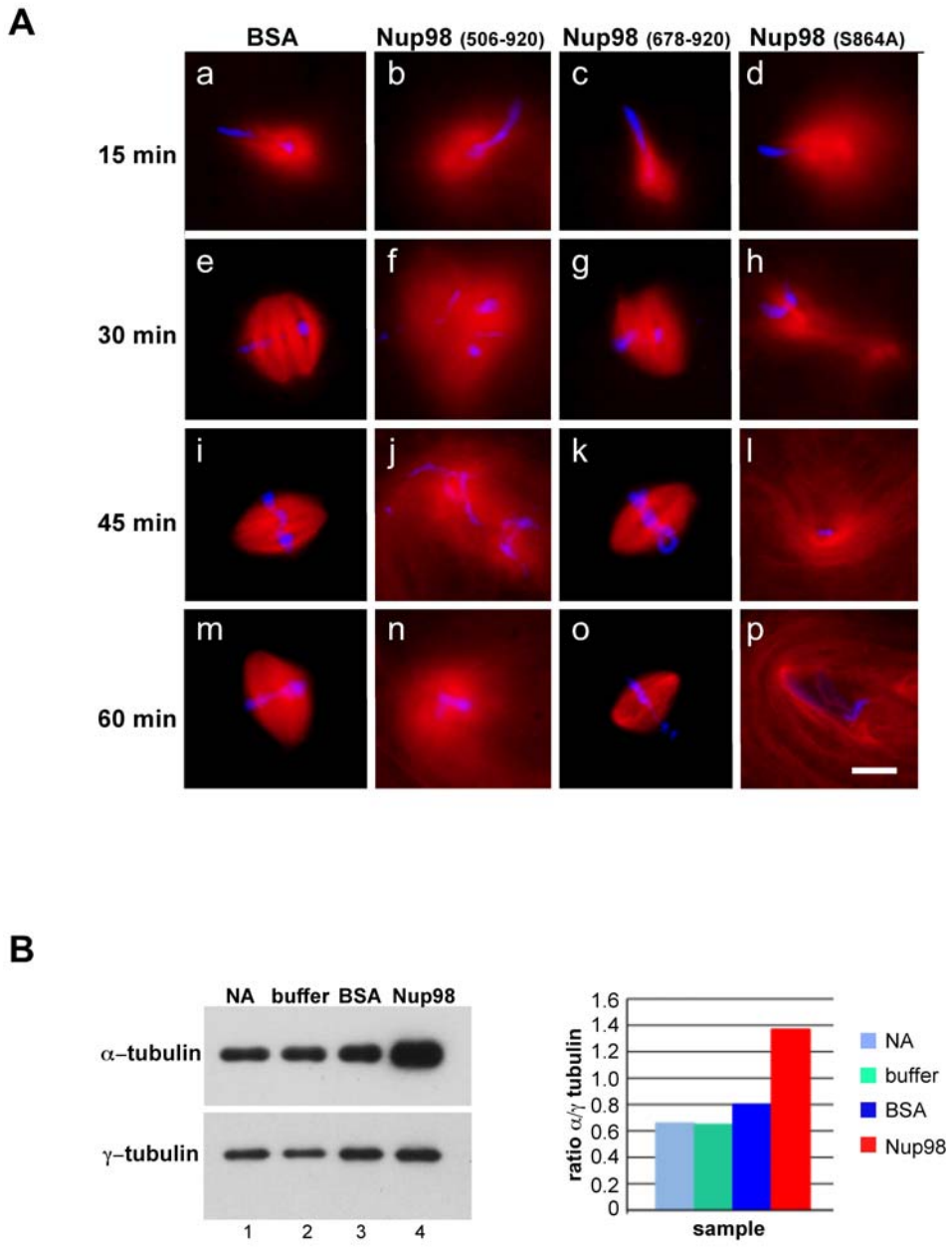

autoproteolysis
excess MT phenotype
FG/GLFG
FG/GLFG
Rae1
binding
920
ND
506
920
+
678
920
−
S864A
506
920
+
506
863
+
506
789
+
506
774
+
506
711
−
506
667
−
173
225
−

Figure 11. **A C-terminal domain fragment of Nup98 causes excess microtubule polymerization during assembly of meiotic spindles. A)** Nup98 C-terminal domain fragments or BSA were added at 6μM at the start of assembly assays. Samples were examined at the indicated time points. Microtubules are labeled with X-rhodamine tubulin (red) and DNA is labeled with Hoechst (blue). Scale bar represents 20 μm. **B*)*** *Left panel*: polymerized tubulin was isolated by and analyzed by immunoblotting for α-tubulin. The γ-tubulin signal serves as a loading control*. Right panel*: the immunoblot at left was quantified and tubulin polymerization is represented as the ratio of α/γ tubulin in each sample.

Figure 11


A
BSA
Nup98 (506-920)
Nup98 (678-920)
Nup98 (S864A)
15 min
30 min
45 min
60 min
a
b
c
d
e
f
g
h
i
j
k
l
m
n
o
p
B
NA
buffer
BSA
Nup98
α–tubulin
γ–tubulin
1
2
3
4
ratio α/γ tubulin
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
sample
NA
buffer
BSA
Nup98

absence of the Nup98 C-terminal domain, tubulin polymers were separated from unincorporated tubulin by pelleting through a glycerol cushion and then immunoblotted to compare amounts of polymerized tubulin (Figure 11B, left). The amount of γ-tubulin present was assessed as a loading control and quantitation of one such experiment is presented (Figure 11B, right). We confirmed that in the presence of the Nup98 C-terminal domain there is indeed an increased amount of polymerized tubulin, suggesting that addition of the C-terminal domain is causing deregulation of microtubule length.

The Nup98 fragment added to these assays contains the domain responsible for autoproteolytic cleavage of Nup98 at residue F863 (aa 710-920)(Rosenblum and Blobel, 1999). This domain is also involved in targeting Nup98 to the NPC through interaction primarily with Nup96, but also with Nup88 (Griffis et al., 2003; Hodel et al., 2002) To determine whether this same domain was sufficient for the observed effect on spindle assembly, we purified the fragment of the Nup98 C-terminus (aa 678-920), which had been used to crystallize the autoproteolytic and NPC-targeting domain (Hodel et al., 2002). When this short C-terminal fragment was added to spindle assembly assays, we obtained normal bipolar spindles that were indistinguishable from controls (Figure 11A, panels c, g, k, o). Therefore, we conclude that a region between residues 506 and 677 of Nup98 is required, although not necessarily sufficient, for the excess microtubule phenotype. Interestingly, this region of Nup98 has no previously assigned function.

Recently, the Nup107 complex, comprised of Nups 107, 160, 133, 96, 85, 43, 37, Sec13, and Seh1, was shown to play a role in assembly of a correct bipolar spindle in Xenopus mitotic extract. In the absence of this complex, bipolar spindles formed but were unstable and subsequently disappeared (Orjalo et al., 2006). A small fraction of the Nup107 complex is physically associated with the mitotic kinetochore where it is thought to promote microtubule attachment (Zuccolo et al., 2007). Given that one member of this complex, Nup96, is a binding partner of the Nup98 C-terminal domain (Hodel et al., 2002; Vasu et al., 2001), we asked whether interaction between Nup98 and the Nup107 complex underlies the Nup98 C-terminal phenotype. For this, we took advantage of a Nup98 mutant which is unable to bind Nup96 (S864A; (Hodel et al., 2002). When this mutant version of the full C-terminal fragment was added to the spindle assembly assay, we obtained the same excess microtubule polymerization (Figure 11A, panels d, h, l, p). A construct truncated at the autoproteolytic site similarly induced excess microtubule polymerization (Figure 10). Thus we conclude that the unregulated microtubule polymerization phenotype is independent of Nup96 and the Nup107 complex.

Through further deletion analysis, we determined that the minimal fragment required for the microtubule phenotype is aa 506-774 of Nup98 (Figure 10). No function has been previously defined for the region between aa 506 and 710; the remainder of the fragment corresponds to the first half of the autoproteolytic domain. Because this smaller fragment proved to be less stable

upon storage following purification, we continued to employ the full C-terminal fragment for most subsequent experiments.

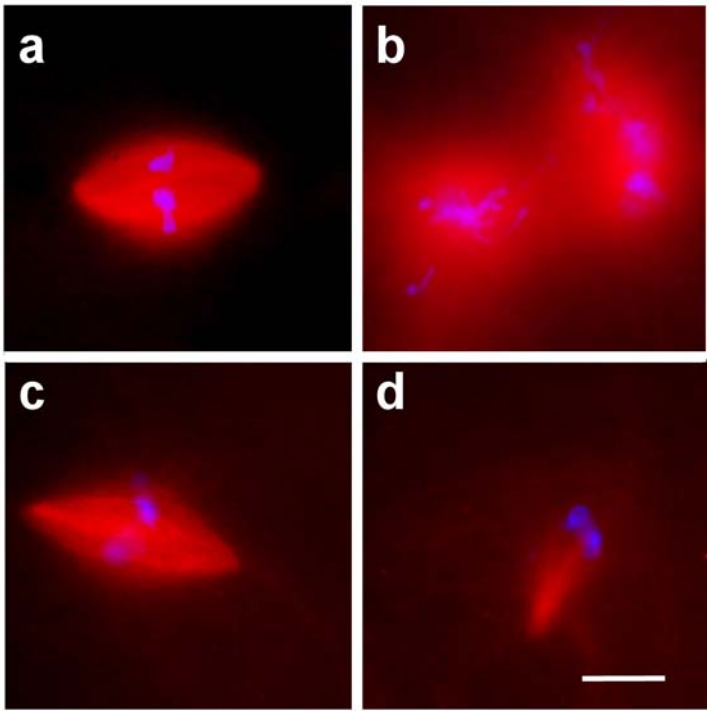
In CSF extracts, bipolar spindles develop from a single centrosome contributed by the sperm chromatin preparation. In dividing cells, the bipolar spindle forms from the pair of centromes that are present as a result of centrosome duplication during S phase. To confirm the effect of the Nup98 C-terminus on spindles formed from replicated centrosomes, we tested the Nup98 C-terminal domain in cycled Xenopus extracts. In these experiments, the CSF extract is first shifted into interphase, allowing nuclei to assemble and replicate both chromatin and centrosomes. The nuclei are then cycled back into mitosis by addition of fresh CSF extract. Spindles formed from cycled extracts showed excess microtubule polymerization and a disrupted spindle structure in the presence of the Nup98 fragment, exactly as seen with non-cycled spindles (Figure 12).

Nup98 and alternate spindle assembly pathways

There are multiple distinct pathways that can work in conjunction to build a mitotic spindle (Kalab and Heald, 2008; O'Connell and Khodjakov, 2007; Walczak and Heald, 2008). In cultured cells, the predominant pathway is thought to be the centrosome-driven or search-and-capture pathway, in which microtubules extend dynamically from the centrosome and search for the kinetochore complex found on the centromeric region of the chromosome . However, there is also a second, chromatin-driven pathway, regulated by the

Figure 12. The Nup98 C-terminal fragment causes excess microtubule polymerization in cycled spindles. Xenopus sperm chromatin was added to CSF extract and the extract was shifted to interphase by addition of CaCl. After 60 min incubation to allow for nuclear formation and centrosome duplication, BSA (panel a), Nup98 C-terminus (panel b), rabbit IgG (panel c), or anti-Nup98 (panel d) was added and the extracts were shifted back into mitosis by addition of one half volume of fresh CSF extract. Indentical results were observed when the protein or antibody was added before the shift into interphase.

Figure 12



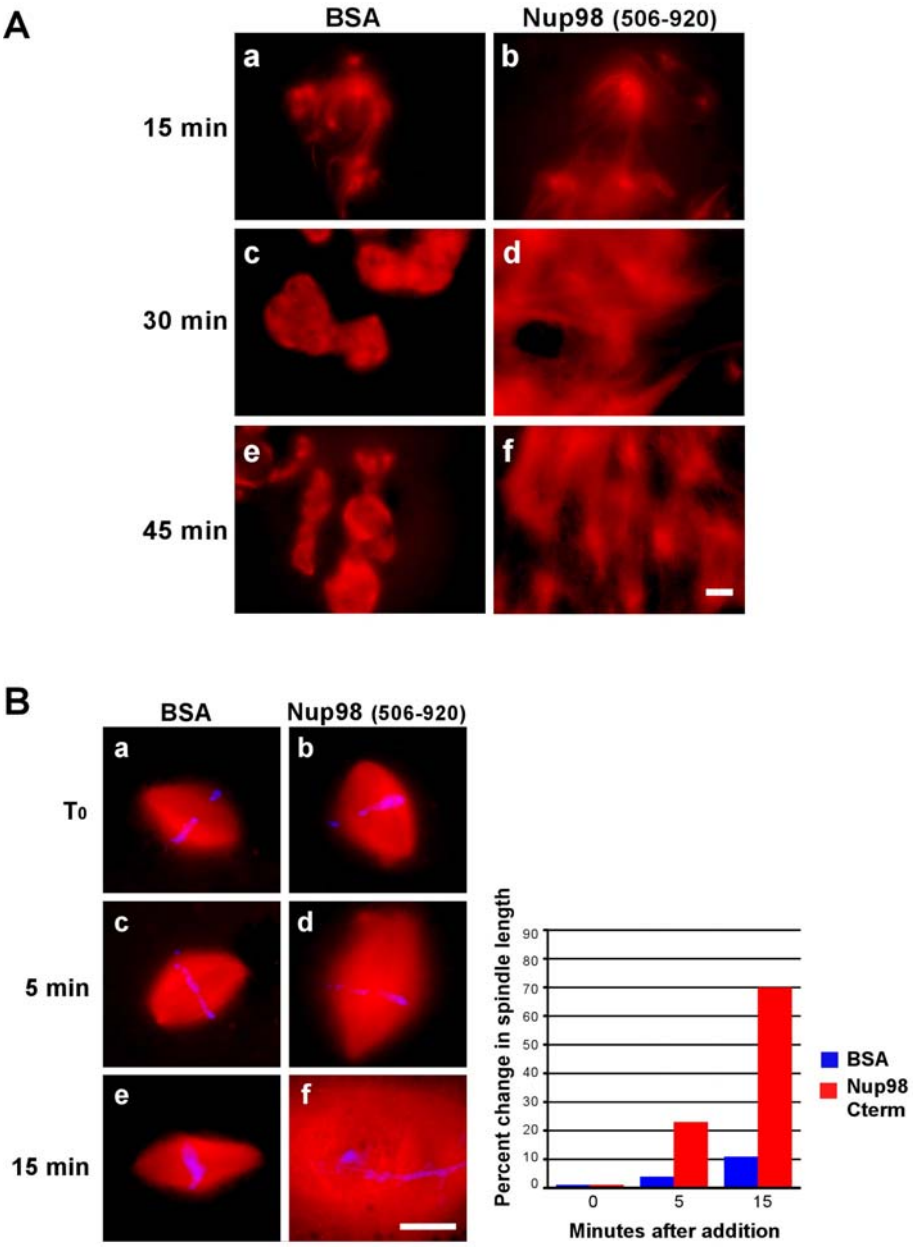
Ran GTPase, in which microtubules polymerize adjacent to chromatin and are then organized by molecular motors and other factors to form a spindle pole. To determine whether addition of Nup98 C-terminus could influence microtubule polymerization initiated by the Ran pathway, we added the Nup98 fragment to a Ran aster assay. Spindle-like Ran asters form upon the addition of a non-hydrolyzing form of Ran-GTP to CSF extract in the absence of centrosomes (Carazo-Salas et al., 1999; Kalab et al., 1999; Ohba et al., 1999; Wilde and Zheng, 1999; Zhang et al., 1999). We found that addition of purified Nup98 C-terminus to Ran aster assays also led to excess polymerization of microtubules (Figure 13A). Asters were larger with more unfocused microtubules, increasing their tendency to aggregate. Thus, the Nup98 fragment can stimulate microtubule polymerization regardless of the manner in which polymerization is initiated.

Importantly, we found that when purified Nup98 C-terminal domain was added to Xenopus egg extract in the absence of either sperm chromatin or Ran-GTP, it is unable to independently initiate microtubule polymerization (data not shown). Therefore, the excess microtubule phenotype is not due to direct initiation of excess microtubule polymers by Nup98. Rather, addition of the C-terminal domain of Nup98 is affecting the dynamics of microtubules once polymerization is initiated by other factors.

The Nup98 fragment clearly perturbed formation of spindles but would an existing, normal bipolar spindle still be sensitive to effects of the Nup98 fragment? To assess this, we preformed spindles in CSF extract, divided the sample into two parts and added either BSA or the Nup98 C-terminus. Strikingly,

Figure 13. The Nup98 C-terminal domain increases microtubule polymerization in Ran asters and in preformed spindles. A) Nup98 C-terminus or BSA was added along with 25 μM RanQ69L to Xenopus extract and Ran aster formation was monitored. Microtubules are labeled with X-rhodamine tubulin (red). Scale bar is 20 μm. **B)** *Left panel*: Spindles were preformed in Xenopus egg extract for 60 minutes. Either BSA or Nup98 C-terminus was then added (T_0) and spindles were monitored over time. Scale bar is 20 μm. *Right panel:* spindle length was measured as pole-to-pole distance. Percent change was calculated as (average spindle length in sample) – (average T_0 length)/ (average T_0 length) x 100. An average of 10 spindles were quantified for each point except the 15 min Nup98 sample in which only 6 intact spindles could be identified.

Figure 13


A
BSA
Nup98 (506-920)
a
b
15 min
c
d
30 min
e
f
45 min
B
BSA
Nup98 (506-920)
a
b
T0
c
d
5 min
e
f
15 min
Percent change in spindle length
90
80
70
60
50
40
30
20
10
0
0
5
15
Minutes after addition
BSA
Nup98 Cterm

within 5 minutes after addition of the Nup98 fragment, the microtubule density appeared to increase and spindles began to elongate from pole to pole (Figure 13B, left). By 15 minutes after addition, while control spindles remained unchanged, the structure of the spindles in the presence of the C-terminal domain of Nup98 was nearly unrecognizable due to the increase in microtubule growth (Figure 13B, left). At 30 minutes, no intact spindles could be identified. Thus, addition of Nup98 C-terminus stimulates excess microtubule growth during assembly of bipolar spindles, Ran asters and in preexisting spindles. Taken together, the data obtained from addition of the Nup98 fragment to extracts or to preformed spindles indicate that Nup98 does not initiate microtubule polymerization but can influence microtubule growth regardless of the pathway through which growth is initiated.

Nup98 antibodies inhibit formation of bipolar spindles but do not affect Ran asters or preformed spindles.

To this point, experiments had been conducted by the addition of exogenous, recombinant Nup98 fragments to the extract. To test for a contribution from endogenous Nup98 in spindle assembly, we set out to alter the level of functional Nup98 protein in the Xenopus extract. Spindles were assembled in the presence of Nup98-specific antibodies to block endogenous protein function. If the Nup98 C-terminal fragment was acting as a dominant negative effector of spindle assembly or function, we anticipated that an antibody that bound this same region might phenocopy the effect of the fragment.

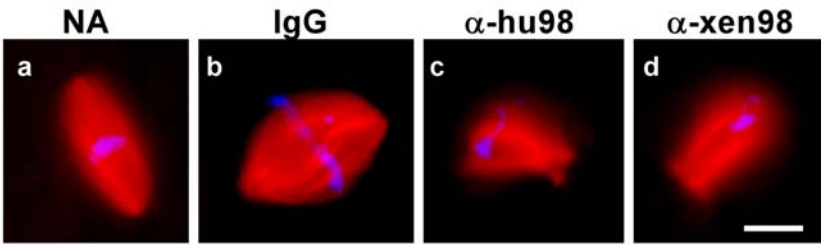
Unexpectedly, in contrast to excess tubulin polymerization, the addition of antibody raised against either Xenopus or human Nup98 C-terminal domain led to a reduction in bipolar spindles and a corresponding increase in stable monopolar spindles up to >50% monopoles (Figure 14A and 14B). Control assays in which the antibody was pre-absorbed with the Nup98 fragment showed no effect of antibody addition (data not shown), demonstrating that the inhibition was due to specific Nup98 binding. Independent western blot and immunofluorescence studies revealed that the human Nup98 C-terminal antibody is directed almost entirely towards epitopes within aa 506-677 (data not shown); there is only minimal recognition of the region from aa 678-920, most likely due to the high structural conservation of the autoproteolytic domain (Robinson et al., 2005). This antibody thus proved to be an ideal reagent for inhibition of Nup98 function.

Using the tubulin spindown assay, we observed a substantial reduction in polymerized tubulin in samples containing Nup98 antibody (Figure 14C). We noted that the level of γ-tubulin was also reduced (Figure 14C, lane 3), which we attribute to a lessened ability of the small half spindles to be recovered through the glycerol cushion. Once again, results were consistent in cycled spindle assays, with a reduction of bipolar spindles and a significant increase in monopolar spindles (Figure 12). The opposing phenotypes obtained from addition of the Nup98 C-terminus and addition of Nup98 C-terminal antibodies indicated that, rather than acting as a dominant negative inhibitor, the C-terminal domain of Nup98 contains a functional activity that promotes microtubule growth.

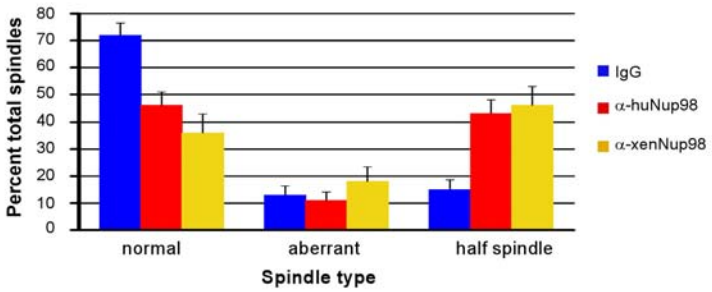
Figure 14. Addition of Nup98-specific antibodies causes a shift to monopolar spindles. A) Antibodies raised to either human or Xenopus Nup98 were added to spindle assays at 200 or 100 µg/ml, respectively. Non-specific rabbit IgG was added at 200 µg/ml. Assays were incubated for 60 minutes. Microtubules are in red, DNA is in blue. Scale bar is 20 µm. **B)** Spindle morphology was quantified with spindles scored as either normal, aberrant (in which DNA was incorrectly aligned or spindle was misshapen), or half spindles. 25-50 spindles were scored in each of 3 independent experiments for a total of 250 spindles. Error bars represent Standard Error of the Proportion (SEP). **C)** Polymerized tubulin was pelleted and analyzed by immunoblotting. The smaller half spindles formed in Nup98 samples pelleted less efficiently leading to decreases in both α- and γ-tubulin.

Figure 14

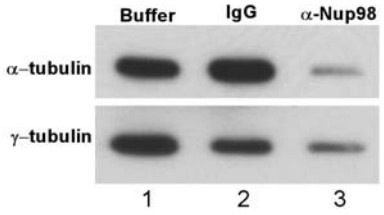
A



B



C



The C-terminal antibodies were then tested in the Ran aster and preformed spindle assays. Although we had observed that addition of antibodies significantly affected bipolar spindle assembly, this same treatment had only minimal affect on Ran aster formation or on the structure of preformed spindles (data not shown; summarized in Figure 15D). This data, taken together with the Nup98 fragment addition data, suggest that although the C-terminal domain can have a dominant affect on microtubules in spindle assembly, Ran asters and preformed spindles, Nup98 is not essential for the Ran pathway of spindle assembly. Additionally, Nup98 is not required to maintain bipolar spindle structure once formed, although the possibility exists that, in the preformed spindle, the Nup98 epitopes are not accessible for antibody binding.

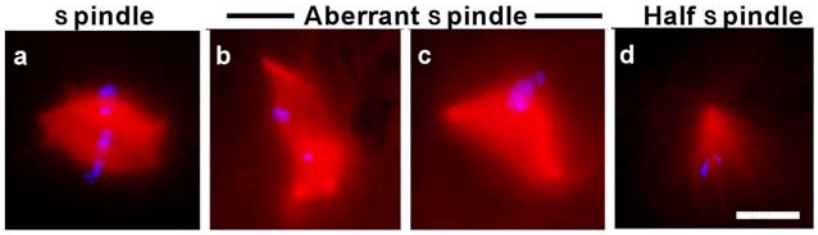
Endogenous Nup98 is required for bipolar spindle assembly in egg extract.

To address the requirement for endogenous Nup98 in spindle assembly more directly, we immunodepleted Nup98 from Xenopus CSF extract and then assessed spindle formation. We observed that, like Nup98 antibody addition during spindle assembly, depletion of Nup98 caused a significant decrease in bipolar spindles with a corresponding increase in stable monopolar spindles (Figure 15A and 15C). Depletion of endogenous Nup98 was confirmed by western blot, which indicated a greater than 90% reduction in Nup98 protein level (Figure 15B). Interestingly, analysis of the Nup98 depleted extract revealed that the Nup98 binding partner, Rae1, was only ~50% co-depleted, suggesting that in

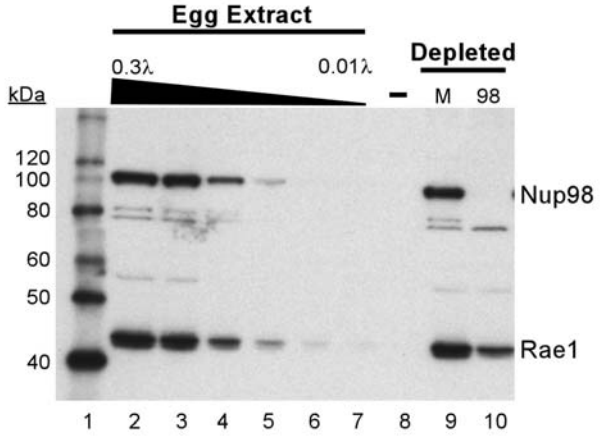
Figure 15. Depletion of Nup98 from Xenopus extracts causes disruption in bipolar spindle assembly which can be restored by the Nup98 C-terminal domain. Extracts were depleted with either nonspecific IgG (Mock), or anti-Nup98 and either BSA (depleted) or purified Nup98 C-terminus (depleted + C-term) was added to a final concentration of 6 μM. Spindles were assembled for 60 minutes. **A)** Spindles assembled following Nup98-depletion and addback of the recombinant C-terminus. Microtubules are in red and DNA is in blue. Scale bar is 20 μm. **B)** The extent of depletion was assessed by blotting 3 μl of Nup98- or Mock-depleted extracts relative to serial two-fold dilutions of Xenopus extract. **C)** Spindle morphology was quantified by scoring spindles as normal, aberrant, or half spindles. Aberrant spindles typically resembled that of 4A, panel c in which DNA was misaligned, but rarely spindles as perturbed as that of Figure 4A, panel b were observed. Three or more independent experiments were scored for each condition with 25-50 spindles per sample for a total of 750 spindles analyzed. Error bars represent SEP. D) Summary of phenotypes observed after manipulation of Nup98 levels in Xenopus egg extract. * In depleted extracts formation of Ran asters required an extra 15 minutes, but asters appeared normal. nd, not done.

Figure 15

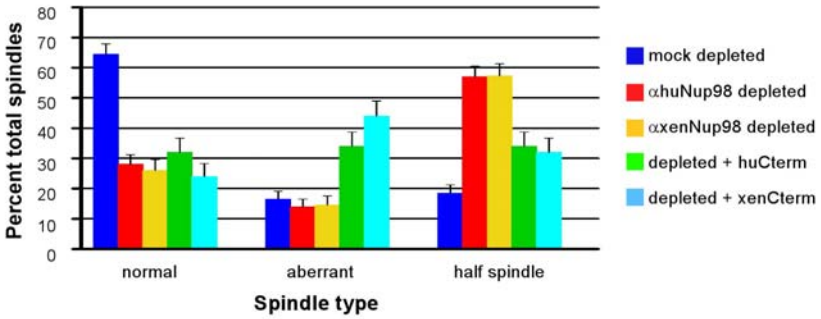
A



B



C



D

Treatment	Assay		
	Spindle assembly	Ran aster formation	Preformed spindles
Nup98 C-term addition	xs MT	xs MT	xs MT
Nup98 Ab addition	monopolar spindles	no effect	no effect
Nup98 depletion	monopolar spindles	+/- *	nd

these mitotic Xenopus egg extracts, Nup98 is not always found in a complex with Rae1 (Figure 15C).

If this region of the Nup98 C-terminus truly stimulates microtubule polymerization, we predicted that the recombinant C-terminal fragment should compensate for the loss of endogenous Nup98. Therefore, we tested whether the Nup98 C-terminus could restore bipolar spindle formation to Nup98-depleted extracts. Strikingly, whereas the Nup98-depleted extract supplemented with BSA contained ~50-60% monopolar spindles as expected, addition of the purified Nup98 C-terminus rescued spindle bipolarity (Figure 15A and 15C). Both purified human and Xenopus Nup98 C-terminal domain fragments were tested, and in both cases, bipolarity of spindles is restored back to control levels. Despite their bipolarity, a fraction of the rescued bipolar spindles were classified as "aberrant" for one of a variety of reasons, including misaligned chromatin, split poles, or general misshapen structure (Figure 15A, panels b and c). These aberrant spindles could be due to the fragile nature of depleted extract, lack of some contribution from another domain of Nup98, or possibly the result of co-depletion of another protein needed for a fully wild-type bipolar spindle. However, the fact that bipolarity is restored provides strong support for our conclusion that the C-terminus of Nup98 is responsible, at least in major part, for a function of Nup98 in bipolar spindle assembly.

Similar to Ran asters formed in the presence of Nup98 C-terminal antibodies, Ran asters formed in Nup98-depleted extract are largely unaffected (data not shown, Figure 15D). In some extracts, we observed a minor delay of ~

15 minutes in formation of the Ran asters when endogenous Nup98 was depleted. However, once the Ran asters formed, their structure was equivalent to Ran asters formed in control extract. This provides a further indication that Nup98 is not required for the Ran pathway of spindle formation. Taken together, our data indicate that Nup98 is critical for assembly of the bipolar spindle when initiated by the chromatin/centrosome. Once a stable bipolar spindle is formed, Nup98 is no longer essential for its maintenance. However, the target of Nup98 regulation of microtubules is present and excess microtubule polymerization can be stimulated by exogenous Nup98 C-terminus. Similarly, Nup98 is not required for the Ran pathway to produce a spindle-like structure, the Ran asters. However, the responsive factor(s) is present and can be hyperactivated by addition of the Nup98 C-terminus.

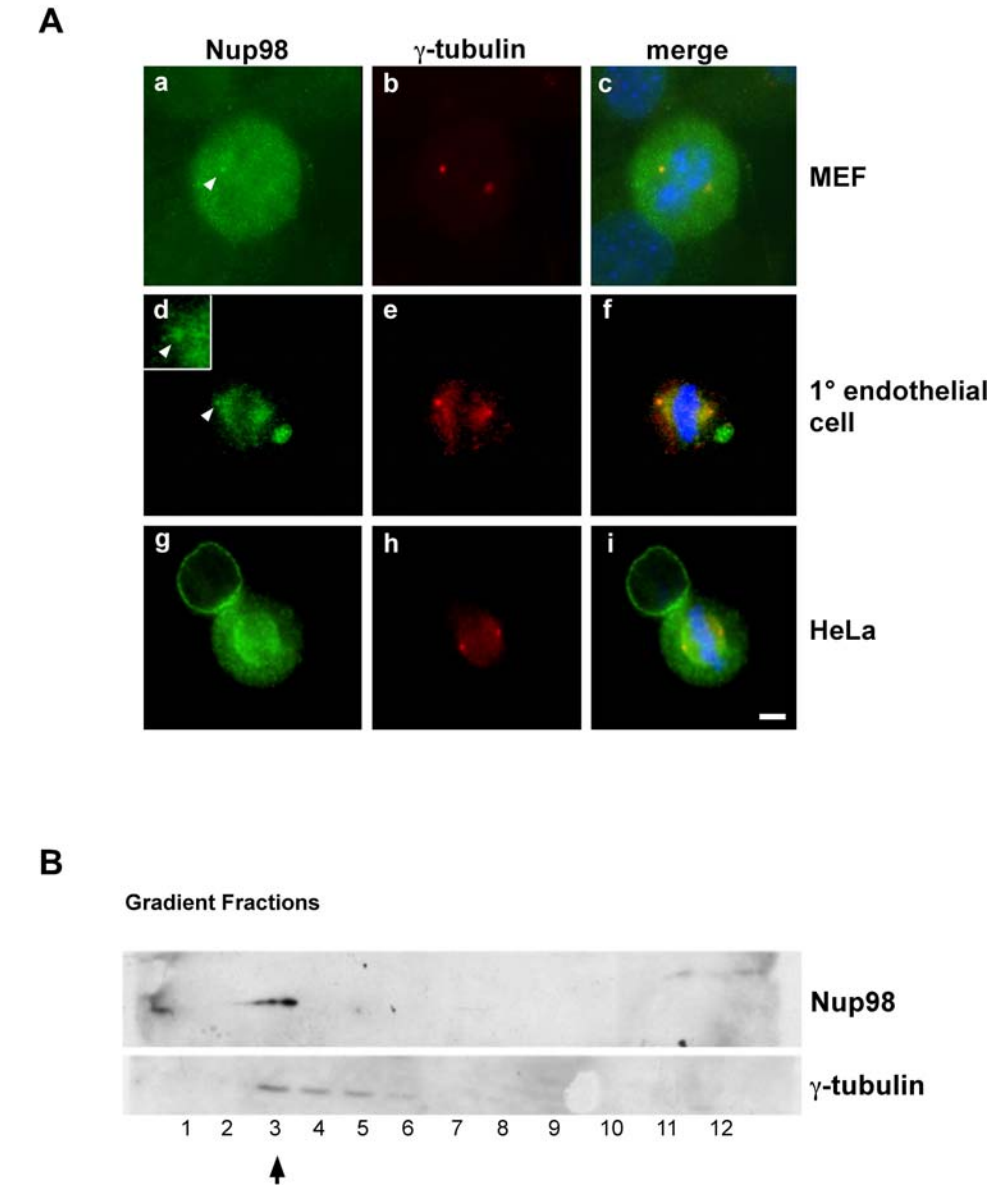
A fraction of Nup98 is associated with centrosomes.

Our lab and others have observed that during mitosis, when the nuclear pore complex is disassembled, the vast majority of Nup98 becomes soluble and dispersed throughout the cell. However, given the apparent contribution of Nup98 to spindle assembly, we were interested in whether there might be a small subpopulation of Nup98 with a specific mitotic localization. In order to investigate this question, we localized Nup98 in cultured cells following a stringent immunofluorescence protocol in which the cells were first permeabilized with 0.5% Triton-X100 for 5 minutes to allow for release of most soluble protein from the cell prior to fixation.

Under these conditions, the mitotic localization of Nup98 was investigated in a variety of cell types, including HeLa cells, human primary endothelial cells, and Chinese hamster ovary cells (CHO). Staining for γ-tubulin functioned as a marker for centrosomes and the spindle apparatus. We detected a very small population of Nup98 protein associating with the mitotic spindle in all of these cell types. Specifically, we found Nup98 colocalized with γ-tubulin on both spindle fibers and centrosomes (Figure 16A). We also observed a similar staining pattern in mouse embryonic fibroblasts that were not permeabilized before fixation (MEFs; Figure 16A, panels a-c). In primary cells (MEFs and endothelial cells) the centrosome association of Nup98 is more prominent than in immortalized, transformed cell lines such as HeLa and CHO (Figure 16A, compare panels a and d to panel g). Because only a small amount of Nup98 is detected at centrosomes, we sought to confirm this association biochemically. Gradient fractions from the purification of primary human lung fibroblast centrosomes (the kind gift of Masayuki Kanai and Kenji Fukasawa) were blotted to assess the presence of Nup98 (Figure 16B). We found that a small fraction of Nup98 did indeed co-purify with γ-tubulin in the centrosome preparation (Figure 16B, lane 3), confirming that a subpopulation of Nup98 interacts with the centrosome or centrosome-associated proteins during mitosis.

Figure 16. A small fraction of Nup98 can be detected at centrosomes. A) Primary MEFs were fixed, permeabilized and stained for Nup98 (green) and γ-tubulin (red). Primary human endothelial or HeLa cells were permeabilized, fixed and stained for Nup98 (green) and γ-tubulin (red). **B)** Sucrose gradient fractions from purification primary lung fibroblast centrosomes were immunoblotted for Nup98 and γ-tubulin.

Figure 16


A
Nup98
γ-tubulin
merge
a
b
c
MEF
d
e
f
1° endothelial cell
g
h
i
HeLa
B
Gradient Fractions
Nup98
γ-tubulin
1 2 3 4 5 6 7 8 9 10 11 12

Mitotic phosphorylations influence the function of Nup98 in spindle assembly.

It has been observed repeatedly that a subset of nucleoporins are phosphorylated during mitosis (Glavy et al., 2007; Macaulay et al., 1995; Onischenko et al., 2007). Indeed, Nup98 was one of the first nucleoporins shown to undergo mitotic phophorylation although individual phosphorylation sites were not mapped. Given the correlation in timing between phosphorylation and nuclear envelope breakdown, it has been presumed that phosphorylation regulates NPC disassembly and/or mitotic nucleoporin functions, but this has not been directly demonstrated. Since we had defined a specific region of Nup98 required for the mitotic stimulation of microtubules, this presented an ideal opportunity to address the possible regulation of this activity by phosphorylation. Our initial experiments indicated that the essential region of the C-terminus was indeed phosphorylated in mitotic extract. When purified GST-fusion proteins were incubated in CSF extract together with γ^{32}P-ATP, we observed that the longer C-terminal construct (aa 506-920) was substantially labeled, whereas neither GST nor the shorter C-terminal construct (aa 678-920) were detectibly labeled under these conditions (Figure 17A).

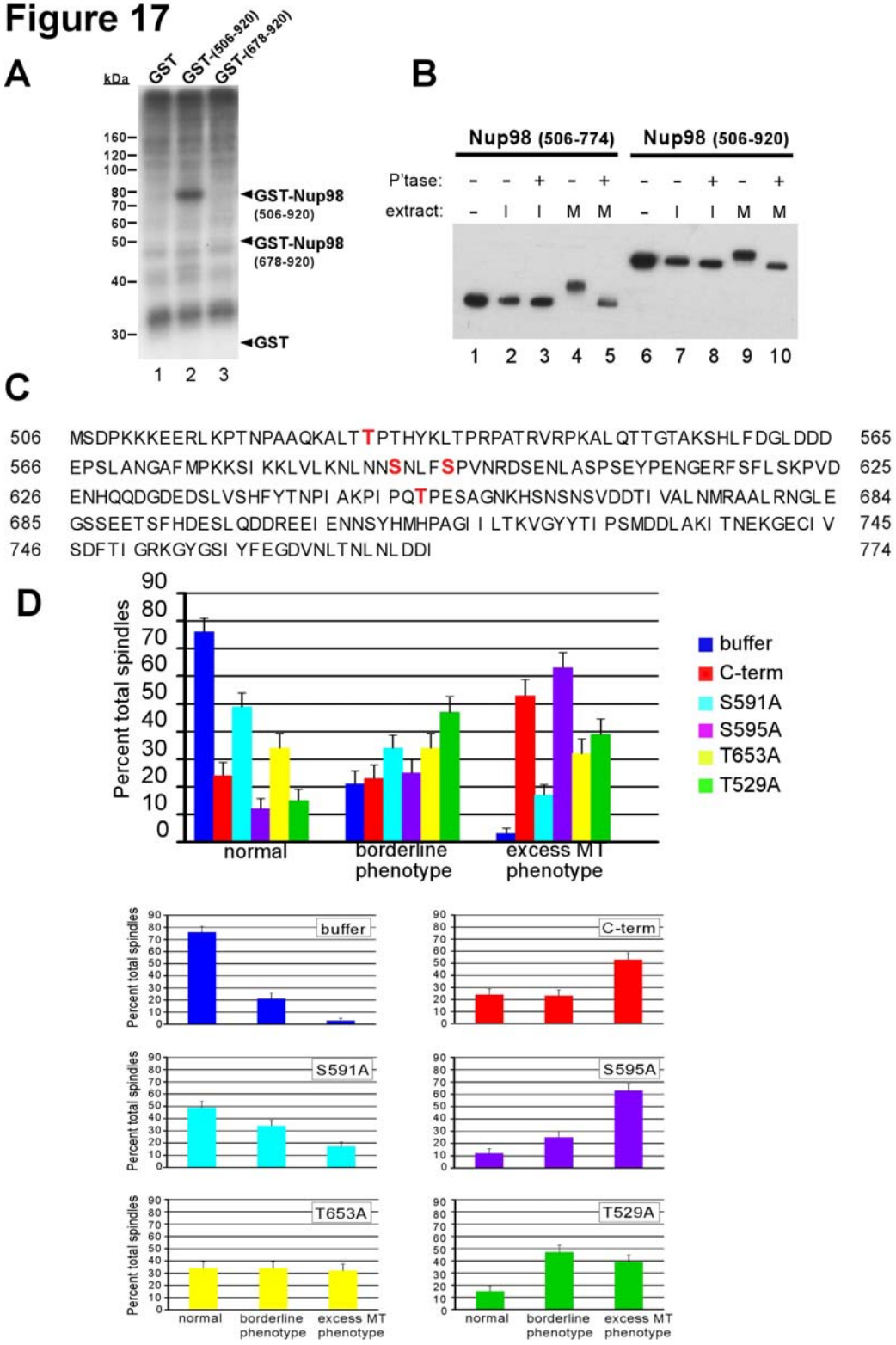
To test whether this phosphorylation was mitosis-specific, both the longer C-term fragment and the minimal fragment of Nup98 required for the excess microtubule phenotype (aa 506-774) were incubated in either interphase or mitotic extract to allow for modification. Nup98 fragments were then recovered from the extract and immunoblotted with antibody to the T7 tag on the

recombinant protein. We found that, in comparison to untreated protein (Figure 17B, lanes 1 and 6) there was a slight upwards shift following incubation in interphase extract, and a substantially greater shift following incubation in mitotic extract (Figure 17B, compare lanes 1, 2, 4 and lanes 6, 7, 9). In both interphase and mitotic samples, the shift was abolished by subsequent treatment with phosphatase (Figure 17B, compare lanes 2 and 3, 4 and 5, 7 and 8, 9 and 10). Thus we conclude that the Nup98 C-terminal region required for excess microtubule polymerization (aa 506-774) is in fact phosphorylated and this modification is substantially increased during mitosis.

In order to test the functional contributions of phosphorylation to Nup98 regulation of microtubule polymerization, we mapped phosphorylation sites by mass spectrometry. Both long and minimal Nup98 C-terminal fragments were incubated in either interphase or mitotic extract and retrieved using the T7 tag at the N-terminus. These fragments were then subjected to mass spectrometry to identify phosphorylated residues. We found multiple phosphorylated serine and threonine residues within the region of interest, but no tyrosine phosphorylation (Figure 18). For functional studies, we chose to focus on mitotic phosphorylation sites within the region of aa 506-774 that were conserved between human and Xenopus Nup98 (Figure 17C). Selected residues were altered to alanine by site-directed mutagenesis in order to abolish their potential for phosphorylation. Because the minimal 506-774 fragment proved to have decreased stability upon freezing, mutations were made and tested within the context of the human Nup98

Figure 17. Phosphorylation influences Nup98 affect on microtubule polymerization. A) The longer Nup98 Cterminal fragment (aa 506-920) is phosphorylated in Xenopus extract, although the shorter fragment (Nup98 678-920) is not. **B)** The minimal C-terminal domain (aa 506-774) or the long C-terminal domain (aa 506-920) were incubated in interphase (I) or CSF (M) extract and immunoblotted. Duplicate samples were treated with phosphatase before analysis. **C)** Sequence of the minimal C-terminal domain. Phosphorylated residues indicated in red were chosen for mutational analysis. **D)** Spindles assembly assays using control or phospho-mutant forms of Nup98 C-terminal domain (505-920). Three independent experiments were quantified for the excess microtubule phenotype, 25-50 spindles per sample for a total of 475 spindles. In the lower panels the results are presented separately for each condition to better visualize the affects of mutants. Error bars represent SEP.

Figure 17


A
kDa
GST
GST-(506-920)
GST-(678-920)
160
120
100
80
70
60
50
40
30
GST-Nup98 (506-920)
GST-Nup98 (678-920)
GST
1 2 3
B
Nup98 (506-774)
Nup98 (506-920)
P'tase: - - + - + - - + - +
extract: - I I M M - I I M M
1 2 3 4 5 6 7 8 9 10
C
506 MSDPKKKEERLKPTNPAAQKALTTPTHYKLTPRPATRVRPKALQTTGTAKSHLFDGLDDD 565
566 EPSLANGAFMPKKSIKKLVLKNLNNSNLFSPVNRDSENLASPSEYPENGERFSFLSKPVD 625
626 ENHQQDGDEDSLVSHFYTNPIAKPIPQTPESAGNKHSNSNSVDDTIVALNMRAALRNGLE 684
685 GSSEETSFHDESLQDDREEIENNSYHMHPAGIILTKVGYYTIPSMDDLAKITNEKGECIV 745
746 SDFTIGRKGYGSIYFEGDVNLTNLNLDDI 774
D
Percent total spindles
90 80 70 60 50 40 30 20 10 0
normal
borderline phenotype
excess MT phenotype
buffer
C-term
S591A
S595A
T653A
T529A
buffer
C-term
S591A
S595A
T653A
T529A
normal
borderline phenotype
excess MT phenotype

aa 506-920 long C-terminal domain. Mutant Nup98 fragments were added to spindle assembly assays to assess whether loss of individual mitotic Nup98 phosphorylations would affect spindle assembly. We observed noticeable differences in the excess microtubule phenotype upon addition of different purified phosphomutants. Independently prepared extracts varied somewhat in their sensitivity to the mutations, suggesting there are additional regulatory events, however in 70% of the 10 or more independent extracts tested for each mutant, we saw consistent and striking effects of the phospho-mutants. Three such experiments were scored for spindle morphology and these results are presented both in aggregate and as individual panels to clarify distinctions (Figure 17D). When serine 591 was mutated to alanine, we observed a significant reduction in the excess microtubule phenotype with a distribution more like that of the buffer control. Conversely, when the nearby serine 595 was mutated to alanine, there was an increase in the excess microtubule phenotype (compare distributions relative to C-term in individual graphs and relative peak heights in the aggregate graph). Mutation of threonines 529 or 653 had less striking effects. The microtubule phenotype was somewhat more or less pronounced but there was not a substantial shift in morphology.

Nup98 C-terminal domain interacts with the microtubule depolymerizing kinesin MCAK.

Addition of the Nup98 C-terminus in the absence of sperm chromatin or RanGTP had not resulted in spontaneous microtubule polymerization. This

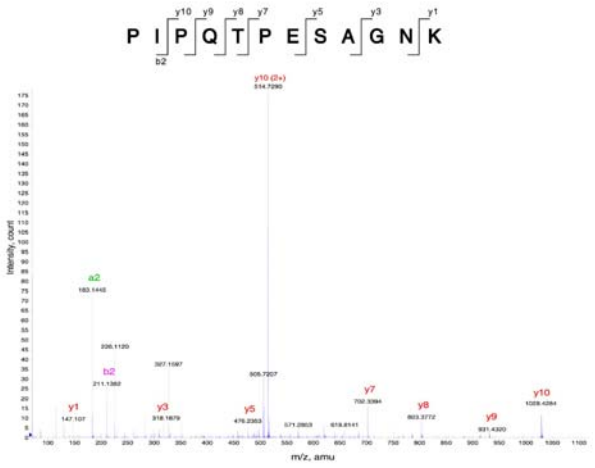
finding suggested that Nup98 could not initiate polymerization, but more likely influenced microtubule dynamics. Given that a small amount of Nup98 was detected at the centrosome, we initially tested the rate of microtubule flux using speckle microscopy (Waterman-Storer et al., 1999) but found that addition of the Nup98 C-terminus did not significantly alter the rate of flux (M.K. Cross, L.A. Cameron, E.D. Salmon and M. A. Powers; data not shown). We then considered a possible influence on plus end microtubule dynamics. Nup98 most likely works through interaction with other regulatory factors rather than direct interaction with microtubules. One such candidate regulatory protein is MCAK (Xenopus XKCM), a depolymerizing kinesin of the kinI/kinesin13 family and the major global regulator of microtubule catastrophe in the egg extract (Desai et al., 1999b; Tournebize et al., 2000; Walczak et al., 1996; Wordeman and Mitchison, 1995). Intriguingly, published studies had established that in the absence of MCAK, excess long microtubules are produced during spindle assembly resulting in “hairy” spindles (Mitchison et al., 2005; Walczak et al., 1996). Conversely, increased levels of MCAK inhibit bipolar spindle formation, and lead to an increase in monopolar spindles and asters (Ohi et al., 2004; Sampath et al., 2004). These phenotypes are the opposite of those observed following manipulation of Nup98; wherein excess C-terminus promotes long microtubules growth and depletion leads to an increase in monopolar spindle. The data together suggested the possibility that Nup98 might act as a negative regulator of MCAK activity during spindle formation.

To investigate this possibility, we performed protein pulldown experiments using purified Nup98 C-terminal fragments. Recombinant proteins were incubated in CSF extract to allow for binding, and then recovered on anti-T7 beads, washed and eluted. Antibody to the tag was used to avoid potential competition between antibody and Nup98-binding proteins. Nup98 C-terminus binding proteins were then probed using an antibody to the Xenopus MCAK homolog, XKCM1 (Figure 19A). Strikingly, we detected association of MCAK with the longer Nup98 C-terminus (aa 506-920) but binding was greatly reduced with the shorter C-terminal domain (aa 678-920) which does not induce the excess microtubule phenotype. The mutant C-terminus (S864A), which also induced excess polymerization, bound MCAK at levels similar to the wild type fragment. We conclude that, whether direct or indirect, interaction between Nup98 and MCAK exhibits the same requirements as does the induction of excess microtubules in the Xenopus spindle assembly assay. We therefore postulate that the Nup98 C-terminal domain acts as a negative regulator of MCAK depolymerization activity and thus facilitates bipolar spindle assembly.

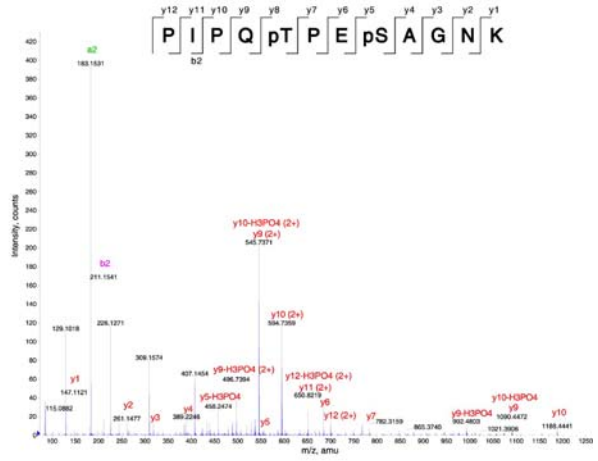
Figure 18. Mass Spectronomy mapping of Nup98 mitotic phosphorylation sites. Mass spectronometric analysis was performed as described in Supplemental Methods. Result are shown for one example LysC digestion peptide. **A)** Spectra for the peptide from untreated control protein. **B)** Spectra for the same peptide obtained from protein incubated in mitotic extract. **C)** Comparison of the human and Xenopus sequence in the region of this peptide. Although two residues, T653 and S656 (shown in red) in the human sample were phosphorylated based on the spectra, only one was conserved in Xenopus and chosen for mutational analysis.

Figure 18

A



B

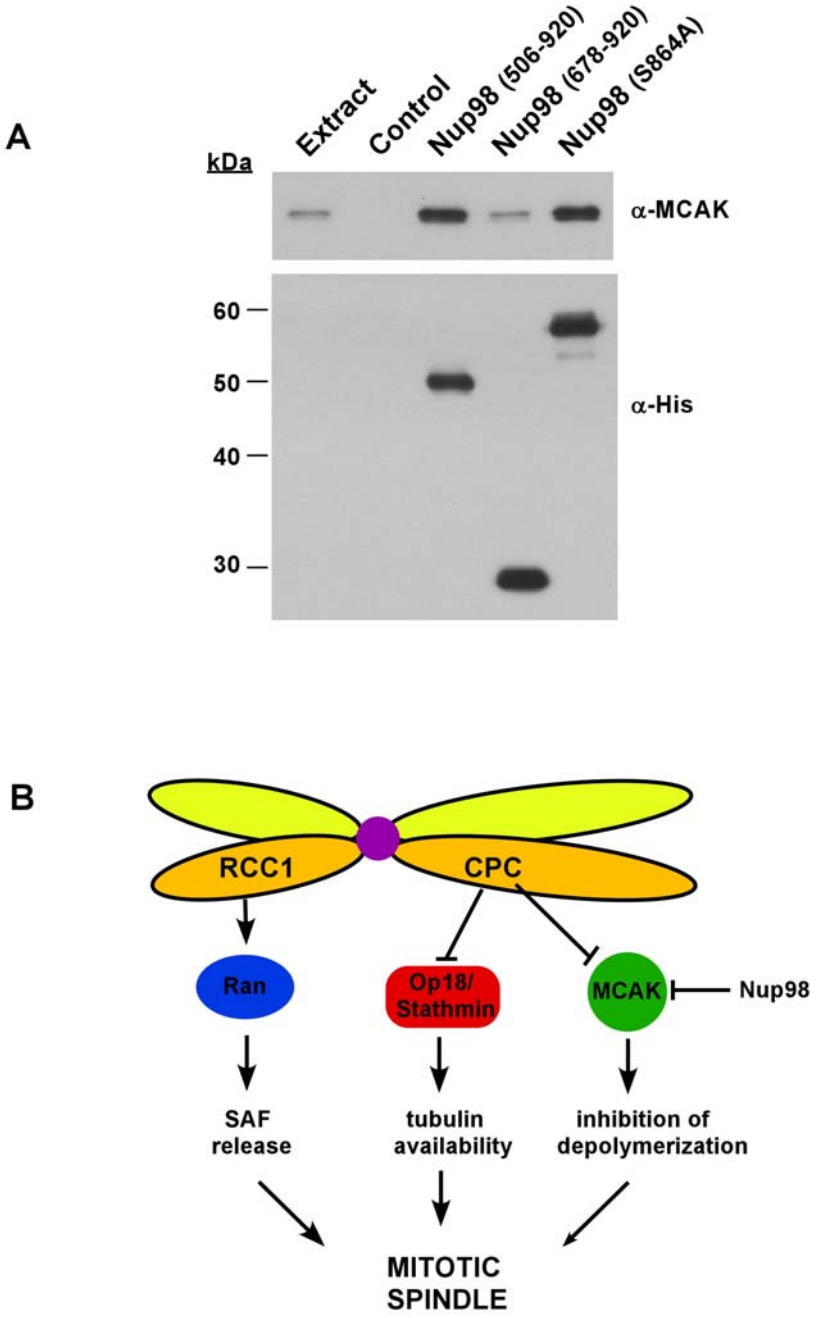


C

human	645	PIAKPIPQTPESAGNK	660
Xenopus	655	PFSRPLPQSQEKKQFN	670

Figure 19. Nup98 interacts with MCAK through the same region required for excess microtubule polymerization. A) Nup98 C-terminal fragments or buffer (Control) were incubated with CSF extract, and immunoblotted with anti-MCAK or anti-His to control for the amount of Nup98 pulled down. Sample lanes contain the material isolated from 4 µl of extract. Extract lane contains 0.1 µl of extract. **B)** Model of Nup98 in spindle assembly.

Figure 19


A
Extract
Control
Nup98 (506-920)
Nup98 (678-920)
Nup98 (S864A)
kDa
α-MCAK
60
50
40
30
α-His
B
RCC1
CPC
Ran
Op18/
Stathmin
MCAK
Nup98
SAF
release
tubulin
availability
inhibition of
depolymerization
MITOTIC
SPINDLE

DISCUSSION

Here we report a novel role for the nucleoporin Nup98 during mitosis. We chose to use the X. laevis egg extract system for these investigations which allowed us to explore potential mitotic function while circumventing effects of Nup98 perturbation on NPC structure or transport. Our findings indicate that Nup98 plays a role in regulation of microtubule dynamics during spindle assembly in the extract. Addition of the Nup98 C-terminus promotes microtubule polymerization and conversely, loss of Nup98 function through depletion or inhibitory antibodies leads to accumulation of monopolar spindles. These observations are the inverse of previously reported results following manipulation of the depolymerizing kinesin, MCAK, in the extract. Our findings led us to propose a model in which Nup98, in addition to the CPC, acts to downregulate MCAK activity during spindle formation (Figure 19).

In the absence of Nup98 we see a substantial shift to half spindles. This result is reminiscent of the phenotype observed with loss of MCAK regulation through either depletion of the CPC or replacement of endogenous MCAK with a non-phosphorylatable mutant (Ohi et al., 2004; Sampath et al., 2004). The CPC is composed of four subunits, Incenp, Survivin, Dasra/Borealin and the kinase Aurora B (reviewed in (Ruchaud et al., 2007). This multimeric complex is localized to the centromere and inhibits MCAK depolymerization activity through phosphorylation at S196 of MCAK (Andrews et al., 2004; Lan et al., 2004). In particular, we noted that our half spindles strongly resemble those produced following depletion of Dasra A from Xenopus extracts (Sampath et al., 2004).

Dasra depletion co-depletes approximately 70% of Incenp and Aurora B; this leads to lack of MCAK phosphorylation, excess MCAK activity and loss of spindle bipolarity. Similarly, depletion of MCAK followed by replacement with non-phosphorylatable, constituitively active MCAK mutants leads to formation of half spindles (Ohi et al., 2004). Both these results suggest that downregulation of MCAK activity is required for spindle bipolarity although the underlying mechanism leading to bipolarity in the egg extract is not entirely clear. More complete (~90%) depletion of the CPC using anti-Incenp led to extreme MCAK activity and left many centrosomes having no attached microtubules at all (Sampath et al., 2004). This is a phenotype that we saw only very rarely in our experiments suggesting that loss of Nup98 does not leave MCAK totally without check on its depolymerization activity.

The monopolar phenotype resulting from depletion of Nup98 could be rescued by addition of the C-terminus alone, leading us to conclude that the C-terminal domain is sufficient to restore bipolarity. This same region of Nup98 interacts, directly or indirectly, with MCAK. We do not yet know whether members of the CPC might be also present in these interactions. Since we do not detect Nup98 at centromeres/kinetochores or along chromosome arms, the sites where the CPC is found, a stable interaction between Nup98 and the CPC seems unlikely, however, this is a subject of ongoing investigation.

Addition of Nup98 to Xenopus extracts is not sufficient to induce spontaneous microtubule polymerization. Similarly, in published reports, depletion or inactivation of MCAK cannot support nucleation of microtubules

around chromatin beads in the absence of the Ran pathway (Sampath et al., 2004). Thus in the absence of Ran-GTP induced release of spindle assembly factors such as TPX2, NuSAP, HURP or Rae1 (Kalab and Heald, 2008), inhibition of MCAK-mediated depolymerization is not sufficient to stabilize microtubules. In keeping with these reports, we find that inhibition of MCAK activity by addition of the Nup98 C-terminus is not sufficient to induce microtubule polymerization in the absence of RanGTP.

Conversely, Nup98 is not required for Ran pathway-induced formation of asters and spindle-like structures; these structures form essentially normally following depletion of Nup98 or in the presence of inhibitory Nup98 antibodies. Similarly, anti-Incenp depletion of the CPC and thus activation of MCAK does not prevent formation of Ran asters or spindles (Sampath et al., 2004). It may be that the high level of Ran-GTP added in these assays leads to sufficient release of spindle assembly/microtubule stablizing factors that MCAK activity is locally overridden.

We were unable to detect any Nup98 at the centromere or kinetochore using three independent antibodies directed against the Xenopus Nup98 C-terminus, full length Xenopus Nup98 (Powers et al., 1995b), or the Nup98 GLFG domain (Fukuhara et al., 2005). Nonetheless, the formal possibility remains that there is a small but highly dynamic population of Nup98 associated with the centromere that we are unable to detect. However, we do observe a small amount of Nup98 associated with the centrosome, a localization that was most noticeable in primary cells. Interestingly, a small amount of MCAK has also been

reported to be present at the spindle pole in both cultured cells and egg extracts (Zhang et al., 2008). In Ran-induced spindle formation, this fraction of MCAK contributes to proper focusing of the poles and is regulated by the Aurora A kinase (Zhang et al., 2008). This was interpreted as an indication that not only flux, but microtuble plus end dynamics, are required to correctly organize the spindle pole.

MCAK is also found in the cytoplasm of the mitotic cell. It is possible that Nup98, the majority of which is dispersed throughout the mitotic cell, normally acts as a cytoplasmic regulator of the soluble pool of MCAK. Cytoplasmic MCAK could help to keep the spindle focused by promoting catastrophe in non-kinetochore attached microtubules. However, as at the kinetochore and spindle pole, MCAK activity must be kept under tight regulation to maintain the proper balance of depolymerization. Soluble Nup98 could interact with MCAK and aid in maintenance of the appropriate level of activity.

Nup98 can be isolated from highspeed supernatent (HSS) of both interphase and mitotic egg extracts in a complex with Rae1. Indeed these two proteins can be largely co-depleted from HSS with WGA-sepharose (Blevins et al., 2003; Macaulay et al., 1995). In contrast, in crude CSF extracts, we found that only approximately 50% of Rae 1 was co-depleted with Nup98. Blower and colleagues (Blower et al., 2005) previously described the association of Rae1 with a large multiprotein complex containing RNA-binding proteins as well as RNA. This complex plays an important role in spindle assembly and further, may aid in partitioning critical transcripts into daughter cells (Blower et al., 2007).

Nup98 was not directly shown to be present in this complex and thus its contribution in this context is somewhat uncertain.

Here we report a very distinct phenotype following Nup98 depletion from that observed after depletion of Rae1 from the extract. Following Rae1 depletion (which was estimated to remove ~60-70% of the protein), spindle formation was strongly inhibited and only sparse, highly bundled microtubules were found (Blower et al., 2005). This is in contrast to the stable monopolar spindles that we observe in Nup98 depletions. We did not find significant numbers of sperm chromatin without associated microtubules, nor did Nup98 depletion decrease the formation of Ran asters, as did Rae1 depletion. Addition of excess recombinant Rae1-binding domain, which competes with Nup98 for Rae1 binding (Blevins et al., 2003), had no effect on spindle formation, suggesting that interaction between these two is not absolutely essential for this process. Most importantly, following Nup98 depletion, we were able to restore spindle bipolarity with recombinant Nup98 C-terminus which lacks the Rae1 binding site. Taken together, we feel these results argue strongly for independent Nup98 and Rae1 roles in mitotic spindle assembly.

Interestingly, one mitotic role shared by these two binding partners seems to be in regulation of the APC/C. Studies from the Van Deursen laboratory established that Nup98 and Rae1 together are required for binding to the cdh1-form of the APC/C in cultured cells (Jeganathan et al., 2005). This binding unexpectedly promotes stabilization of the securin protein, which had formerly been thought to be a specific target of the cdc20-APC/C, and thus contributes to

delay in the metaphase to anaphase transition. It is uncertain whether this regulation contributes to the transition to anaphase in the egg extract, but this will be an important future question.

In order to explore potential regulation of mitotic nucleoporin function, we identified multiple phosphorylation sites within the minimal Nup98 region required for affects on spindle assembly. Mitosis-specific sites were selected for additional analysis based on their conservation between human and Xenopus. Additional phosphorylation sites were identified but not pursued here due to location outside the minimal region, lack of conservation, or phosphorylation in interphase extract. However these sites will be tested subsequently for other potential functions. It is interesting that the sites we chose to investigate were identified by two different phosphorylation prediction algorithims (Zhou et al., 2004) to be potential sites for cyclin-dependent kinases, albeit with varying degrees of homology to the concensus sequence. Indeed, we found that the long but not the short fragment can be strongly phosphorylated in vitro by purified cyclinB/cdk1 (M.A.P, unpublished). Interestingly, one prediction algorithim suggests S591 as a potential substrate of plk1, and T653 is predicted to also have high potential for phosphorylation by MAPK, which is highly active in the egg extract.

Mutation of two of the chosen residues, S591 and S595 altered the extent of microtubule polymerization induced by the Nup98 C-terminus. Mutation of these residues had opposing effects on the excess microtubule phenotype, with S591A substantially reducing the phenotype and S595A somewhat enhancing

the phenotype. We have not detected significant changes in the ability of these mutant C-terminal domains to bind MCAK, however due to the close proximity of these residues to each other, it is possible that there is interplay between their phosphorylation states which may complicate this analysis and will require further experimentation. Phosphorylation-induced changes in conformation within this region of Nup98 seem unlikely since preliminary structural analysis suggests that the region N-terminal to aa 710 is disordered.

In conclusion, from this and other studies, a novel mitotic checkpoint seems to be materializing in which the relocalization and functional contribution of nucleoporins to the assembling spindle serves as a signal that the nuclear envelope is disassembled. This ensures that a stable mitotic spindle does not form while the nuclear envelope is still intact. Nup98 is one of the first nucleoporins released from the NPC in prophase, leaving the pore even before nuclear envelope breakdown is completed (Dultz et al., 2008). In this way, Nup98 becomes rapidly available to contribute to regulation of MCAK and promote spindle formation.

EXPERIMENTAL PROCEDURES

Preparation of Xenopus CSF extract and Spindle Assembly Assay

CSF extract was prepared as described (Desai et al., 1999a). To concentrate the extract, eggs were spun through 750 μl of Nyosil oil (Nye Lubricants, Inc., Fairhaven, MA) before crushing. Demembranated sperm chromatin were isolated as described (Powers et al., 2001). Spindle assembly assays were performed in CSF extract essentially as described (Desai et al., 1999a). The extract was supplemented with 145 nM X-rhodamine tubulin (Cytoskeleton, Inc., Denver, CO) and sperm chromatin at 500/μl. Nup98 C-terminal fragment or BSA was added to 20 μl of this mix at a final concentration of 6 μM. For antibody addition, affinity purified anti-human Nup98 C-terminal domain (Griffis et al., 2002) was added at 200 μg/ml and affinity purified anti-Xenopus Nup98 C-terminus was added at 100 μg/ml. As a control, non-specific Rb IgG (Calbiochem: La Jolla, CA) was used at equivalent concentrations. Assays were incubated at room temperature and 2 μl aliquots were combined with 2μl of fix (60% glycerol, 1X MMR, 1 mg/ml Hoechst, 10% formaldehyde). Samples were analyzed using a BX60 microscope (Olympus, Tokyo, Japan) with an 8-bit camera (Dage-MTI, Michigan City, IN) and IP Lab software (Scanalytics, Fairfax, VA). Antibody specificity controls were prepared by pre-incubating 4 μg of antibody with ~2.5 μg purified Nup98 C-terminus before addition to the assay mix. Spindles were quantified as described in Figure Legends. Error bars are calculated as Standard Error of the Proportion (SEP) using the equation: $\sqrt{(P(1-P)/n)}$ where P= percent of sample and n= total number of spindles.

Tubulin Spin down Assay

Tubulin spin downs were performed as described (Budde et al., 2001), with some modifications. The extract was supplemented with X-rhodamine labeled tubulin as above and sperm chromatin at 5000/µl. Nup98 C-terminal fragment or BSA was added to 25 µl of this mix and assays were incubated at room temperature for 30 minutes. Assays were diluted by triturating in 0.5 ml buffer (30% glycerol, 1X BRB80 (80 mM K-Pipes, pH 6.8, 1 mM $MgCl_2$, 1 mM EGTA), 1% Triton X-100) and layered onto a 1 ml cushion (40% glycerol, 1X BRB80). Polymerized tubulin was pelleted in a microcentrifuge for 10 minutes at 13,200 rpm, RT. The supernatant was aspirated, and the interface was washed with water. The wash and most of the cushion were aspirated, and the sample was spun for 3 minutes as above. The remaining supernatant was removed and tubulin pellets were resuspended in 200 µl gel sample buffer (SB). Two microliters of each sample were resolved on a 10% SDS-PAGE gel and immunoblotted for α tubulin (Sigma-Aldrich; 1:10,000) or γ-tubulin (Sigma-Aldrich; 1:5,000).

Depletions and protein add back

Depletions were performed as described (Desai et al., 1999a) with some modifications. Antibodies were bound to Affi-prep protein A beads (Biorad, Hercules, CA) at 8 µg/25 µl beads, blocked and washed as described. For depletions, 25 µl of antibody-bound beads were rotated with 200 µl extract, 4^0 C,

30 minutes. The partially depleted extract was transferred to a fresh set of antibody beads, and rotated for another 30 minutes. For protein add back, only one 45 minute depletion was used. Either BSA or Nup98 C-terminal fragment was added to the depleted extract at a concentration of 1.5 μM.

Ran Aster Assay

Ran aster assays were performed as described (Wilde and Zheng, 1999) with some modifications. ZZ-RanQ69L (the kind gift of K. Weis, UC Berkeley) was added to Xenopus CSF extract at 25 μM. The extract was supplemented with X-rhodamine tubulin as above, and Nup98 C-terminus or BSA was added at a final concentration of 6 μM. Samples were fixed and analyzed as above.

Expression and purification of recombinant proteins

Nup98 C-terminal fragments, AA 505-920, AA 676-920, and AA 505-920 S864A were subcloned into the pET 28a vector and expressed as N-terminal His-tagged proteins in *E. coli* BL21(DE3) cells. Truncated Nup98 C-terminal fragments were generated by substitution of a stop codon for residues 668, 712, 775, or 864 by Stratagene Quickchange. The cells were grown to an $O.D._{600}$ ~0.6 and induced with 0.5 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG) at 37^0 C for 3 hours. The proteins were purified and dialyzed at 4° into 20 mM Hepes (pH 7.5), 150 mM NaCl. Glycerol was added to 5% and the protein was aliquoted, flash frozen, and stored at -80^0 C. His-ZZ-RanQ69L was expressed in *E. coli* M15 pREP cells, purified as above, and dialyzed into CSF-XB buffer.

Immunofluorescence localization of mitotic Nup98

To detect mitotic localization of Nup98 in primary endothelial cells and in cell lines, we followed a protocol developed by the Salmon lab (Canman et al., 2000) (http://www.bio.unc.edu/Faculty/Salmon/lab/protocolsimmunofluorescence.html) except that methanol-free formaldehyde (Polysciences, Warrington, PA) was used in place of glutaraldehyde. Cells were mounted with Vectashield (Vector Laboratories, Burlingame, CA). MEFs were fixed and then permeabilized as previously described (Griffis et al., 2002). The following antibodies were used: anti-Nup98 GLFG monoclonal (Sigma-Aldrich; 1:500 in MEFs and endothelial cells, 1:200 in HeLa and CHO cells), anti-γ tubulin (Sigma-Aldrich; 1:250 in primary cells, 1:8000 in cell lines), goat anti-rat Alexa 488 (Invitrogen, 1:2000), goat anti-mouse Alexa 555 (Invitrogen, 1:16,000).

Centrosome purification

Centrosomes were prepared from primary human lung fibroblasts as described (Moudjou and M., 1998). Following the discontinuous sucrose gradient, fractions were collected from the bottom of the gradient, diluted with 1 ml buffer (10 mM PIPES, pH 7.2) and pelleted. Protein pellets were resuspended in SB and immunoblotted.

Protein pulldowns, and lambda phosphatase treatment for phosphorylation studies

For radioactive labeling, GST-Nup98 fragments were added to CSF extract in the presence of 267 µCi/ml γ32P-ATP (10mCi,3000 Ci/mmol; Amersham;) and incubated for 20 min, RT. Protein was recovered on glutathione beads, washed, and eluted in SB.

For analysis of gel shifts or phospho-site mapping, the Nup98 fragments were added to 100 µl of CSF extract and incubated for 60 minutes at RT. Samples were diluted in 1 ml of XB with protease and phosphatase inhibitors (XB-PI; 5mM NaPyrophosphate, 5mM NaF, 2mM NaOrthovanadate, and 10 µg/ml of Chymostatin and Leupeptin), spun at 7,500 x g, 4° for 1 minute, and the supernatant from each was added to 150 µl anti-T7 beads (MS/MS) or 15 µl His-bind resin (gel shifts). Samples were incubated at 4^0 for 60 minutes with rotation. T7 beads were washed 1 time with XB-PI, 2 times with XB-PI + 0.5M KCL, and 1 time with XB-PI. Bound protein was eluted with SB, resolved by SDS-PAGE, and detected with Imperial stain (Pierce). The desired bands were excised for MS/MS analysis.

His-bind resin was washed 4 times with XB salts + protease and phosphatase inhibitors, or if the samples were to be treated with lambda phosphatase, the last two washes were in XB-PI minus the phosphatase inhibitors. For phosphatase treatment, 50 µl of 1X phosphatase buffer and 1µl of lambda phosphatase (New England Biolabs) were added to beads and incubated

at 30^0 C for 30 minutes. Digestion was stopped with 50 µl of 2X SB. Proteins were detected by immunoblotting with anti-T7 antibody (Novagen) at 1:10,000.

In-gel digestion and mass spectrometry analysis of Nup98 phosphoproteins

Following SDS-PAGE, the Nup98 protein bands stained with Coomassie Brilliant Blue were subjected to in-gel reduction with dithiothreitol followed by alkylation of the cysteine residues by iodoacetamide. The proteins were in-gel digested (24 h at 37 °C) using sequencing grade trypsin (Promega, Madison, WI) or Lys-C endopeptidase (WAKO Biochemicals, Richmond, VA) and the peptides were extracted using 60% v/v acetonitrile, 10 mM in ammonium hydrocarbonate, pH 8.6. After enzymatic digestion, the samples were dried down completely in a SpeedVac rotary evaporator and reconstituted by first adding 10 ul of a solution of 1% acetonitrile/0.05% formic acid/0.025% aqueous trifluoroacetic acid followed by vortexing. The sample was then spun down in a microcentrifuge and an aliquot of 2 ul (20% of total digest) was used for nanoLC-ESI-MS/MS analysis.

The system used for the analysis was an Ultimate 3000 nanoHPLC system (Dionex, Sunnyvale, CA) with an autosampler and column switching for on-line desalting. A 0.3 x 5 mm PepMap-C18 cartridge (Dionex) was used for trapping/desalting. A nanobore column (0.075 x 150 mm PepMap-C18, 100 Å pore size, 3 um particle size; Dionex) was used as the analytical column. The LC effluent was directly sprayed into the QSTAR XL (Applied Biosystems, Foster City, CA) system using a Proxeon Biosystems (Odense, Denmark)

nanoelectrospray source. Peptide elution from the column was affected using an acetonitrile gradient from 2% v/v to 80% v/v containing 0.1% aqueous formic acid as a counter ion. The flow rate was set at 250 nL/min and the total LC run time was 140 min including 20 min equilibration.

The QSTAR XL was operated in the information dependant acquisition (IDA) mode as described (Khwaja et al., 2007). Briefly, for each cycle, a single MS spectrum was acquired followed by up to 4 MS/MS spectra based upon observed ions in the MS spectrum. The MS spectrum was acquired over the m/z range of 350 to 1600 with the accumulation time set to 1 s. Each MS/MS spectrum was acquired over the m/z range of 50 to 1600 using Q2 pulsing and an accumulation time of 2.5 s. Precursors were determined by each cycle's MS spectrum from the m/z range of 375 to 1300 with a threshold of 30 counts.

The data from each sample was processed by ProteinPilot software (Applied Biosystems) using the software setting which puts special emphasis on finding potential phosphorylation sites. The search engine used the Uniprot/SWISSPROT database as the protein database source.

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Chapter 3

Microtubule Flux

Microtubule flux, otherwise known as treadmilling, occurs when tubulin subunits are added to the plus end of a microtubule polymer and are balanced by loss of tubulin subunits on the minus end (Margolis and Wilson, 1978). This process was first observed during *in vitro* experiments with purified tubulin, but eventually tubulin flux was detected within spindle microtubules as well (Mitchison et al., 1986; Mitchison, 1989). In the spindle, tubulin subunits could be seen fluxing from the kinetochore to the spindle pole, and this poleward flux could be inhibited with non-hydrolyzable ATP analogs, suggesting that one or many molecular motors may be involved in the process (Sawin and Mitchison, 1991). More recent work in *Xenopus* egg extracts has provided evidence that the plus-end directed kinesin, Eg5, is required for both poleward flux and bipolarity (Kapoor et al., 2000; Mitchison et al., 2004; Miyamoto et al., 2004). However, in cells, inhibition of Eg5 had only minimal effects on flux, and instead, the depolymerizing kinesin Kif2a, which localizes to microtubule minus ends, drives flux in mammalian cells (Cameron et al., 2006). Microtubule depolymerization by kinesins at the minus end is counterbalanced by CLASP, a microtubule plus end-associated protein that stimulates polymerization (Maiato et al., 2003; Maiato et al., 2005; Maiato et al., 2004; Maiato et al., 2002). Poleward flux is important for moving chromosomes during chromosome segregation and for maintaining spindle length.

Investigating flux

Many tools have been developed over time to study microtubule flux within cells. Early experiments used tubulin analogs to map the site of tubulin incorporation into fibers within the spindle. Cells were injected with biotin-labeled tubulin subunits, which loaded onto the microtubule polymer near the kinetochore and traveled towards the pole (Mitchison et al., 1986). DIC microscopy and fluorescence imaging of microtubules were also utilized to study microtubule dynamics *in vivo*. There were several drawbacks to these early methods; DIC requires relatively flat samples and could not be used in thicker areas of dense microtubules such as mitotic spindles, and fluorescent microscopy of microtubules in live cells was difficult due to photodamage of the cells (Cassimeris et al., 1988 ; Schulze and Kirschner, 1988). More sophisticated protocols emerged soon thereafter, in which cells were microinjected with either rhodamine-labeled tubulin or tubulin labeled with a photoactivatable fluorescent probe (Gorbsky et al., 1988; Mitchison, 1989). Labeled tubulin subunits incorporate into the spindle structure, and areas of fluorescence could either be bleached or photoactivated using lasers. The resultant area of modified tubulin would then contrast with the surrounding spindle fibers, and using time-lapse microscopy, movement of those tubulin subunits could be followed.

Fluorescent speckle microscopy was discovered serendipitously during experiments in which cells were being microinjected with rhodamine tubulin to study microtubule dynamics during cellular migration (Waterman-Storer and Salmon, 1997). Cells injected with relatively low amounts of rhodamine tubulin

contained microtubules with varying fluorescence intensity along the length of the microtubule polymer due to random incorporation of labeled and unlabeled tubulin subunits. This resulted in a “speckled” appearance that could be used in conjunction with time-lapse microscopy to investigate the kinetics of microtubule dynamics and tubulin subunit turnover through space and time (Waterman-Storer et al., 1998). Speckle microscopy has since become a more general tool for studying dynamics of other cellular polymers such as actin, as well as proteins associated with these polymers (Bulinski et al., 2001; Kapoor and Mitchison, 2001; Ponti et al., 2003).

Fluorescent speckle microscopy requirements

In order to utilize fluorescent speckle microscopy (FSM) as a tool for studying flux, very low amounts of labeled tubulin subunits must be used. If 10% or higher of the total tubulin concentration available for polymerization is labeled, the microtubule polymer will appear evenly labeled (Danuser and Waterman-Storer, 2006). This is due to the high density of tubulin heterodimers in the microtubule polymer, which results in over 1000 tubulin heterodimers per micron in an image. Thus, if too many labeled tubulin subunits incorporate into the polymer, they are not resolved at the level of the light microscope and appear as a continuously labeled polymer. However, if the concentration of labeled tubulin is too low, the speckles are too sparse, making analysis of microtubule dynamics impossible; generally addition of ~0.5%-2% labeled tubulin results in optimum speckle imaging (Danuser and Waterman-Storer, 2003).

One drawback to using small amounts of labeled tubulin is that the resultant speckles are very dim, so another important requirement for FSM is a sensitive imaging system. FSM was originally developed using wide-field epifluorescent microscopy, but has since been adapted for both spinning disk confocal and total internal reflection fluorescent microscopy (TIRF) (Adams et al., 2004; Adams et al., 2003; Waterman-Storer et al., 1998). Cellular microtubule dynamics occur very quickly, and are not easily captured by a traditional scanning confocal microscope. The spinning disk in a spinning disk confocal system splits the laser into multiple beams that can collect information simultaneously from a large region. TIRF microscopy eliminates background fluorescence by selectively exciting a small region of fluorophores in a sample, leading to greater contrast between speckles and background noise. Furthermore, with both spinning disk confocal and TIRF microscopy, data can be collected quickly, which avoids photobleaching of fluorescence.

FSM analysis

In the beginnings of FSM, speckles were tracked through time by hand, which was inefficient and prone to error (Salmon et al., 2002; Schaefer et al., 2002; Watanabe and Mitchison, 2002). However, kymograph analysis allowed for easily obtainable values on average velocity and direction of speckles (Waterman-Storer et al., 1998). To generate a kymograph, a thin rectangular region parallel to the movement of the speckles is selected from a timelapse image (Figure 20A). This same region is then extracted from each image in the

timelapse and aligned in sequence vertically. This allows for comparison of speckle movement through time, and rates of movement can be calculated. In the work described below, we employed kymograph analysis to asses whether Nup98 C-terminal domain altered the tubulin flux rate. Newer, even more advanced tools for mapping large subsets of speckle movement through time are being developed, however they are beyond the scope of this dissertation and hence will not be discussed.

FSM and Nup98

Early in our study of Nup98 in mitosis, we observed long microtubules when adding the Nup98 C-terminal domain (aa 505-920) to spindle assembly assays in *Xenopus* egg extract. We hypothesized that these long microtubules might be the result of a decrease in the tubulin flux rate, which in *Xenopus* is ~2 μm/min (Maddox et al., 2003). Furthermore, addition of Nup98 antibodies to spindle assembly assays appeared to greatly reduce the length of microtubule polymers, which suggested an increase in tubulin flux.

In order to further investigate the hypothesis that Nup98 was affecting tubulin flux, we utilized fluorescent speckle microscopy analysis of spindles formed in the egg extract. Spindles were formed in the presence of either BSA or the Nup98 C-terminal domain and speckle-levels of rhodamine-labeled tubulin, and either a spinning disk confocal microscope or a wide-field microscope was used to generate time lapse images. In other experiments, BSA and the Nup98 C-terminal region were added to spindles preformed in the extract.

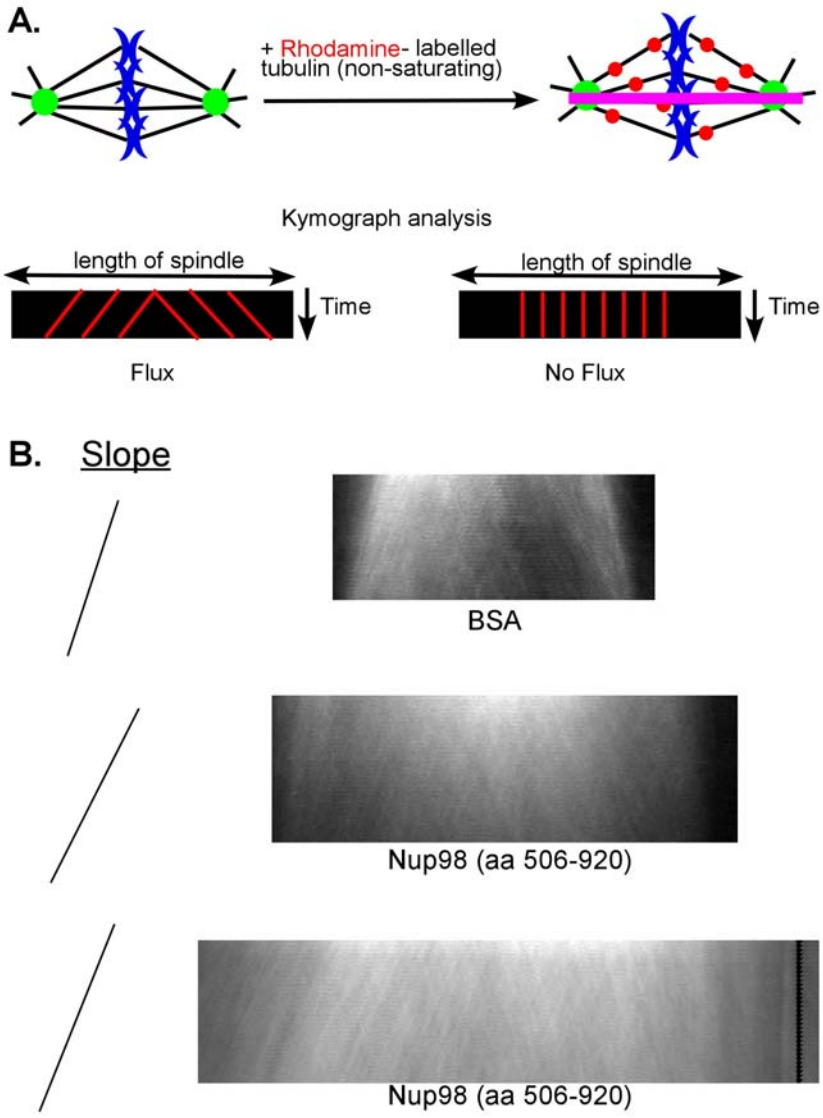
Speckles fluxing from the kinetochore towards the pole result in a diagonal line from the center of the kymograph towards either end of the kymograph. A loss of tubulin flux results in a kymograph containing completely vertical lines, representing speckles that do not move over time. If a reduction of tubulin flux occurs without loss of flux entirely, the slope of the line becomes steeper, representing slower movement of the speckle through the microtubule polymer.

Kymographs generated from spindles formed in the presence of Nup98 C-terminal domain proved to be difficult to analyze due to the lack of well-defined spindle poles. However, experiments in which the proteins were added to spindles preformed in extract produced analyzable data. Kymograph analysis of one such experiment using wide-field microscopy is shown in Figure 20, panel B. These spindles were preformed in extract before being exposed to excess Nup98 C-terminal domain, and the images collected show evidence of an increased distance between the two spindle poles and increased microtubule density when compared to spindles exposed to BSA. This is in keeping with the preformed spindle experiments presented in Chapter Two (Figure 13B). Comparison of the resultant slope of speckle movement between the control kymograph and the kymographs from spindles containing Nup98 C-terminus are identical, which is evidence that tubulin is moving from the center of the spindle to the spindle pole at the same rate.

In subsequent experiments, we determined that the effect of antibody addition on microtubule polymers was due to the presence of thimerosol, a preservative agent used to inhibit bacterial growth in our affinity purified

Figure 20. Kymograph analysis of speckle microscopy. A) Cartoon diagram of kymograph analysis. Non-saturating amounts of rhodamine-labeled tubulin (red) are added to spindles, and time-lapse images are taken. A slim rectangular region (orange bar) is selected from the first image and each subsequent image, and a kymograph is generated from the montage of these images. **B)** Kymographs of spindles formed in *Xenopus* egg extract and treated with either BSA or Nup98 C-terminal domain.

Figure 20


A.
+ Rhodamine- labelled tubulin (non-saturating)
Kymograph analysis
length of spindle
Time
Flux
length of spindle
Time
No Flux
B. Slope
BSA
Nup98 (aa 506-920)
Nup98 (aa 506-920)

antibodies. Thimerosol is a potent inhibitor of microtubule assembly *in vitro* (Brunner et al., 1991). Our experiments show that addition of small amounts of thimerosol to egg extract inhibits microtubule polymerization, and when added to preformed spindles in the extract, leads to a highly reproducible 25% reduction in spindle length from pole to pole (Cross and Powers, unpublished).

When retested in spindle assembly assays, Nup98 C-terminal inhibitory antibodies to which thimerosal had not been added resulted in an increase in monopolar spindles. Tubulin flux in spindles formed in *Xenopus* egg extract begins upon bipolarization of the spindle, and thus, monopolar spindles do not flux (Mitchison et al., 2004). Furthermore, antibody addition had no effect on preformed spindles in the extract.

Our conclusion from the speckle microscopy studies is that Nup98 does not alter tubulin flux. Kymograph analysis of protein addition to preformed spindles clearly demonstrates that, although Nup98 C-terminus addition generates long microtubules, the tubulin dimers move polewards through these spindles at approximately the same rate as controls. While the shortened spindles generated by thimerosal addition to preformed spindles fit with a model in which flux was altered, the lack of any affects to preformed spindles when thimerosal is absent no longer supports the flux model. However, this analysis led us to investigate other mechanisms by which excess microtubule polymerization may occur, and as discussed in Chapter Two, we now propose a model in which the C-terminus of Nup98 contains a functional domain that can regulate the microtubule depolymerizing kinesin, MCAK.

Chapter 4

Conclusions

In the research presented here, we have provided evidence for a novel function of Nup98 during mitosis. Our data support a role for Nup98 in assembly of correct bipolar spindle structure through regulation of microtubule dynamics. We have observed that endogenous Nup98 is required for forming a bipolar spindle; when Nup98 is immunodepleted from *Xenopus* egg extract, an increase in monopolar spindles is observed. Furthermore, this mitotic function of Nup98 is governed through a domain within the C-terminus that has no previously attributed function. This activity of Nup98 can be constrained by addition of inhibitory antibodies directed specifically towards the C-terminal domain, and spindle bipolarity can be restored to Nup98 depleted extracts by addition of purified Nup98 C-terminal domain.

There are multiple pathways that lead to bipolar spindle formation. In mammalian cells, bipolarity is primarily achieved through a centrosome-driven "search and capture" mechanism. However, chromatin can also drive spindle assembly through a pathway regulated by the small GTPase Ran. The Ran driven pathway is most prevalent in cellular systems lacking centrosomes, such as *Xenopus* oocytes, but other labs have shown that this pathway does exist in cells containing centrosomes as well and may make important contributions even in these cells (Kalab et al., 2006; Khodjakov et al., 2000). Our experiments establish that Nup98 is not required for the Ran pathway of spindle assembly. RanGTP induced microtubule polymerization can occur in egg extracts depleted

of Nup98. However, addition of the C-terminal domain of Nup98 to Ran asters still has a dominant effect, leading to an increase in microtubule polymerization.

The regulation of microtubule dynamics in the vicinity of the spindle is critical for forming a bipolar spindle structure. Two prominent microtubule destabilizers, Op18/Stathmin and MCAK, are inhibited during spindle assembly through phosphorylation by Aurora B, a member of the chromosomal passenger complex (Figure 19B) (Gadea and Ruderman, 2006; Kelly et al., 2007; Sampath et al., 2004). Op18/stathmin functions by sequestering free tubulin heterodimers, thus lowering the free tubulin concentration available for microtubule polymerization and promoting catastrophy, and MCAK is a microtubule depolymerizing kinesin (Cassimeris, 2002). Lack of phosphoregulation of these proteins inhibits correct bipolar spindle formation (Budde et al., 2001; Ohi et al., 2004).

Our model, proposed in Chapter Two, suggests that Nup98 regulates mitotic microtubule dynamics through MCAK (Figure 19B). The correct regulation and localization of MCAK activity is essential for spindle formation and biplolarity. We observed that the long microtubules resulting from excess Nup98 activity are identical to the long microtubules that occur when MCAK activity is overly inhibited (Walczak et al., 1996). Inhibition or depletion of Nup98 leads to an increase in monopolar spindles. Similarly, an increase in MCAK activity, through loss of regulation by the CPC, also results in an increase in monopolar spindles (Ohi et al., 2004; Sampath et al., 2004). The size and microtubule density of our monopolar spindles are especially reminiscent of the monopolar spindles formed

in extracts depleted of Dasra, a component of the CPC (Sampath et al., 2004). Therefore, our model proposes that Nup98 interacts, either directly or indirectly, with MCAK to downregulate the depolymerizing activity of MCAK. Indeed, we were able to detect an interaction between Nup98 and MCAK in pulldown experiments with the Nup98 C-terminal domain.

Through immunofluorescence and co-purification, we discovered a small fraction of Nup98 associated with the centrosome. In support of this, proteomic characterization of Drosophila centrosomes found Nup98 associated with these structures (Habermann, 2008). We have not determined at this time whether centrosomal localization is specifically required for the mitotic function of Nup98. Interestingly, there is a small fraction of MCAK localized to poles of spindles and Ran asters (Walczak et al., 1996; Wordeman and Mitchison, 1995; Zhang et al., 2008). A recent study by the Walczak lab demonstrated that regulation of MCAK microtubule depolymerizing activity at the poles by Aurora A is necessary for bipolarization of Ran asters, although this regulation has not been studied for chromatin or centrosome-driven spindles. Thus, Nup98 may interact with MCAK at the centrosome to promote spindle assembly.

The novel mitotic function of Nup98 that I describe here may be regulated in part by mitotic phosphorylation. The region of the Nup98 C-terminal domain involved in this mitotic function is hyperphosphorylated in mitotic egg extracts. Mass spec analysis of the long Nup98 C-terminal fragment incubated with interphase or mitotic egg extract identified multiple mitosis-specific phosphorylation sites conserved between human and Xenopus Nup98. For a

subset of these sites, mutation to a non-phosphorylatable amino acid led to changes in the level of excess microtubule phenotype. In particular, mutation of 591 led to a reduction of the microtubule phenotype and mutation of 595 led to somewhat of an increase in the microtubule phenotype. The sites chosen were all suggested by prediction algorithms to be phosphorylated by mitosis-specific kinases.

Future Directions

There are two distinct directions in which this project can progress from here. One important direction is to continue investigations into the molecular mechanisms involved in the mitotic function of Nup98. We have identified an interaction with MCAK; it will be important to establish whether this is a direct interaction and to identify potential additional binding partners that may be involved in this activity of Nup98. A second direction we would like to further explore is the regulation of this function of Nup98. Our data suggest that the mitotic activity of Nup98 is influenced, at least in part, by phosphorylation, although it is clear that this phosphoregulation is complex and potentially involves crosstalk between multiple residues. Thus, additional experimentation is planned to further our understanding of the function of phosphorylated residues we have identified.

Binding partners

In a pulldown of the Nup98 C-terminus from *Xenopus* egg extract, we identified the mitotic centromere associated kinesin MCAK. We will initially test the potential direct regulation of MCAK activity by Nup98 using an *in vitro* microtubule depolymerization assay. When purified MCAK is incubated *in vitro* with taxol-stabilized microtubules, the microtubules are depolymerized (Ems-McClung et al., 2007). To determine whether Nup98 can directly inhibit the depolymerizing function of MCAK, we will add purified Nup98 C-terminal domain to this *in vitro* assay. If the interaction is direct, this will quickly and definitively reveal the mechanism. In parallel, we will continue investigation of the C-terminal pulldowns for other mitotic interacting proteins. Initally, we will take a candidate approach and probe this pulldown for INCENP, Survivin, Dasra/Borealin and Aurora B, all members of the CPC that regulates MCAK activity.

Beyond these known MCAK regulators, it will be important to investigate the possible association of novel proteins that may bind the Nup98 C-terminal domain in a cell cycle-specific manner. To begin to pursue this question, we sent the C-terminal domain for two-hybrid binding analysis, in hopes of identifying novel binding partners. This analysis found only one confident binding interaction between the C-terminus and the nucleoporin Pom121. While this was a novel interaction and an interesting result for the lab, it seems unlikely to be related to this project. We plan on continuing pulldowns of the C-terminal domain from both interphase and mitotic *Xenopus* egg extracts, and through mass spec will identify proteins that bind specifically during mitosis. I have generated a pulldown

protocol that results in a very clean pulldown and eliminates the majority of background from proteins binding non-specifically to beads. This protocol will be instrumental in performing the interphase/mitotic comparison pulldowns.

Mitotic binding partner identification will allow for further elucidation specific molecular mechanisms underlying the mitotic function of Nup98. Once binding partners have been identified, various experiments can be performed to further investigate the functions of these interactions. Inhibiting interaction between Nup98 and binding partners or co-depleting Nup98 and binding partners from the extract, are experiments that may produce information on molecular mechanism.

Phosphorylation

Although we have already obtained interesting results from modification of phosphorylation sites identified in our initial mass spec analysis, there is much to be done to expand this part of the project. It is clear that residues S591 and S595 appear to be involved in the novel mitotic function of Nup98. Given our model, the most straight forward hypothesis would be that phosphorylation of these residues affects binding of the C-terminus to MCAK. However, we have not observed any substantial change in MCAK binding with the S591A and S595A phosphomutants in pulldown experiments. Given the close proximity between these two residues, it is possible that the phosphorylation status of one can influence the phosphorylation status of the other. Futhermore, in 30% of experiments, there was no change in the microtubule phenotype when S591 or

S595 were mutated, indicating that this phosphoregulation is a complex mechanism that we don't fully understand.

Ongoing mass spec analysis has identified additional phosphorylated residues that were not detected in our initial experiments. Further, there were phosphorylated residues that did not meet our criteria for the initial studies, but are nonetheless of interest. Thus, a future direction is to continue mutation of mitosis-specific phosphorylation sites to determine if additional residues can affect the excess microtubule phenotype.

Mitotic phosphorylation is also thought to be responsible, at least in part, for initiating disassembly of the nuclear pore complex at mitosis. In analysis of nuclear pore disassembly, Nup98 was found to be the first nucleoporin to disassociate during mitosis (Dultz et al., 2008). Thus, it will be interesting to characterize our phosphomutants within a cellular context, to see if they are still capable of associating with the pore. In particular, we detected phosphorylation of S822, which is outside of our region of interest, but is in an appropriate site within the C-terminal structure to potentially regulate association with the pore. As well, these phosphorylations may affect the localization of the small subpopulation of Nup98 we identified at the centrosome. All of these are important questions that can be pursued in the future.

Final thoughts

Nup98 and Rae1 during mitosis

A subfraction of Nup98 is found in a complex with its binding partner Rae1 during mitosis (Pritchard et al., 1999). As discussed in the introduction to this dissertation, the Nup98-Rae1 complex has been previously implicated in two distinct roles during mitosis. Evidence from mouse models supports a role for this complex in inhibition of the APC/C during metaphase (Jeganathan et al., 2006; Jeganathan et al., 2005). As well, Rae1 was found in complex with a variety of other spindle assembly factors and RNPs, and this study implied that Nup98 may be involved in the sequestration of this complex by Importin β (Blower et al., 2005). However, neither of these mitotic functions of Nup98 is well understood, and it has not been clearly demonstrated that Nup98 contributes to either the Rae1-RNP complex or Rae1-NuMA interaction.

It is clear from the data presented in Chapter Two that the novel functional domain of Nup98 that I have described in this dissertation is contained within the C-terminus. Restoration of bipolar spindles in Nup98-depleted extract by addition of purified C-terminal domain suggests that this function does not require interaction between Nup98 and Rae1. Addition of excess purified Rae1-binding domain to spindle assembly assays, which has been shown to compete with endogenous Rae1 for Nup98 binding, resulted in wild-type spindle formation (Blevins et al., 2003). This result again suggests that interactions between Rae1 and Nup98 are not required for proper bipolar spindle assembly in *Xenopus* egg extract.

Implications for Nup98 in cancer

The NUP98 gene is involved in multiple chromosomal translocations that have been identified in leukemia patients (Borrow et al., 1996; Nakamura et al., 1996). Such leukemogenic fusion proteins lack the C-terminal domain of Nup98, and thus, the level of the regulatory domain described in this dissertation is reduced by 50% in leukemic cells. It has been proposed that one leukemogenic mechanism of these fusion proteins is to activate and repress genes, given that the N-terminus of Nup98 can bind to histone modifiers and several fusion partner proteins are capable of binding to chromatin (Bai et al., 2006; Kasper et al., 1999). However, loss of this MCAK regulatory domain may serve as an additional contributing mechanism.

Recently, our lab localized the Nup98-HoxA9, Nup98-HoxD13, and Nup98-PMX1 homeodomain fusion proteins in mitotic cells. At low levels of expression, these proteins were found adjacent to kinetochores (Xu and Powers, submitted). At higher expression levels, fluorescent puncta spread along the chromosome arms. We have been unable to detect a kinetochore localization of endogenous Nup98, which is somewhat unexpected given the molecular interaction between Nup98 and MCAK. However, given the kinetochore localization of these fusion proteins, it is possible that they are indicative of a highly transient association of the full-length Nup98 with the centromere that is somehow stabilized in the context of these leukemic fusion proteins. This question is currently under investigation in the laboratory.

Model of Nup98 in mitosis

Although we have not yet detected a stable interaction of Nup98 with the centromere region of the spindle where a subfraction of MCAK is known to reside, there are multiple ways in which Nup98 may promote the spatial inhibition of the microtubule depolymerizing activity of MCAK. It is possible that Nup98 regulates the correct positioning of MCAK regulatory protein complexes such as the CPC. Centromeric localization of the CPC results in phosphorylations of MCAK that inhibit its microtubule depolymerizing activity. Dasra, a component of the CPC, is required for efficient binding of the CPC to chromatin, and our Nup98 depletion phenotype is very similar to that obtained after Dasra depletion (Sampath et al., 2004). Although we have attempted to investigate MCAK phosphorylation levels using a phospho-specific MCAK antibody, we have been unable to detect changes in phospho-MCAK levels due to low sensitivity of the antibody in our hands.

It is also quite possible that, instead of affecting phosphorylation status of MCAK, Nup98 is regulating the level of active MCAK at the centromere by relocalizing it away from the spindle. Little is known about the regulation of soluble MCAK within the extract, and Nup98 may be bind to MCAK in the cytoplasm to regulate the level of MCAK activity at the kinetochore. Future experiments will determine the details of MCAK regulation by Nup98.

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