FG NUCLEOPORINS COORDINATE MULTIPLE TRANSPORT PATHWAYS THROUGH THE NUCLEAR PORE COMPLEX

By

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ABBREVIATIONS

Δ – deletion
ΔC – deletion of cytoplasmic face Nup FG domains: nup42ΔFG nup159ΔFG
ΔN – deletion of nuclear face Nup FG domains: nup1ΔFXFG nup2ΔFXFG nup60ΔFXF
5-FOA – 5-flouororotic acid
6xHis – six histidine epitope tag
dsRed – red fluorescent protein derived from *Discosoma* sp.
EM – electron microscopy
ER – endoplasmic reticulum
FG – phenylalanine-glycine
FRET – fluorescence resonance energy transfer
FXFG – phenylalanine-any amino acid-phenylalanine-glycine
GFP – green fluorescent protein
GLFG – glycine-leucine-phenylalanine-glycine
GST – glutathione-S-transferase
HDEL – histidine-aspartate-glutamate-leucine (amino acid sequence for ER retention)
hnRNP – heterogeneous ribonucleoprotein particle
IP_6 – inositol hexakisphosphate
Kap – karyopherin
mmp – more minimal pore
kDa, MDa – kiloDalton, megaDalton
MBP – maltose binding protein
Min – minute
miRNA – micro RNA
mmp, – more minimal pore
mRNA – messenger RNA
mRNP, messenger ribonucleoprotein particle
ms – millisecond
NE – nuclear envelope
NES – nuclear export signal
NLS – nuclear localization sequence
NPC –nuclear pore complex
Nup – nucleoporin
poly$(A)^+$ – poly-adenylated
rRNA – ribosomal RNA
SC – synthetic complete yeast growth medium
SEM – standard error of the mean
snRNA – small nuclear RNA
SR protein – RNA binding protein enriched for serine-arginine
tRNA – transfer RNA
YPD – 1% yeast extract, 2% peptone, 2% dextrose yeast growth medium

Standard one-letter and three-letter amino acid code is used throughout this document. These abbreviations are:

A	Ala	Alanine
C	Cys	Cysteine
D	Asp	Aspartic acid
E	Glu	Glutamic acid
F	Phe	Phenylalanine
G	Gly	Glycine
H	His	Histidine
I	Ile	Isoleucine
K	Lys	Lysine
L	Leu	Leucine
M	Met	Methionine
N	Asn	Asparagine
P	Pro	Proline
Q	Gln	Glutamine
R	Arg	Arginine
S	Ser	Serine
T	Thr	Threonine
V	Val	Valine
W	Trp	Tryptophan
X		any
Y	Tyr	Tyrosine

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CHAPTER I

INTRODUCTION TO NUCLEOCYTOPLAMIC TRANSPORT

The problem of organelle compartmentalization

The subcellular compartmentalization of eukaryotic cells into organelles establishes functional and regulatory separation between activities of the cell. Each organelle, however, must communicate and coordinate activities with other organelles in the cell. Such coordination requires the movement of molecules between the cytoplasm and a given organelle, and this transport process is accomplished by the use of regulatory transport pores, channels, and translocons. Precise and unique transport channels and strategies are employed for transport of molecules into each cellular organelle (SCHNELL and HEBERT 2003).

For example, the nuclear envelope (NE) encompasses chromatin and forms a physical discontinuity between the sites of transcription and translation, and thus is a barrier between the nuclear and cytoplasmic compartments. A result of this separation is additional levels at which gene expression can be regulated, but also is a need for efficient inter-compartmental signaling and trafficking. Nuclear pore complexes (NPCs) span the NE barrier. As one of the most complex and dynamic molecular translocons, the NPC regulates all movement of molecules in and out of the nucleus (SUNTHARALINGAM and WENTE 2003; TERRY *et al.* 2007; TRAN and WENTE 2006). Cellular signaling pathways often use regulated subcellular localization of factors between the nucleus and

cytoplasm to coordinate activation responses (HOOD and SILVER 1999; TERRY *et al.* 2007).

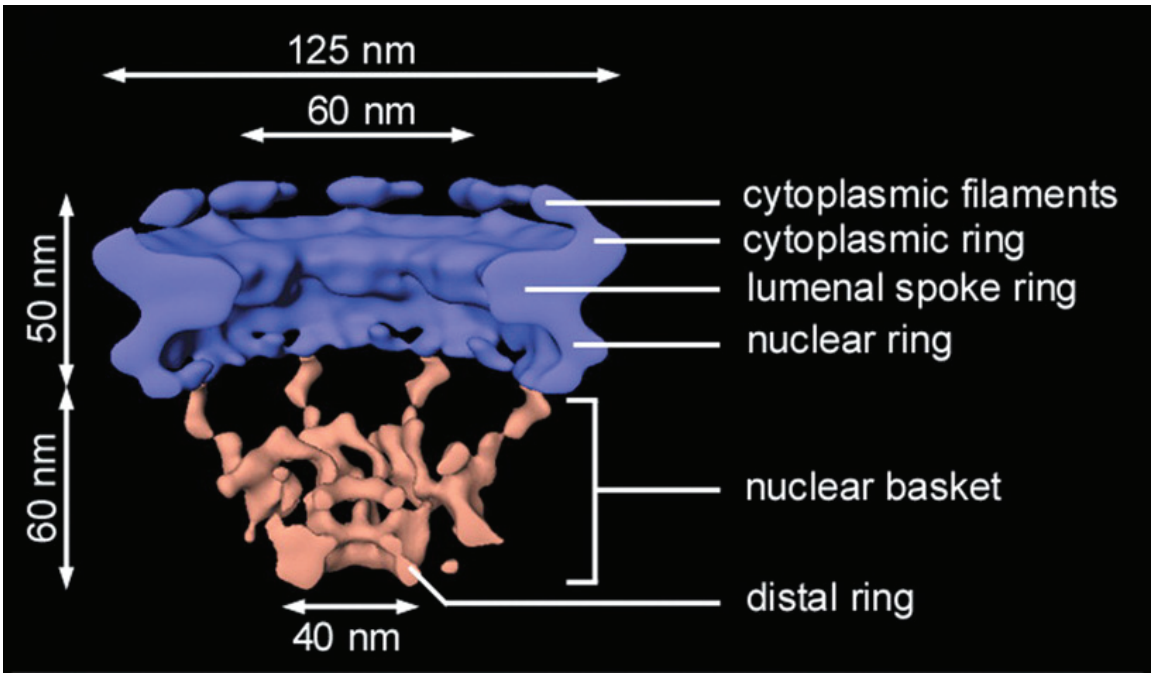
In this introductory chapter, I will discuss the cellular functions and properties of the NPC, describe the properties and functions of FG-Nups, discuss the dynamic interactions between transport receptors and FG-Nups, and illustrate proposed models of the transport mechanism. Ultimately, I will build this into a discussion of the complexities of nucleocytoplasmic transport and discuss how regulation of transport is critical in both normal and disease cell biology and physiology.

Nuclear pore complexes

NPC protein composition and robustness of transport

NPCs are large, selective transport channels assembled from multiple copies of ~30 different protein components, collectively termed nucleoporins (Nups) (Figure 1-1A) (CRONSHAW *et al.* 2002; ROUT and AITCHISON 2001; ROUT *et al.* 2003; ROUT *et al.* 2000; SUNTHARALINGAM and WENTE 2003). The NPC proteome includes transmembrane Nups, which anchor the NPC in the NE, structural Nups, and FG-Nups (Figure 1-1B) (SUNTHARALINGAM and WENTE 2003). The latter is characterized by repeats of Phe-Gly (FG), and these proteins have specific and essential roles in transport through the NPC (discussed below). Recent high through-put modeling studies find that the NPC is built from repeating structural modules (ALBER *et al.* 2007). This repetitive structure is built by sequential assembly of multiple copies (presumably in multiples of eight owing to the symmetry of the NPC) of each Nup. Structurally, NPCs have an asymmetric shape with

A.



B.

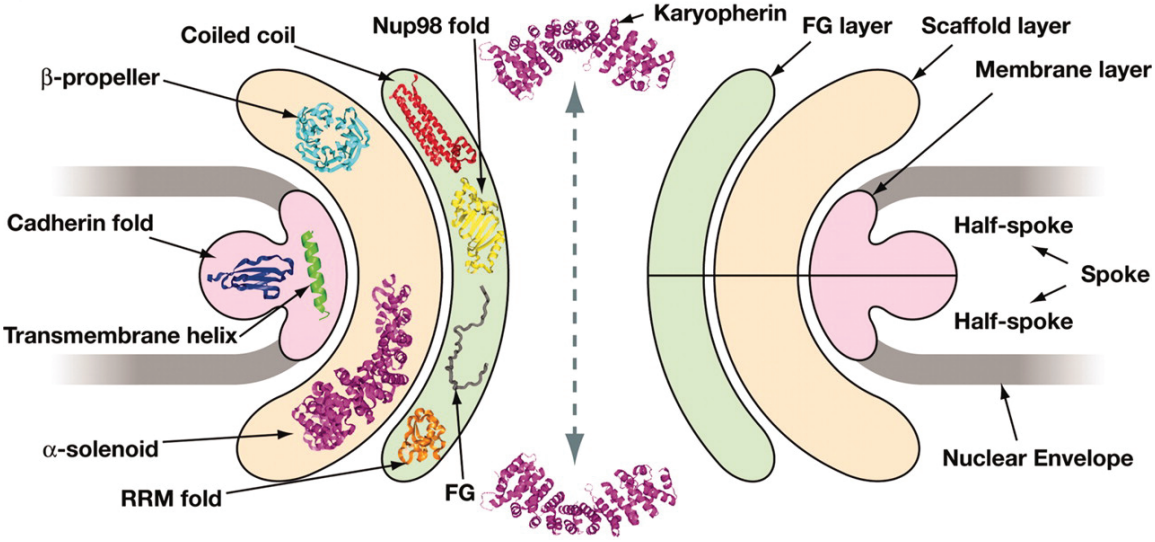


Figure 1-1. The Nuclear Pore Complex (NPC) is a multi-layered transport channel. (A). Cryo-electron tomography imaging of nuclear pore complexes of *Dictyostelium* reveals the structural features of the NPC. Reprinted from *Beck et al. 2004. Science.* (B) Schematic diagram of the protein layers that build the NPC, including the NE-associated (membrane) layer, a structural or scaffold layer, and an FG layer. Protein folding motifs common to each layer are shown as ribbon diagrams. Reprinted from *Devos et al. 2006. PNAS.*

unordered filaments extending from the cytoplasmic face of the pore (Figure 1-1A). The filaments of the nuclear side of the NPC converge into a basket structure (BECK *et al.* 2004; BECK *et al.* 2007; DENNING *et al.* 2003; VASU *et al.* 2001). Overall, the NPC has eight-fold rotational symmetry (AKEY and RADERMACHER 1993; ALBER *et al.* 2007; BECK *et al.* 2004; HINSHAW *et al.* 1992; KISELEVA *et al.* 2003). Remarkably, this overall structure and many of the components are conserved throughout eukarya (AKEY and RADERMACHER 1993; ALBER *et al.* 2007; BECK *et al.* 2004; BROHAWN *et al.* 2008; CRONSHAW *et al.* 2002; HINSHAW *et al.* 1992; KISELEVA *et al.* 2003; MANS *et al.* 2004; ROUT *et al.* 2000).

The fully assembled NPC is capable of rapid and selective transport of macromolecules. The rapidity of transport is well illustrated by consideration of the process of ribosome biosynthesis in HeLa cells: during a ~24 hour cell cycle, a HeLa cell doubles its contents, including synthesizing ~10^7 ribosomes (GORLICH and MATTAJ 1996; RIBBECK and GORLICH 2001). Assembly of these ribosomes requires steps of mRNA export, translation of ribosomal proteins, nuclear import of those proteins (~$8x10^8$ ribosomal proteins to assemble ~10^7 ribosomes), nucleolar assembly of ribosomal subunits, and, finally, export of those ribosomal subunits. Thus, each of the ~2800 NPCs in a HeLa cell must import ~100 ribosomal proteins and export ~3 ribosomal subunits per minute. All of this transport must be accomplished in the context of all of the other factors that are imported and exported from the nucleus: histones, transcriptional regulatory factors, transcription and replication machinery, intracellular and extracellular signaling regulators, *et cetera* (GORLICH and KUTAY 1999). Similarily, *Saccharomyces cerevisiae*, which has ~75-150 NPCs per cell (WINEY *et al.* 1997), is estimated to actively

transport 50-250 messenger (m)RNA transcripts per NPC per minute, along with 10-20 ribosomal subunits and up to 1,000 transfer (t)RNAs per pore per minute (HURT *et al.* 2000). In addition to transporting of all of these distinct types of RNA, these NPCs are also simultaneously trafficking large numbers of protein cargoes (GORLICH and KUTAY 1999). Thus, trafficking through the NPC is quite robust and efficient.

The NPC permeability barrier paradox

The ability of the NPC to so rapidly transport these appropriate molecules while selectively precluding inappropriate molecules from entering the nucleus is one of the mysteries of this biological machine. Transport is selective in that the NPC regulates passage of large molecules, but is permeable to movement of ions, small metabolites, and small proteins by free diffusion (SUNTHARALINGAM and WENTE 2003). Amazingly, although the NPC faithfully impedes transport of molecules larger than the ~40kDa permeability limit - it is an effective selective barrier (FRIED and KUTAY 2003). Molecules smaller than this permeability limit diffuse through at a rate that is inversely proportional to their size (PAINE *et al.* 1975). On the other end of the spectrum, the vertebrate NPC has been shown to transport signal-bearing gold particles up to 39nm in diameter (FELDHERR and AKIN 1997; PANTE and KANN 2002), as well as transporting similarly large-sized physiological cargoes, including ribosomal subunits (JOHNSON *et al.* 2002; KOHLER and HURT 2007) and Balbiani ring mRNPs (DANEHOLT 2001a). For these and other cargoes larger than the permeability barrier limit, the assistance of a transport receptor is required for movement through the NPC. Receptor-bound molecules greater than this barrier limit size move through the pore at a rate that approaches the rate of

diffusion (GILCHRIST *et al.* 2002). Thus, the NPC does not significantly slow the passage of appropriate, transport-competent large molecules. Paraxodically, binding Nups actually accelerates transport efficiency. The rate of transport through the NPC for similarly sized molecules is quite different if one of them binds Nups. Specifically, the transport receptor Ntf2 enters the nucleus ~30-fold faster than GFP, even though these two molecules are of similar size (SIEBRASSE and PETERS 2002). Therefore interactions between transport complexes and the NPC must be transient and in a manner that does not slow the movement of the transport complex through the NPC. Although molecules under the diffusive permeability barrier size limit can move across the NPC independent of a receptor, it is interesting to note that there are no known essential factors that rely solely on diffusion. This underscores the functional efficiency and importance of receptor-facilitated nucleocytoplasmic transport. Moreover, this barrier must remain selective while flexing to accommodate cargo-receptor complexes that vary over several orders of magnitude (*e.g.,* sizes from ~30kDa proteins to the ~30MDa Balbiani ring messenger ribonucleoprotein particle (mRNP)) (SUNTHARALINGAM and WENTE 2003; WURTZ *et al.* 1990). It appears that trafficking through the NPC is bidirectional (FELDHERR *et al.* 1984).

The integrity of the NPC is necessary to maintain the permeability barrier. For example, in *S. cerevisiae*, deletion of the structural proteins Nup170 or Nup188 results in NPCs that are "leaky" to diffusion of larger molecules than wild-type pores permit (SHULGA *et al.* 2000). Both of these proteins are linked to structural roles in NPC assembly, and, at least in the case of the *nup170*Δ (Δ, deletion) mutant, NPC assembly is impaired, preventing incorporation of a subset of structural Nups and FG-Nups (KENNA

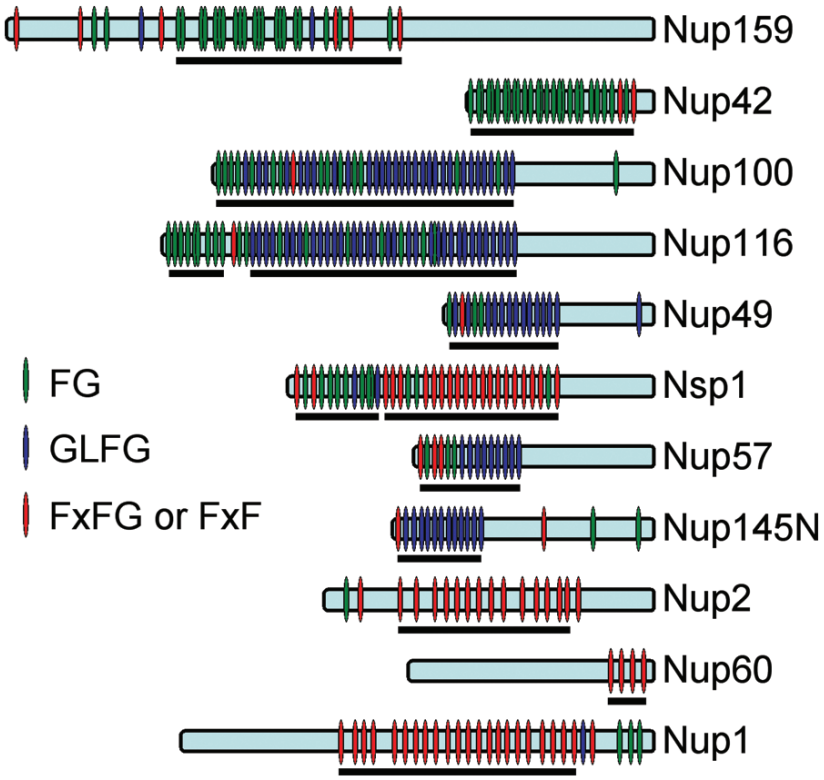
et al. 1996; NEHRBASS *et al.* 1996). Thus, Nup deletions from the NPC can alter permeability barrier integrity. Further, the barrier that remains in these cells has increased sensitivity to aliphatic alcohols (SHULGA and GOLDFARB 2003). This suggests that: (a) the barrier integrity is partially compromised in certain Nup deletion cells; and (b) the barrier is likely maintained by hydrophobic interactions between FG-Nups (PATEL *et al.* 2007). Certain cells use alterations to the nuclear permeability barrier to regulate transport. The filamentous fungus *Aspergillis nidulans* partially disassembles its NPC, removing both structural and FG-Nups, in a cell-cycle dependent manner (DE SOUZA *et al.* 2004; OSMANI *et al.* 2006). The direct consequence is that these nuclei have a relaxed permeability barrier, and this is correlated with nuclear import of cell cycle machinery; presumably the nuclear entry of these cell cycle regulators is controlled at the level of the NPC permeability barrier.

FG-Nucleoporins

FG-Nups: repeat motifs and sequence composition

The FG-Nups are a unique protein family with a variety of functions related to regulating nuclear import and export. Specifically, we hypothesize that FG domains contribute to both the permeability barrier and to the active translocation mechanism. At the primary amino acid sequence level, FG-Nups have domains with clusters of repeats of Phe-Gly followed by a characteristic spacer sequence (Figure 1-2) (ROUT and WENTE 1994). The core repeat unit of each FG-repeat is either Phe-Gly (FG), Gly-Phe-Leu-Gly (GLFG), or Phe-any-Phe-Gly (FXFG) (ROUT and WENTE 1994). In addition to bearing

(A)



(B)

MFGFSGSNNG
FGNKPAGSTG
FSFGQNNNNTNTQPSASG
FGFGGSQPNSGTATTGG
FGANQATNT
FGSNQQSSTGG
GLFGNKPALGSLGSSSTTASGTTATGT
GLFGQQTAQPQQSTIGG
GLFGNKPTTTTG
GLFGNSAQNNSTTSG
GLFGNKVGSTGSLMGGNSTNTSNMNAG
GLFGAKPQNTTATTG
GLFGSKPQGSTTNG
GLFGSGTQNNNTLGGGGLGQ

Figure 1-2. FG-repeats cluster as FG-domains in FG-Nups.
(A) Schematic diagram of the 11 FG-Nups in S. cerevisiae. Each FG repeat is represented by an oval (Green, FG; Blue, GLFG; Red, FXFG). The region deleted in our FGΔ mutants is indicated by the black bar. Reprinted from *Strawn et al. Nature Cell Biology 2004.*
(B) The primary amino acid sequence for the GLFG domain of Nup57. This sequence corresponds to the black bar in part (A) under Nup57, and is the region deleted in our FG Δ mutant collection.

subtly different core repeats, the spacer sequences between FG, GLFG, and FXFG repeats differ slightly. FXFG-repeats are enriched for Ser and Thr, and tend to be highly charged; GLFG-repeats are devoid of acidic residues and are enriched for Asn and Gln. Repeats with an FG core appear to be more degenerate and may have either spacer type. Others have subcategorized FG core repeats further (*e.g.*, PSFG; (PATEL *et al.* 2007)), but these repeats do not have unique spacer sequences, and thus we group them with the FG class of repeats.

FG-Nups are anchored in subcomplex structures throughout the NPC (ROUT *et al.* 2000; SUNTHARALINGAM and WENTE 2003), and their flexible filaments may occupy/reach a dynamic range of topological positions (DENNING *et al.* 2003; FAHRENKROG *et al.* 2002; LIM *et al.* 2006b). In *S. cerevisiae*, three FXFG repeat-containing Nups are found exclusively on the nuclear basket face of the NPC – these are Nup1, Nup2, and Nup60 (Table 1-1) (ROUT *et al.* 2000). The FG-Nups Nup42 and Nup159 are components of the cytoplasmic fibrils. The FXFG repeat-containing Nup Nsp1, as well as the GLFG-containing Nup49 and Nup57 are distributed centrally or symmetrically in the NPC. The GLFG-containing Nups Nup100 and Nup116 are biased towards the cytoplasmic face of the pore, whereas Nup145N, also a GLFG repeat Nup, localization is biased towards the nuclear face of the pore (ROUT *et al.* 2000). Given the homology of both sequence and function of Nup100, Nup116, and Nup145N, and their apparent evolutionary relationships (MANS *et al.* 2004; WENTE *et al.* 1992) (see below), we consider the net distribution of these to be effectively symmetrical (STRAWN *et al.* 2004). The FG-Nups of higher eukaryotes also arrange in distinct structural locations within the pore (SUNTHARALINGAM and WENTE 2003).

Table 1-1. Properties and homologues of FG-Nups.[d,e]

S. cerevisiae	Essential in *S. cerevisiae*	Localization (*S. cerevisiae*)	Repeat motif(s)	Abundance per pore (*S. cerevisiae*)	Number of FG repeats (*S. cerevisiae*)	Vertebrate	Abundance per pore (vertebrate)	*C. elegans*	*D. melanogaster*
Nup42	No	Cytoplasmic	FG	8	28	hCG1/ NLP1	16	—	—
Nup159	Yes	Cytoplasmic	FG	8	25	Nup214	8	npp-14	DNup214
Nup49	Yes	Symmetric	GLFG	16	17	Nup58, Nup45	48	—	Nup58
Nup57	Yes	Symmetric	GLFG	16	15	Nup54	32-48	npp-1	Nup54
Nsp1	Yes	Symmetric	FG, FXFG	32	12, 22	Nup62	16	npp-11	Nup62
Nup100	No	Cytoplasmic-bias	GLFG	8	44	Nup98	8	npp-10	Nup98
Nup116	No	Cytoplasmic-bias	GLFG	8	9, 40				
Nup145N	No	Nuclear-bias	GLFG	16[c]	13				
Nup1	No[a]	Nuclear	FXFG	8	22	Nup153	8	npp-7	Nup153
Nup2	No	Nuclear	FXFG	8[c]	14	Nup50	32	—	—
Nup60	No	Nuclear	FXF	8	4	–		—	—
—	—	Symmetric, Integral membrane	FG	—	23[b]	Pom121	8	—	—
—	—	Cytoplasmic	FXFG	—	21[b]	Nup358/ RanBP2	8	npp-9	Nup358

a. Nup1 is essential in certain *S. cerevisiae* genetic backgrounds (DAVIS and FINK 1990).
b. number of repeats in *Homo sapiens* protein.
c. Estimate
d. Estimates of localization and abundance from (CRONSHAW *et al.* 2002; ROUT *et al.* 2000).
e. Homologues based on summaries in (HETZER *et al.* 2005; SUNTHARALINGAM and WENTE 2003).

In higher eukaryotes, FG-domains are glycosylated. Glycosylation is specifically mediated by O-linked GlcNAc transferase, which attaches an N-acetylglucosamine (GlcNAc) moiety to Ser or Thr (MILLER *et al.* 1999). This O-linked glycosylation of Nups is not essential for proper Nup localization at the NPC (JINEK *et al.* 2004). These O-linked GlcNAc residues are present on the cytoplasmic surface of FG-Nups and may play a role in substrate docking and translocation through the NPC (DAVIS and BLOBEL 1987; FINLAY and FORBES 1990; GREBER and GERACE 1992; MILLER *et al.* 1999) or may regulate the phosphorylation state of specific O-glycosylated Nups (MILLER *et al.* 1999). The biological importance of glycosylation of FG-Nups in metazoan cells and the impact of these post-translational modifications on nucleocytoplasmic transport is unclear.

NPC functions mediated by FG-Nups

FG-Nups are involved in a number of NPC functions, including receptor-mediated transport, permeability barrier integrity, gene gating, and transport directionality. FG domains have been studied extensively for their role in interacting with transport receptors during nucleocytoplasmic transport (ALLEN *et al.* 2001; ALLEN *et al.* 2002; RYAN and WENTE 2000; STRAWN *et al.* 2001; TERRY and WENTE 2007) (AITCHISON *et al.* 1996; ALLEN *et al.* 2001; ALLEN *et al.* 2002; BRADATSCH *et al.* 2007; DAMELIN and SILVER 2000; KATAHIRA *et al.* 2002; MARELLI *et al.* 1998; ROUT *et al.* 1997; SEEDORF *et al.* 1999; STRASSER *et al.* 2000; STRAWN *et al.* 2004; STRAWN *et al.* 2001), and are required in specific combinations for efficient transport (STRAWN *et al.* 2004; TERRY and WENTE 2007). The complexity and redundancy of FG-Nups within the NPC has made it difficult to study their roles *in vivo* in metazoans. Genetic manipulation

of the yeast model system, however, has proved an effective system for studying the role of FG-Nups. Despite their potential roles in terminal events of nuclear export (or in initial events in nuclear import), the cytoplasmic filament Nups and their FG domains are dispensible (STRAWN *et al.* 2004; WALTHER *et al.* 2002). In addition, direct swapping of the FG-domains between *S. cerevisiae* Nup1 (FXFG domain; nuclear basket-localized) and Nup159 (FG domain; cytoplasmic filament-localized) does not cause any detectable perturbations of transport (ZEITLER and WEIS 2004). Indeed, cells with deletions of all asymmetric FG-domains (those of Nup1, Nup2, Nup60, Nup42, and Nup159) in *S. cerevisiae* are viable and has no significant transport defects (STRAWN *et al.* 2004). In stark contrast, the central or symmetrically distributed FG domains are required in specific combinations (STRAWN *et al.* 2004; TERRY and WENTE 2007). In addition to their role in mediating canonical transport through the central NPC channel, FG-Nups are necessary for transport of inner nuclear membrane proteins (KING *et al.* 2006).

At least some aspects of transport directionality may be mediated by FG-Nups (ULLMAN *et al.* 1999), although it is argued that the primary determinant for directionality is, instead, the Ran GTP/GDP gradient (NACHURY and WEIS 1999). For both the Kap95/Kap60 import and mRNA export pathways, domains adjacent to FG repeats coordinate termination of transport and release of transporting complexes from the NPC (STEWART 2007a; TRAN and WENTE 2006). Additionally, FG-Nups are critical components of the permeability barrier, and NPCs lacking specific FG domains are "leaky", permitting diffusion of inappropriate molecules (PATEL *et al.* 2007). Other structural (non-FG) Nups are also important for maintenance/establishment of the permeability barrier (GALY *et al.* 2003; SHULGA *et al.* 2000), though it is possible that

deletion of structural Nup(s) in these experiments also impairs assembly of the full complement of FG-Nups at these NPCs. Of note, the extent to which FG-domains contribute to the permeability barrier and the resilience of this barrier to partial deletion is presently debated within the transport field. Resolving this discrepancy is an important future direction and is discussed further in Chapter 4. FG-Nups are also linked to gene gating, the process of chromatin association with NPCs (CASOLARI *et al.* 2004), though it is not clear whether this association is through their FG-repeat domain or through functions of non-FG domains of these Nups. As a whole, this diversity of functional roles underscores the importance of FG-Nups to the NPC, but also increases the complexity of studying the function of FG-Nups.

Evolutionary conservation of FG-Nups

The conservation of FG-repeat motifs across Nups and in multiple species facilitated the early cloning and characterization of this protein family. FG repeat motifs in organisms from yeast to mammals are recognized specifically by monoclonal antibodies to NPC proteins (ARIS and BLOBEL 1989), indicating that these motifs are indeed conserved. Unlike most of the other folds and motifs in the NPC, there is no clear prokaryotic ancestor for FG domains, which makes understanding their evolutionary appearance complex (MANS *et al.* 2004). Evolutionary modeling studies identified repetitive folds and motifs among Nups and suggest that Nups arose from gene duplication and diversification events over evolutionary time (DEVOS *et al.* 2004). These studies also hypothesize that Nups are related to coated vesicle components, which, like Nups, stabilize highly curved surfaces (BROHAWN *et al.* 2008; DEVOS *et al.* 2004).

Protein structure prediction analysis of Nups finds very few motifs – predominantly alpha-helices and beta-sheets (DEVOS *et al.* 2006). This high level of redundancy and duplication also suggests that the evolution of the NPC and diversification of Nups has been quite rapid. Coiled-coil motifs are predicted to anchor most of the FG-Nups into the NPC (DEVOS *et al.* 2006), while the unfolded FG domains are flexible (DENNING *et al.* 2003; FAHRENKROG *et al.* 2002; LIM *et al.* 2006b).

Between yeast and metazoans, some Nups are highly conserved in both sequence and structure, while others have divergent sequences yet retain similar tertiary structures (MANS *et al.* 2004). Structural elements and subcomplex shapes are, overall, maintained in such a way that the ultrastructure of NPCs is highly similar between divergent species (AKEY and RADERMACHER 1993; ALBER *et al.* 2007; BECK *et al.* 2004; HINSHAW *et al.* 1992; KISELEVA *et al.* 2003; MANS *et al.* 2004). An interesting example of gene duplication and divergence is illustrated by the *S. cerevisiae* FG-Nups Nup100, Nup116, and Nup145 versus their vertebrate counterparts Nup96 and Nup98. Evidence for evolutionary gene duplication events among these three Nups comes from genomic sequences; the same tRNA and transposon sequence elements are adjacent to both the *NUP100* and *NUP116* loci (WENTE *et al.* 1992), and the N-terminal GLFG repeats of Nup145 are similar to the sequence of repeats in Nup100 and Nup116 GLFG domains (WENTE and BLOBEL 1994). A second line of evidence for evolutionary gene duplication and divergence among these three Nups comes from examining the protein domain organization (RYAN and WENTE 2000). The *S. cerevisiae* Nup145 polypeptide is a precursor to two proteins found in the NPC; the peptide is autocatolytically cleaved post-translationally to Nup145N (size ~65kDa) and Nup145C (size ~80kDa) (ROSENBLUM and

BLOBEL 1999; TEIXEIRA *et al.* 1997; WENTE and BLOBEL 1994), which each assemble into different substructural positions in the NPC (HODEL *et al.* 2002). Remarkably, this unusual event is conserved; the cleavage motif and event also occurs with the vertebrate homologs Nup96/Nup98, which are transcribed and translated as a ~194kDa fusion polypeptide (FONTOURA *et al.* 1999). The uncleaved Nup96/Nup98 fusion protein is impaired for assembly into the NPC (HODEL *et al.* 2002), thus raising interesting questions about whether this proteolytic processing event may be involved in a regulatory step of NPC biogenesis or in preventing premature activity linked to either of these peptides.

Structural features of FG-Nups

Biophysical studies demonstrate that FG-domains are natively unfolded and flexible within the NPC (DENNING *et al.* 2003; DENNING *et al.* 2002; FAHRENKROG *et al.* 2002; LIM *et al.* 2006b). As extensive unfolded protein domains, the FG-domains are characterized by a large hydrodynamic (Stokes) radius, enrichment in amino acid residues associated with structural disorder, high flexibility, and proteolytic sensitivity (DENNING *et al.* 2003; DENNING *et al.* 2002; DOKUDOVSKAYA *et al.* 2006). Although unfolded regions are predicted in a substantial portion (~30%) of the *S. cerevisiae* proteome, the FG-domains are particularly large unfolded sequences (DUNKER *et al.* 2001). Unfolded protein domains favor binding to multiple partners and can facilitate rapid association and dissociation rates (TOMPA 2005). The flexibility of these domains likely favors repeated collisions with multiple binding partners, and means that an FG-domain is accessible from multiple directions. In support of the flexibility of FG-

domains, immuno-electron microscopy (EM) with an antibody specific to the FG-domain of *Xenopus* Nup153 finds that this domain occupies multiple topological positions (FAHRENKROG *et al.* 2002), while the non-FG domains of Nup153 are anchored at specific points in the NPC. While some have suggested that FG domains alter their topology (PAULILLO *et al.* 2005) or collapse (LIM *et al.* 2007b) upon transport receptor binding, how this contributes to the transport mechanism remains unclear. FG-Nups are anchored into specific locations and subcomplexes of the NPC by their non-FG domains, and deletion of these domains results in mis-targeting, *e.g.* (BAILER *et al.* 1998; HO *et al.* 2000a).

The four steps of receptor-facilitated translocation through the NPC

Properties of receptor-facilitated transport

Nuclear import and export of signal-containing cargoes larger than the permeability barrier limit is generally facilitated by a transport receptor (GORLICH and KUTAY 1999), and the process of translocation involves four steps: (1) cargo-receptor complex assembly; (2) translocation through the NPC; (3) termination of transport and cargo release; and (4) recycling of the transport receptor (Figure 1-3) (STEWART 2007a). First, a receptor-cargo complex must form. Each of the cargos that uses a transport receptor must display a nuclear localization signal (NLS) or nuclear export signal (NES) sequence (CHOOK and BLOBEL 2001; PEMBERTON and PASCHAL 2005). This signal is recognized and bound by a specific transport receptor. These signal sequences are both necessary and sufficient for interaction with a Kap for transport. Second, the transport

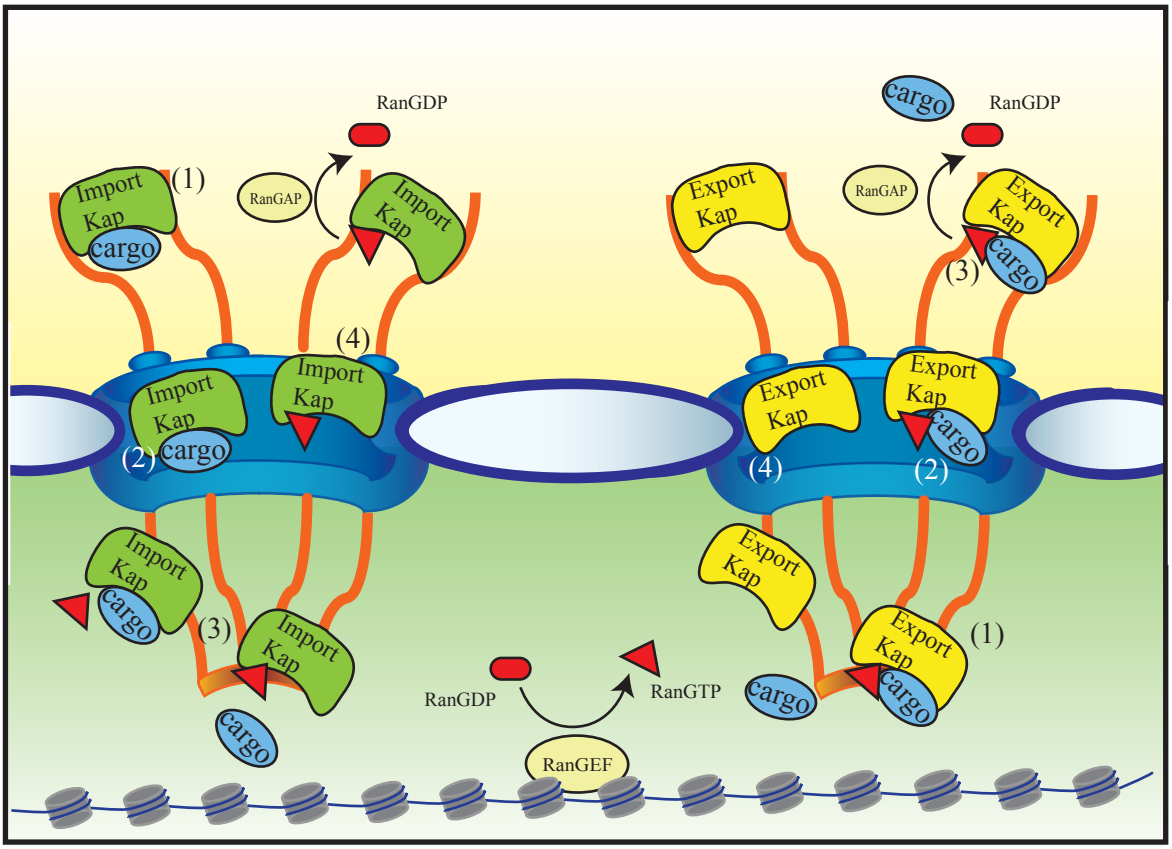


Figure 1-3. Nucleocytoplasmic transport requires four steps:
A complete cycle of nuclear import by a Kap is shown on the left; nuclear export is shown on the right.
(1) Formation of a transport receptor-cargo complex. A signal-bearing cargo is recognized by and binds a specific transport receptor. For nuclear export, the Kap-cargo complex is a heterotrimer with RanGTP.
(2) Translocation through the NPC. The Kap-cargo complex moves through the NPC by interacting with a subset of NPC proteins, the FG-Nups (not shown).
(3) Complex disassembly. Nuclear import complexes are disassociated by binding of RanGTP. Nuclear export complexes disassemble as RanGAP hydrolyzes RanGTP to RanGDP.
(4) Kap recycling. Import Kaps, bound to RanGTP, are recycled through the NPC. Export Kaps traverse the NPC empty.
Nuclear (chromatin-associated) RanGEF maintains high nuclear pool of RanGTP.
The transport receptor Ntf2 (not shown) imports RanGDP into the nucleus to maintain the RanGDP-RanGTP gradient.

receptor, now bound to a signal-containing cargo, interacts with a subset of Nups to mediate movement through the NPC and thus carry the cargo between the nucleus and cytoplasm (ROUT *et al.* 2000; SUNTHARALINGAM and WENTE 2003). The Nups directly involved in this process are the FG-Nups. Transport receptors therefore serve as a molecular bridge between FG-Nups and a cargo molecule to allow efficient nuclear import and export. Third, the receptor-cargo complex is disassembled at the far side of the NPC. Finally, the transport receptor is recycled for another round of transport. In order to mediate these steps, transport receptors include three key functional features: (1) binding to signal-containing cargoes; (2) binding to NPC components (specifically, FG-domains) to facilitate transport; and (3) mechanism for directionality, cargo release, and recycling of the transport receptor (PEMBERTON and PASCHAL 2005). This dissertation focuses primarily on the second step of this process – receptor-cargo complex interaction with the NPC. Recent work has offered many insights into the nature of the FG-Nups, their interactions with transport receptors, and their regulatory roles in transport, disease, and development. Understanding the structural, functional, and regulatory properties of FG-Nups has provided new insights into a novel paradigm for selective barrier structures and for the mechanism of regulated and efficient nucleocytoplasmic transport (see below).

Step 1: formation of a receptor-cargo complex

The karyopherin family of transport receptors

The major family of transport receptors is the karyopherins (Kaps), which includes 14 known members in *S. cerevisiae* and more than 21 identified in humans (GORLICH and KUTAY 1999; HAREL and FORBES 2004; MACARA 2001; MOSAMMAPARAST and PEMBERTON 2004). The structure of Kaps is an arch built of typically 20 HEAT repeats (PEMBERTON and PASCHAL 2005) (COOK *et al.* 2007). Structurally, a HEAT repeat is paired antiparallel alpha helices connected by a short loop. HEAT repeats are found in other cellular factors, including those from which they derive their name: **h**untingtin, **e**longation factor 3, '**A**' subunit of protein phosphatase A (PR65/A), and **T**OR1 lipid kinase (ANDRADE *et al.* 2001). The array of tandem HEAT repeats in Kaps is highly flexible, and this flexibility potentially allows Kaps to adapt to carry a variety of cargoes and/or to interact with differently spaced FG-repeats (CHOOK and BLOBEL 2001; CINGOLANI *et al.* 2002; CINGOLANI *et al.* 1999; CONTI *et al.* 2006; LEE *et al.* 2000; LEE *et al.* 2003a; LEE *et al.* 2003b; PEMBERTON and PASCHAL 2005).

Extensive domain mapping and structural studies have characterized the inner face of the N-terminus of Kaps as the binding site for RanGTP, the Kap directionality and release factor (see below) (COOK *et al.* 2007). Cargo binding is adjacent to this area on the inner face of the arched Kap structure, though recent structural studies have identified multiple possible cargo binding sites on at least one Kap (CHOOK and BLOBEL 2001; CINGOLANI *et al.* 2002; CONTI 2002; COOK *et al.* 2007). Additionally, each Kap may make distinct contacts with each of its cargoes, further complicating the identification of

Kap-cargo interaction domains (CINGOLANI *et al.* 2002; CINGOLANI *et al.* 1999; LEE *et al.* 2003a; PEMBERTON and PASCHAL 2005). The outer backbone of each Kap is the platform for interaction with FG-Nups during transport. Specifically, crystallographic and modeling studies show that the Phe side chain of an FG repeat fits into hydrophobic pockets formed by the HEAT repeats of each Kap (BAYLISS *et al.* 2000b; BAYLISS *et al.* 2002b). Multiple FG-binding sites have been identified on the outer face of Kaps (BAYLISS *et al.* 2000b; BAYLISS *et al.* 2002b; BEDNENKO *et al.* 2003; ISGRO and SCHULTEN 2005; ISGRO and SCHULTEN 2007b). Because there are likely multiple FG-binding sites on a single Kap, it is possible that transport through the NPC is accomplished by pivoting through FG-Nups by binding with different hydrophobic pockets on the Kap. Additionally, although FG-Kap binding is measured to be low-affinity (e.g., ~100nM-1uM) (BEN-EFRAIM and GERACE 2001; PYHTILA and REXACH 2003), there are multiple FG-repeats on each FG-Nup and multiple FG-binding sites on each Kap; therefore, avidity of binding sites may also contribute to transport.

These structural paradigms are predicted to be true for both import and export Kaps (PEMBERTON and PASCHAL 2005), although a slight variation is used in transport via yeast (y)Kap95-Kap60 (vertebrate (v)Importin β-Importinα). Kap95-Kap60 functions as a heterodimer for nuclear import of cargoes bearing specific NLSs, including the basic classical (c)NLS, such as that of SV40 large-T antigen (STEWART 2007a). The cNLS-cargo binds on the inner surface of Kap60. The Importin Beta Binding (IBB) domain of Kap60 extends and interacts with the inner surface of Kap95 (Figure 1-4B, C) (KOBE 1999; WEIS *et al.* 1996). This trimer of Kap95-Kap60-cargo is an import-competent complex. Termination of Kap95-Kap60 nuclear import is a multi-step process: the cargo-

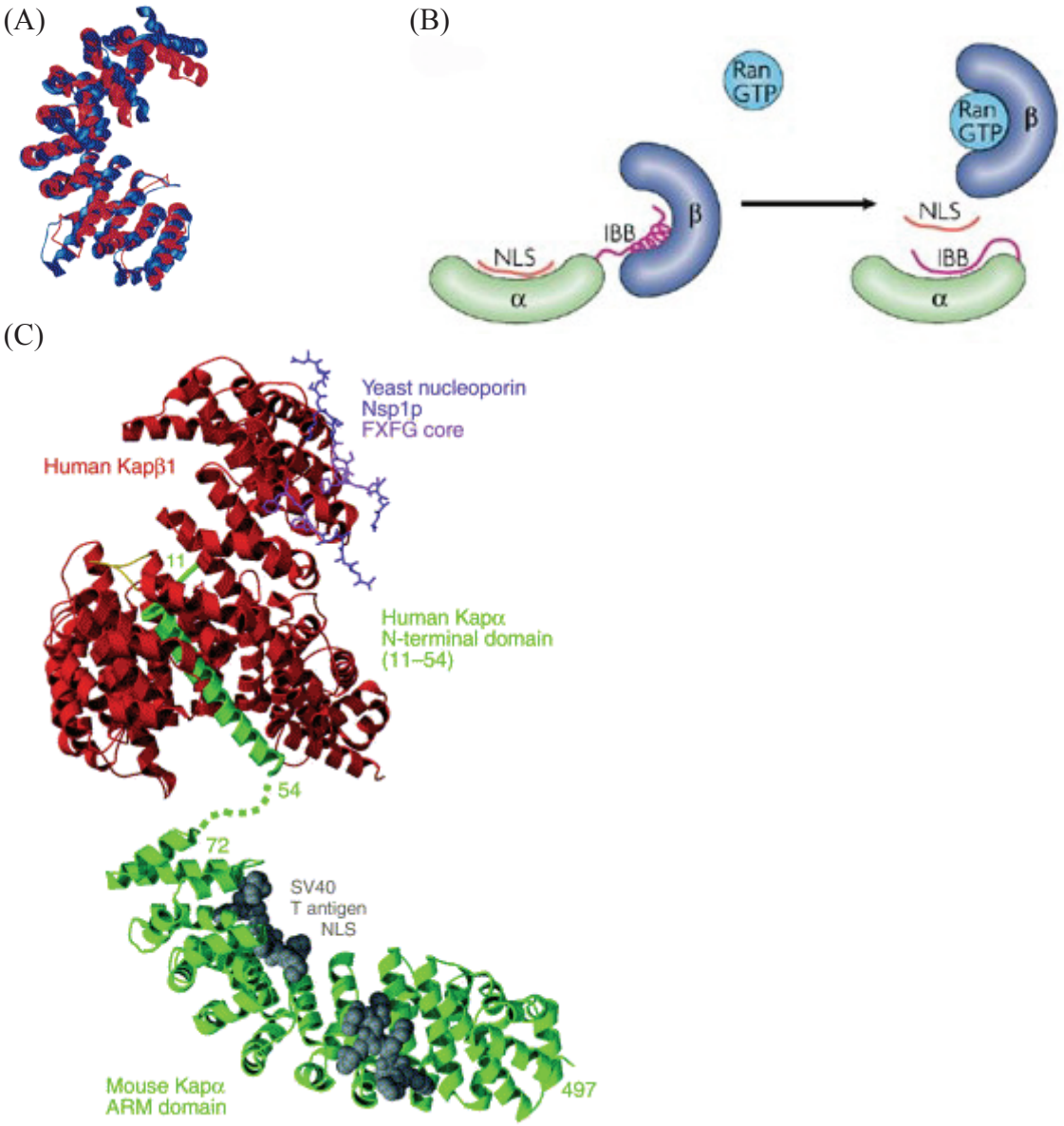


Figure 1-4. Structural insights into Kap95-Kap60 transport.
(A) Karyopherins are highly flexible. This ribbon diagram overlay shows the conformational shift in vImpβ between the empty (blue) and RanGTP-bound (red) forms.
(B) Diagram of the events during termination of nuclear import via Kap95 (Impβ) and Kap60 (Impα). An NLS-containing cargo interacts with Impα, which in turn binds to Impβ via its Importin Beta Binding (IBB) domain. Nuclear RanGTP contributes to destabilizing this complex once it traverses the NPC. This results in cargo release (free NLS), RanGTP-Impβ complex ready for recycling, and Impα with IBB bound in place of NLS. Adapted from *Stewart. 2007 Nat. Rev. Mol. Cell Biol.*
(C) Structural model of the import complex. The SV40 NLS (gray) binds the inner face of Impα (Kapα/Kap60) (green). The IBB of Kapα binds the central inner face of Kapβ (Impβ, Kap95) (red). On the outer surface of Kapβ, an FXFG peptide (purple) is shown interacting with binding sites between helices. Reprinted from *Chook & Blobel. 2001. Curr. Opin. Struct. Biol.*

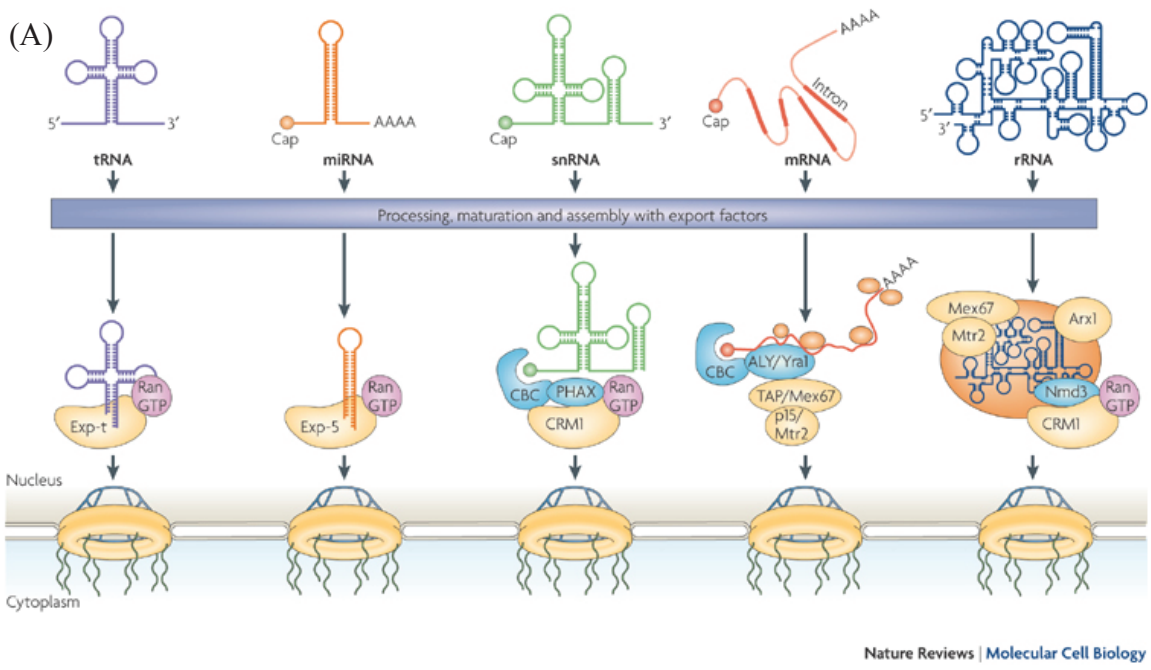
Kap60 complex dissociates from Kap95 via RanGTP binding to Kap95 (LEE *et al.* 2005; VETTER *et al.* 1999). This readies Kap95-RanGTP for nuclear export/recycling. However, the mission of cargo delivery is not yet accomplished. Dissociation of the cargo from Kap60 requires another series of catalytic events involving Cse1, RanGTP, and Nup2 (STEWART 2007a). Cse1 is a Kap family member and directly binds Kap60 to mediate its recycling back to the cytoplasm (MATSUURA and STEWART 2004). Import via Kap95-Kap60 is thus twice as energetically expensive as is any other Kap: nuclear export/recycling of Kap95 and Kap60 requires hydrolysis of two RanGTP molecules (MACARA 2001). Although energetically expensive, the use of Kap60 as an adaptor broadens the dynamic range of import above that which Kap95 can accomplish alone (MACARA 2001; RIDDICK and MACARA 2007).

Direct binding between Kaps and specific RNAs has also been identified (KOHLER and HURT 2007). tRNA binds directly to a dedicated Kap, vExportin-t (yLos1) (HELLMUTH *et al.* 1998; KUTAY *et al.* 1998). Micro(mi)RNA precursors and small nuclear (sn)RNAs are also transported through NPCs in a Kap- and Ran-dependent manner, although for snRNA export, interaction with a Kap is bridged by adaptor proteins that recognize structural features of the snRNAs (BOHNSACK *et al.* 2004; IZAURRALDE *et al.* 1995; KOHLER and HURT 2007; LUND *et al.* 2004; YI *et al.* 2003). Interaction between transport receptors and signal-containing cargoes can be regulated by several means of surface accessibility changes, signal masking, and/or post-translational modification of NLS and NES signals (TERRY *et al.* 2007). Additionally, some Kaps seem to be dedicated to transport of a specific family of cargoes, *e.g.,* Kap123 mediates nuclear import of most ribosomal proteins (ROUT *et al.* 1997) and Exportin-t is dedicated

to tRNA transport (HELLMUTH *et al.* 1998; KUTAY *et al.* 1998). It is interesting to note that there are specific receptors for each of the varieties of RNA – including tRNA, snRNA, mRNA, and miRNA - that must transport through the NPC (Figure 1-5A) (KOHLER and HURT 2007). This was convincingly demonstrated by competition experiments in which a *Xenopus* oocyte nucleus is microinjected with an excess of any one class of RNA. Nuclear export of each single class of RNA is saturable (presumably as the export receptor becomes limiting in the system flooded by cargo), but classes of RNA do not compete with each other for export (BATAILLE *et al.* 1990; JARMOLOWSKI *et al.* 1994; NAKIELNY and DREYFUSS 1999; POKRYWKA and GOLDFARB 1995). Additionally, the transport receptors for each of these classes of RNA discriminate between mature and precursor RNA forms – each of these classes of RNA is transcribed as a precursor and undergoes a series of regulated maturation events – the export receptor only associates with and exports the properly processed, mature RNA form (KOHLER and HURT 2007; NAKIELNY and DREYFUSS 1999). Thus, the assembly of nuclear export complexes is a highly regulated process. While Kaps are the transport receptor for most proteins and RNAs (including rRNA, tRNA, miRNA, snRNA), bulk mRNA export employs a non-karyopherin transport receptor.

Mex67-Mtr2 (TAP-p15) is a transport receptor for mRNA

Nuclear export of mRNA is a critical step in the continuum of gene expression from transcription to translation. As the NE spatially separates these two cellular processes, efficient and regulated nuclear mRNA export is critical to rapid/proper gene expression. Nuclear export of mRNA is linked to proper pre-mRNA processing and



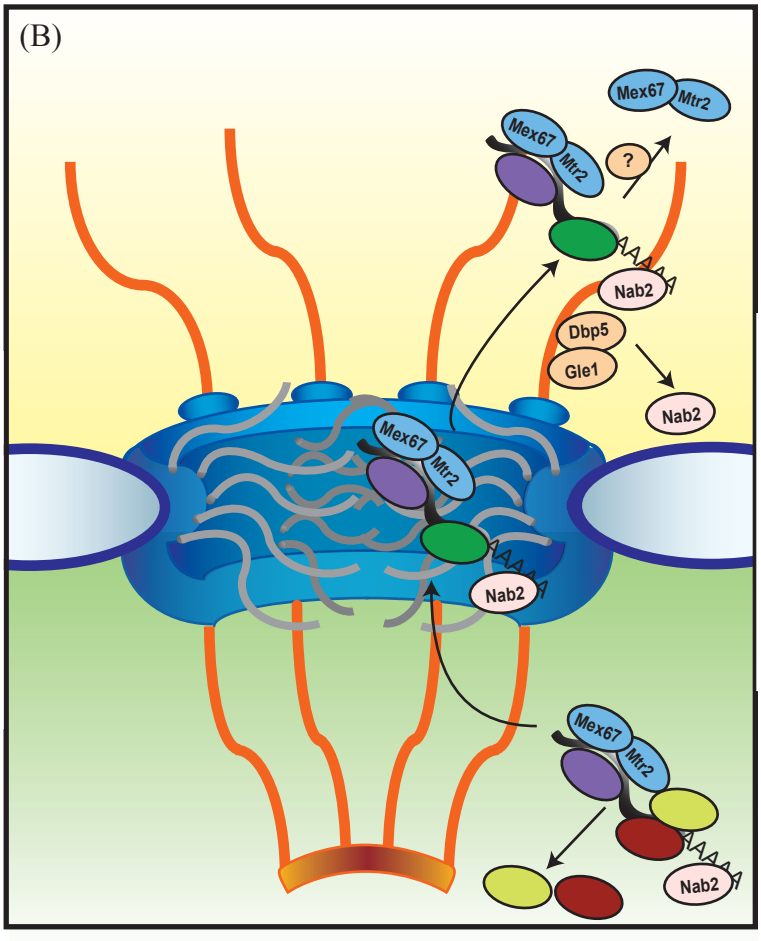


Figure 1-5. Nuclear export of RNA requires specific transport receptors and regulatory steps.
(A). Transport receptors recognize and bind - either directly or through adaptor proteins - mature, processed forms of RNA in the nucleus. Specific transport receptors facilitate nuclear export of tRNA, miRNA, snRNA, mRNA, and rRNA through the nuclear pore complex. Reprinted from *Kohler & Hurt. 2007. Nat Rev Mol Cell Biol.*
(B). During the continuum from transcription through nuclear export, the protein composition of mRNPs is dynamic and changes as the transcript matures.

requires ongoing transcription (IGLESIAS and STUTZ 2008). As a pre-mRNA matures through transcription, 5' capping, poly(A)$^+$ tail processing, and splicing, the composition of proteins bound to the mRNA is continually changing (IGLESIAS and STUTZ 2008). This protein-mRNA complex is known as an mRNP. The mRNA export receptor is the heterodimer Mex67-Mtr2 (*S. cerevisiae*; in metazoans, TAP/NXF-p15/NXT) (KATAHIRA *et al.* 1999; SANTOS-ROSA *et al.* 1998; SEGREF *et al.* 1997). Mex67-Mtr2 in yeast and TAP-p15 in metazoans are each essential for bulk mRNA export (HEROLD *et al.* 2001; KATAHIRA *et al.* 1999; SANTOS-ROSA *et al.* 1998; SEGREF *et al.* 1997; TAN *et al.* 2000; WIEGAND *et al.* 2002). Temperature-sensitive alleles of *mex67* have defects in mRNA export, and at steady-state, Mex67 co-localizes with Nups along the nuclear rim (HUH *et al.* 2003; SEGREF *et al.* 1997). Likewise, Mtr2 localizes to the nuclear rim, and temperature-sensitive alleles of *mtr2* have mRNA export defects and genetic interactions with mRNP and NPC proteins (SANTOS-ROSA *et al.* 1998). Mex67-Mtr2 meets the criteria for a *bona fide* mRNA transport receptor: Mex67-Mtr2 have genetic interactions with NPC components (SEGREF *et al.* 1997; STRASSER *et al.* 2000), bind mRNA (LUND and GUTHRIE 2005; SANTOS-ROSA *et al.* 1998), shuttle through the NPC (KATAHIRA *et al.* 1999), and physically interact with FG-Nups (ALLEN *et al.* 2001; STRASSER *et al.* 2000; STRAWN *et al.* 2001; TERRY and WENTE 2007).

Structure and conservation of the mRNA export receptor

Mex67-Mtr2 and TAP-p15 are structurally distinct from the Kap family of transport receptors and function independent of the RanGTP system (CLOUSE *et al.* 2001; GRANT *et al.* 2002; GRANT *et al.* 2003; HEROLD *et al.* 2000; SENAY *et al.* 2003).

Interestingly, although Mex67-Mtr2 and TAP-p15 have similar biological roles in mediating nuclear mRNA export, genetic evidence suggests that these receptor heterodimers have co-evolved; Mex67 cannot be functionally replaced by TAP, nor can Mtr2 by p15. However, a double null mutant *mex67*Δ *mtr2*Δ *S. cerevisiae* can be partially complemented by co-expression of TAP and p15 (KATAHIRA *et al.* 1999). A small subset of human mRNAs are exported independently of TAP-p15, and many viral mRNAs also use export mechanisms independent of TAP-p15 (for review, see (CULLEN 2003; KOHLER and HURT 2007)). The export-competent mRNP moves through the NPC, presumably by serial interactions between Mex67-Mtr2 and FG-repeats. Mex67-Mtr2 has been demonstrated to bind to at least nine different FG-Nups (ALLEN *et al.* 2001; STRASSER *et al.* 2000; STRAWN *et al.* 2001; TERRY and WENTE 2007). Structurally, there are at least two FG binding sites on Mex67-Mtr2 and also on TAP-p15 (GRANT *et al.* 2002; GRANT *et al.* 2003; SENAY *et al.* 2003). One of these FG binding sites has structural similarity to Ntf2, while the other is similar to a ubiquitin associated motif (BRAUN *et al.* 2001; BRAUN *et al.* 2002; FRIBOURG *et al.* 2001; FRIBOURG and CONTI 2003; GRANT *et al.* 2002; GRANT *et al.* 2003). Within TAP, the two FG binding sites are structurally different motifs. Interestingly, TAP mutants with two Ntf2-like motifs or two UBA-like motifs are competent for mRNA export; TAP truncations with just one FG binding motif are non-functional (BRAUN *et al.* 2002; CULLEN 2003). Likewise, a mutation in Mex67 that uncouples the Mex67-Mtr2 heterodimer causes mRNA export defects (SANTOS-ROSA *et al.* 1998; SEGREF *et al.* 1997). This supports a model wherein successful TAP translocation through the NPC requires multiple FG binding sites and

reinforces the notion that avidity is a driving mechanism for FG-transport receptor interactions in the nucleucotyoplasmic transport mechanism.

Cargoes and signal sequences[1]

The signals displayed by cargo molecules and recognized by transport receptors are NLSs and NESs, and these have classically been defined as primary amino acid motifs that are both necessary and sufficient for transport (FRIED and KUTAY 2003). However, it is now clear that such signals are composed of primary sequence and secondary/tertiary structural elements, and that they are present in both proteins and RNAs. The precise sequence and the substructural context of an NLS or NES defines its specificity for the various Kaps (FRIEDRICH *et al.* 2006). Furthermore, the receptor-protein and receptor-RNA interactions that mediate cargo localization are governed by multiple post-transcriptional and post-translational modifications (TERRY *et al.* 2007). These modifications allow for specific transport regulation of individual cargo.

mRNP protein composition

The protein composition of an mRNP is highly dynamic, with changes occurring throughout the life of a single transcript (IGLESIAS and STUTZ 2008). This phenomenon was classically illustrated by immuno-EM studies of the Balbiani ring mRNP in salivary glands of *Chironomus tentans*. The Balbiani ring mRNP, a massive ~40kbp transcript, is visible by EM and undergoes directional nuclear export, with its 5' terminus leading

[1] Some material in this paragraph reprinted from Terry LJ, Shows EB, Wente SR. Science. 2007 Nov 30;318(5855):1412-6.

(DANEHOLT 2001a; DANEHOLT 2001b). Immuno-EM analysis of the protein composition of this mRNP shows that specific factors bind to or are displaced from the mRNP at defined points in the continuum of mRNP processing and export. In addition to compositional changes on a single mRNP, the protein composition of mRNPs varies among mRNA transcripts. For example, the mRNA-binding proteins Nab2, Npl3, and Nab4 differentially associate with subsets of specific transcripts (GUISBERT *et al.* 2005). Nab4 is preferentially associated with transcripts linked to amino acid metabolism, while Npl3 is enriched on transcripts of ribosomal proteins. Transcript-specific differences in mRNP composition have also been reported in mammalian cells (GAMA-CARVALHO *et al.* 2006). Of note, dissociation and reassociation of hnRNPs from/to transcripts has been noted following cell lysis (MILI and STEITZ 2004); thus, these results must be interpreted with prudence. Further mRNP composition differences are found when comparing intron-containing and intron-less mRNAs; following splicing, components of the exon junction complex remain associated with the splice site (ISKEN and MAQUAT 2007). Export of intron-containing (*i.e.*, still unspliced/incompletely processed) mRNAs is blocked by two NPC-associated proteins, Mlp1 and Mlp2 (GALY *et al.* 2004; GREEN *et al.* 2003). Together, these factors serve as a quality control mechanism to preclude leakage of unspliced mRNAs from the NPC.

In addition to dynamic mRNP events in the nucleus, there is differential export of subsets of mRNPs at the level of the NPC. Under conditions of heat shock, global poly(A)$^+$RNA export is blocked and heat shock mRNA transcripts are preferentially exported (SAAVEDRA *et al.* 1996). During this stress response, specific NPC-binding sites are required for nuclear export of heat shock response messages (SAAVEDRA *et al.* 1997;

STUTZ *et al.* 1997). Export of heat shock mRNAs, but not of global mRNAs, is defective in a *nup42*Δ *S. cerevisiae* null mutant. Thus the NPC has the capacity to regulate nuclear export of specific sets of mRNAs.

mRNA export receptor regulation

Recruitment of Mex67-Mtr2 to the mRNP is a critical and regulated step in the nuclear mRNA export process. Such regulation is necessary given that pre-mRNA transcripts must undergo a number of processing steps to generate mature mRNAs ready for export and translation. These processes include transcription, processing (5' capping, 3' end formation, splicing, and/or editing), and quality control events, each of which is tightly linked to mRNA export mechanisms (HIERONYMUS and SILVER 2004). During each of these coordinated processes, the set of proteins bound to the pre-mRNA is continually and dynamically remodeled (Figure 1-5B) (BURATOWSKI 2005; DANEHOLT 2001b; FASKEN and CORBETT 2005; HIERONYMUS and SILVER 2004; JENSEN *et al.* 2003; VINCIGUERRA and STUTZ 2004). During this mRNA maturation process, serial recruitment of binding proteins to the mRNP ultimately builds an mRNP that is competent for nuclear export. An mRNP is a large and complex cargo and presents a special problem for nuclear export. Additionally, the need for regulated export – to prevent leakage of incompletely processed mRNAs into the cytoplasm – is evidenced by the existence of multiple regulatory mechanisms in the nucleus. Several factors have been linked to the regulated recruitment of Mex67-Mtr2 to nuclear mRNPS. The yeast SR (Ser-Arg) protein Npl3 is recruited co-transcriptionally to pre-mRNAs (GILBERT *et al.* 2001; LEI and SILVER 2002). SR proteins are a family of conserved phospho-proteins that

regulate mRNA stability, translation, and export via cycles of phosphorylation and dephosphorylation (KOHLER and HURT 2007). Phosphorylation of Npl3 in the cytoplasm (by the kinase Sky1) directs nuclear import of Npl3 via the Kap receptor Mtr10 (GILBERT *et al.* 2001). Phospho-Npl3 associates with nascent transcripts, and as the mRNP matures, Npl3 is dephosphorylated by nuclear Glc7 (GILBERT and GUTHRIE 2004). This dephosphorylation promotes interaction of Npl3 with Mex67-Mtr2, thereby loading the mRNA export receptor onto the transcript by way of Npl3 as a signal for export competence. Successful export of an mRNP carries Npl3 into the cytoplasm, where re-phosphorylation by Sky1 disassembles Npl3 from Mex67-Mtr2 (GILBERT *et al.* 2001), thus readying both Mex67-Mtr2 and Npl3 for a new round of mRNP regulation/export. Similar cycles of phosphorylation and dephosphorylation are implicated in regulating TAP-p15 recruitment to mRNPs in metazoan cells (HUANG *et al.* 2003). Other heterogenous nuclear ribonucleoproteins (hnRNPs) have also been demonstrated to recruit Mex67-Mtr2 to maturing transcripts. The *S. cerevisiae* hnRNP Yra1 (ALY/REF in metazoans) also interacts with Mex67-Mtr2 and may recruit the heterodimer to the mRNP during gene expression (LEI *et al.* 2001; STRASSER *et al.* 2002; ZENKLUSEN *et al.* 2001). Clearly there are multiple mechanisms for efficient and regulated recruitment of the transport receptor to the maturing mRNP, underscoring the importance of this regulatory step in gene expression. In addition, this redundancy suggests that there may be multiple Mex67-Mtr2 heterodimers bound on a single transcript. However, the stoichiometry of Mex67-Mtr2 on a given transcript remains unknown. Does a larger mRNA transcript require more receptors for nuclear export? Recent studies have found that 60S ribosomal subunits – very large export cargoes, like mRNPs – use multiple

receptors for export (BRADATSCH *et al.* 2007; KOHLER and HURT 2007; YAO *et al.* 2007). Successful maturation of a pre-mRNA into an mRNA and assembly with a proper set of hnRNPs readies an mRNP for nuclear export. This process is highly regulated by nuclear and NPC-associated factors.

For both mRNA export via Mex67-Mtr2 and all Kap-facilitated transport, formation of a receptor-cargo complex is the first step required for nucleocytoplasmic transport.

STEP 2: translocation of receptor-cargo complexes through the NPC

Structural features of transport receptor interaction with FG-Nups

To move through the NPC, all of these transport receptors – including Kaps, Mex67-Mtr2, and Ntf2 – must interact with Nups. Despite their structural diversity, each of these transport receptors interacts with the same subset of Nups, the FG-Nups (TRAN and WENTE 2006).

Affinity and avidity of interactions

Transport receptor interaction with FG-Nups is a critical determinant to nucleocytoplasmic translocation, and current evidence supports a model of multiple low-affinity binding events between a transport receptor and FG-Nups during translocation. Interactions between transport receptors and FG domains are weak (typically micromolar binding affinities) and are likely transient (PYHTILA and REXACH 2003; TIMNEY *et al.* 2006). In fact, transport receptor mutants with increased affinity for binding FG repeats,

such as the *ntf2-N77Y* mutant, impair nucleocytoplasmic transport (LANE *et al.* 2000; QUIMBY *et al.* 2001). This result demonstrates the necessity of rapid, low-affinity interactions between transport receptors and FG repeats for proper and efficient transport. Biochemical approaches have demonstrated that every FG-Nup in *S. cerevisiae* is capable of binding at least one transport receptor, and each transport receptor can bind at least one FG domain (Table 1-2). Overall, each transport receptor appears to have a preference for binding specific FG-Nups or repeat-types (*e.g.*, (ALLEN *et al.* 2001; ALLEN *et al.* 2002; RYAN and WENTE 2000; STRAWN *et al.* 2001; TERRY and WENTE 2007) (AITCHISON *et al.* 1996; ALLEN *et al.* 2001; ALLEN *et al.* 2002; DAMELIN and SILVER 2000; MARELLI *et al.* 1998; ROUT *et al.* 1997; SEEDORF *et al.* 1999; STRAWN *et al.* 2004; STRAWN *et al.* 2001)). Avidity of FG repeats does, indeed, impact Kap95 binding to purified FG domains *in vitro* (PATEL and REXACH 2008). The functional importance of avidity of FG repeats within a given domain has not been examined *in vivo*, but is an important consideration for future studies (see Chapter 4).

Hydrophobic binding pockets of transport receptors

Multiple crystallographic studies of the interaction between an FG-repeat and transport receptor show that the Phe residue of the FG-repeat is buried in a hydrophobic pocket on the outer face of the transport receptor (Figure 1-4C) (BAYLISS *et al.* 2000a; BAYLISS *et al.* 2002a; BAYLISS *et al.* 2000b; BAYLISS *et al.* 2002b; BAYLISS *et al.* 1999; FRIBOURG *et al.* 2001; GRANT *et al.* 2002; GRANT *et al.* 2003; SENAY *et al.* 2003). This paradigm applies to Kaps, Ntf2, Mex67-Mtr2 and TAP-p15. Thus, these studies provide direct evidence for the role of the individual FG-repeat unit in interacting with a transport

Table 1-2. Documented interactions between transport receptors and FG-Nups.

		Import Karyopherins								
		Kap95-Kap60	**Pse1 (Kap121)**	**Kap122**	**Kap119 (Nmd5)**	**Kap104**	**Kap123**	**Kap114**	**Kap108 (Sxm)**	**Mtr10**
Nup42	FG	5, 20, 36, 39				5, 36	5, 36			5
Nup159	FG	18, 17, 7	34		31		41		17, 56	18
Nup49	GLFG	11, 22, 36	36			5	36			5
Nup57	GLFG	11, 36	35, 36			11, 5	5, 36		36	5
Nsp1	FG, FXFG	11, 17, 39	34, 39				39		17	
Nup100	GLFG	11, 20, 49, 36, 39, 22	36			36	36		36	
Nup116	FG, GLFG	11, 50, 49, 51, 36, 23, 39, 38	30, 34, 36	36, 38		11, 36	36, 38		36	36
Nup145 (NorC)	GLFG	11	30, 57			11, 5				5
Nup1	FXFG	18, 11, 5, 17, 7, 21, 22, 15, 23, 36, 47, 14, 23, 41, 39, 20, 40, 48	5, 30	13	31	5	5, 40	12	17	5, 18
Nup2	FXFG	18, 17, 21, 19, 14, 47, 36, 52, 16, 41, 15, 58	30	13	31		14, 15, 19, 41			
Nup60	FXF	19, 14, 53, 19, 36, 16, 41, 15, 22					14, 16, 36, 21			

Table 1-2, continued.

		Import/Export	Export Karyopherins			Unknown	Other transport receptors		
		Msn5	**Xpo1 (Crm1)**	**Los1**	**Cse1**	**Kap120**	**Ntf2**	**Arx1**	**Mex67-Mtr2**
Nup42	FG	36	8,9, 36, 55					1, 36	
Nup159	FG		5,6,7,36, 37, 55					1	27, 42
Nup49	GLFG	36	9, 36			36		1	
Nup57	GLFG	36	9, 36			36	54	2	27, 45
Nsp1	FG, FXFG		4	24, 25			32, 33, 54	1	27, 42
Nup100	GLFG	36	36					1,2, 36	36, 29
Nup116	FG, GLFG	30, 36	36			36, 38		1,2	28, 29, 27, 42, 45, 46, 38
Nup145 (NorC)	GLFG	30	5				54		44
Nup1	FXFG	30	5				32, 54	1	27, 43
Nup2	FXFG	30		24, 26	10		32	1	
Nup60	FXF							1	27, 43, 41

receptor. *In vitro* assays demonstrate that hydrophobic residues may be substituted in FG repeats (e.g., F to W or F to Y) with only modest effects on Kap95 binding, while mutagenesis of F to A in repeats abolished binding (PATEL and REXACH 2008). Interestingly, at least one Kap forms distinct interactions with different FG repeats (BAYLISS *et al.* 2002b). Such subtle structural differences may thus dictate the preference of a receptor for specific FG-binding sites. Additional factors may also contribute to binding site specificity, though, including adjacent non-FG binding sites, the substructural location of the FG-domain within the NPC, contributions from spacer regions, and the occupancy of neighboring FG-binding sites, and sorting out the potential contributions of each of these has been difficult. Due to the flexibility from the inherently unfolded FG peptides used in crystallization studies to date, interactions between the spacer regions and transport receptors have not been visualized at the atomic/structural level. As structural studies have thus far been unable to resolve interactions between a spacer region and a receptor, it is unclear what role these sequences might play.

Imaging of single-molecule transport in a permeabilized cell system (YANG *et al.* 2004) demonstrated that a receptor-cargo complex does not proceed through the NPC in a directed or linear fashion, but rather than it moves about in a Brownian motion fashion, potentially engaging in multiple NPC-receptor interactions during its ~10 ms transport time. Given this time scale and the motion of the complex visualized in the NPC, these studies are consistent with multiple, low-affinity interactions occurring between FG repeats and the transport complex. Despite the apparent low affinity of FG-receptor interactions, there remains preference for specific binding domains, though the mechanistic determinants of these preferences remain elusive. For example, in *S.*

cerevisiae, Mex67 and Kap95 interact preferentially with different domains of Nup116 (STRAWN *et al.* 2001), indicating that there are *in vivo* subtle differences between domains and also suggesting that a single FG-domain could provide binding sites for multiple transport factors, though it is not known if these binding events could be simultaneous. In addition, as all known transport receptors have more than one binding site for FG repeats on their surface, it is like that the avidity of FG repeats within the NPC and in interacting with these receptors is an important contribution to the transport mechanism. Furthermore, recent mathematical and computational modeling predicts that transport receptors may have more FG binding sites than previously detected (ISGRO and SCHULTEN 2005; ISGRO and SCHULTEN 2007a; ISGRO and SCHULTEN 2007b). These observations – made in molecular dynamics simulations – must be verified biochemically, but again suggest that avidity of transport receptor – FG binding must be considered in proposed models of the transport mechanism. Thus, although it is clear that multiple, stochastic, low-affinity interactions between transport receptors and FG repeats occur during transport, there are additional factors influencing transport.

Step 3: termination/release of transport

The small GTPase Ran

Following movement through the center of the NPC, a transport receptor-cargo complex must be disassembled to deliver the cargo to the destination compartment. For both Kap- and Mex67-Mtr2-mediated transport, this step is coordinated by a nucleotide switch mechanism. The termination of a single nuclear import or export event by a Kap is

mediated by the RanGTP cycle. The small GTPase Ran is a member of the Ras superfamily of proteins and, as such, functions as a binary molecular switch between GDP- and GTP-bound forms (WENNERBERG *et al.* 2005). Ran is essential for assembly and disassembly of transport complexes and as such provides directionality to Kap-mediated nucleocytoplasmic transport. The nucleotide-bound state of Ran is spatially regulated by the Ran GTPase Activating Protein (RanGAP) and the Ran Guanine Nucleotide Exchange Factor (RanGEF) proteins (SUNTHARALINGAM and WENTE 2003). RanGAP is localized to the cytoplasm, and thus the cytoplasm is a RanGDP-rich environment. The RanGEF is nuclear-localized and thus the predominant nuclear form of Ran is in the GTP-bound state.

Ran-mediated disassembly of transport complexes

Nuclear import complexes of a Kap and an NLS-containing cargo are dissociated in the nucleus by RanGTP, which binds the Kap and displaces the cargo. For example, two domains of transportin (yKap104) – the N-terminus and a C-terminal acidic loop - interact with Ran (CHOOK and BLOBEL 2001). This C-terminal acidic loop is likely also involved in cargo recognition and binding (PEMBERTON and PASCHAL 2005), and mutations in this loop uncouple RanGTP binding and cargo release. Thus a model for RanGTP-mediated disassembly of a transportin-cargo complex emerges: transportin-cargo moves through the NPC and reaches the nuclear compartment; RanGTP binds the N-terminus of transportin, weakening the association between transportin-cargo; RanGTP competes with cargo for binding to the C-terminal loop and this ultimately releases the cargo from transportin. Thus the cargo is released into the nucleus and transportin is

ready for recycling for the next round of transport. A multi-step release mechanism has also been structurally characterized in catalyzing termination of Kap95/Kap60 nuclear import (CINGOLANI *et al.* 1999; LEE *et al.* 2005; STEWART 2007a), and Importin β truncation mutants that cannot bind nuclear RanGTP are locked at the NPC (KUTAY *et al.* 1997b). Nuclear export complexes are a heterotrimer of an export Kap, an NES-containing cargo, and RanGTP. This export trimer moves through the NPC and is disassembled at the cytoplasmic face of the NPC, where RanGAP activates hydrolysis of RanGTP to RanGDP. Further evidence for the role of the Ran gradient as the critical determinant of transport directionality comes from a study of permeabilized tissue culture cells: reversal of the Ran gradient, by addition of a Ran mutant locked in the GTP-bound state, causes transport receptors to carry cargoes in the opposite direction (NACHURY and WEIS 1999). Thus the small GTPase Ran is a critical determinant of the transport direction and is required for terminating nuclear import and export events.

Termination of mRNA export

Like Kap-mediated transport, nuclear export of mRNA requires a nucleotide hydrolysis mechanism to ensure directionality of transport (TRAN and WENTE 2006). This appears to be accomplished by the proteins Dbp5 and Gle1, which act together with the small molecule inositol hexakisphosphate (IP_6) (ALCAZAR-ROMAN *et al.* 2006a; WEIRICH *et al.* 2006). Dbp5 is an RNA-dependent ATPase and is a member of the DEAD-box family of RNA helicases (CORDIN *et al.* 2006). Current models suggest that these factors work cooperatively to remodel the composition of the exporting mRNP at the cytoplasmic face of the NPC (TRAN *et al.* 2007). This has the potential to release the

mRNP from the NPC by altering its protein content to resemble that of a cytoplasmic mRNP, rather than a transport-competent mRNP. Additionally, as Mex67 is specifically a target of this remodeling event (LUND and GUTHRIE 2005), the activity of Dbp5-Gle1-IP_6 to remove Mex67 at the cytoplasmic face of the NPC could ready Mex67 for recycling and subsequent rounds of nuclear transport. Serial removal of Mex67 and hnRNPs from the exporting mRNP may function as a ratchet-like mechanism to direct the mRNP out of the NPC and prevent retro-translocation (STEWART 2007b).

Nucleoporins contributing to directional transport

Although nucleotide hydrolysis systems appear to be the driving determinant of transport directionality and termination of a transport event (ENGLMEIER *et al.* 1999; NACHURY and WEIS 1999; RIBBECK *et al.* 1999), non-FG binding sites on FG-Nups are utilized by at least two different transport pathways. Termination of Kap95-Kap60 nuclear import - that is, cargo release and preparation of Kap95 and Kap60 for recycling - involves non-FG binding sites on Nup1 and Nup2 (GILCHRIST *et al.* 2002; GILCHRIST and REXACH 2003; LIU and STEWART 2005; MATSUURA *et al.* 2003; MATSUURA and STEWART 2005; PYHTILA and REXACH 2003; SOLSBACHER *et al.* 2000). Similarly, termination of mRNA export involves Nup42 and Nup159, which are binding platforms for the mRNP-associated proteins Gle1 and Dbp5, respectively (HODGE *et al.* 1999; MURPHY *et al.* 1996; MURPHY and WENTE 1996; SCHMITT *et al.* 1999; STRAHM *et al.* 1999; WEIRICH *et al.* 2004). For both mRNA export and Kap95-Kap60 import, termination of transport and disassembly of transport complexes thus involves both asymmetric FG binding sites and flanking, high-affinity, non-FG binding sites. We

speculate that coupling between FG and non-FG binding sites contributes to efficiency and directionality of transport.

Step 4: transport receptor/factor recycling

Recycling of Karyopherins

A successful Kap-mediated transport event releases cargo in the destination compartment and frees the Kap for recycling and reuse for another round of transport. This recycling phase – moving back to the cytoplasmic face for an import Kap or to the nuclear face for an export Kap – is poorly understood. Crystallography and other structural modeling studies suggest that the helical pitch of a Kap may shift/change whether the Kap is free, bound to cargo, bound to RanGTP, or bound to both cargo and RanGTP (Figure 1-3A) (STEWART 2007a). Such a shift could potentially alter the binding between the Kap and the NPC. This could in turn alter what subset of NPC binding sites are utilized by the Kap during transport. A model in which a Kap uses different NPC binding sites for import vs. export is attractive – this way, a recycling Kap would not interfere with its cargo-bound transporting form – and is an interesting concept for future study.

Recycling of mRNA export factors

Following termination of mRNA export, a large number of factors presumably must be recycled. Remodeling of mRNP protein composition at the termination of mRNA export likely readies many RNA-binding shuttling proteins for recycling. For

example, the RNA-binding protein Nab2 is targeted by Dbp5-Gle1-IP_6 for removal from the mRNP (TRAN *et al.* 2007). Nab2 is subsequently re-imported to the nucleus by Kap104 for ensuing rounds of mRNP export (AITCHISON *et al.* 1996). Phosphorylation signals direct recycling of many SR-proteins, which are then re-imported into the nucleus by specific transport receptors (KOHLER and HURT 2007). Mex67-Mtr2 can presumably mediate its own recycling by way of binding FG domains.

Recycling of Ran via Ntf2

Since both the export/recycling step of an import Kap and the export of an export Kap-cargo complex carry RanGTP out of the nucleus – at a rate of efflux estimated at more than 10^5 molecules per second per nucleus (GORLICH *et al.* 2003; SMITH *et al.* 2002), there must be a countermeasure to import and supply Ran to the nucleus. Indeed, RanGDP is imported to the nucleus by a non-Kap transport receptor, Ntf2. Ntf2 is structurally unrelated to Kaps and functions as a homodimer (PEMBERTON and PASCHAL 2005). Ntf2 specifically imports the GDP-bound form of Ran by binding the Switch II domain of Ran, which is conformationally distinct in the RanGTP vs. RanGDP forms (STEWART *et al.* 1998). Each cycle of Ntf2 transport carries two RanGDP molecules into the nucleus to maintain the nuclear pool of RanGTP.

Proposed models of the transport mechanism

Key considerations for models

The complexity of the NPC and the dynamic nature of transporting molecules has made it difficult to define the mechanism of nucleocytoplasmic transport. While multiple models for this have been proposed (Figure 1-6), none completely explain known NPC properties, and these models have been the subject of lively debates recently. Any model of this transport mechanism must explain a number of features, including:

(1) the ability of the NPC to act as a permeability barrier, discriminating against entry of inappropriate macromolecules;

(2) the ability of the NPC to transport receptor-cargo complexes of a dynamic range of sizes;

(3) the ability of the NPC to transport these receptor-cargo complexes rapidly – at a rate that approaches the rate of diffusion, despite the fact that there are binding events between FG-Nups and these receptors (which would paradoxically seem to slow transport);

(4) the ability of small molecules to freely move through the pore via diffusion;

(5) the apparent ability of NPCs to simultaneously transport molecules bidirectionally;

(6) the preference for specific FG binding pathways among transport receptors; and

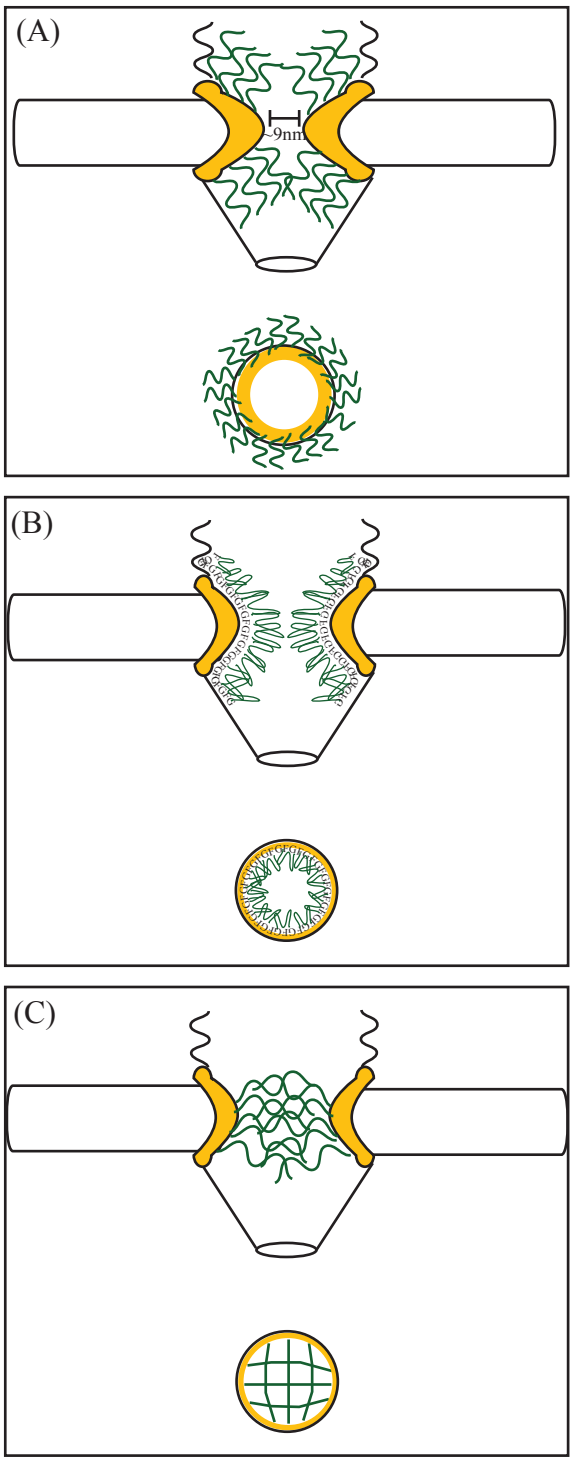


Figure 1-6. Models for the mechanism of NPC selectivity and transport.
The proposed apperance of the NPC from either side view or cross-section through center of NPC is shown for each model. NE, black. FG domains, green. Structural NPC elements, yellow.

(A) Brownian Virtual Gating model. The center of the NPC is a narrow channel, from which FG domains extend to form an entropic barrier to transport. Transport receptors (not depicted) bind these FG domains, overcoming the entropic barrier. By collectling on the NPC periphery, transport complexes increase the probability that they will spontaneously move across the barrier.

(B) Reduction of Dimensionality model. FG repeats form a continuous surface along the inner face of the NPC, and transport complexes pivot along this surface. The spacer sequence between FG repeats loop outward, forming a physical barrier to diffusion of large molecules; transport complexes can transiently displace these as they move along the FG surface.

(C) Selective Phase Partitioning model. Hydrophobic interactions between FG repeats form a physical meshwork with gel-like properties. Transport receptors bind and transiently dissolve the meshwork in order to tanslocate through the NPC.

(7) the resilience of the NPC to deletion of up to 50% of its FG domain mass.

Not only has it been difficult to reconcile these complex properties of the NPC, but it has also been difficult to develop or design experimental systems to validate proposed models of the transport mechanism. Overall, the key differences between proposed models are in the nature of interactions between FG repeats and in the biophysical consequences of FG-receptor interaction. Let us briefly review the tenets of three current proposed models and discuss the experimental evidence for each.

Brownian/Virtual Gate model

The Brownian/Virtual Gate model (Figure 1-6A) suggests that the NPC is an energy/entropy barrier (ROUT *et al.* 2003; ROUT *et al.* 2000). FG domains form an entropic barrier at each face of the NPC in a way that makes barrier passage energetically unfavorable for molecules in a size-dependent manner (*i.e.,* the larger the molecule, the more entropically unfavorable barrier passage is). These FG domains are presumably mobile and unstructured. Transport receptors overcome this barrier by stochastically interacting with FG-Nups, directly increasing the local concentration of receptor-cargo complexes on FG-Nups and therefore also increasing the probability that a given receptor-cargo complex will randomly diffuse through the NPC. In support of this model, a layer of vNup153 FXFG domains is entropically repulsive (LIM *et al.* 2006b; LIM *et al.* 2007b). The topological flexibility of FG-domains viewed by EM is consistent with a model in which FG domains do not stably interact, but this data is inconsistent with a recent study that suggested that FG-domains form a physically rigorous gel (FREY *et al.*

2006). Furthermore, in ensuing experiments, Lim et al. found that addition of vImportin β to this system collapsed this entropic layer, as is predicted for the virtual gating model (LIM *et al.* 2007a). The Brownian/Virtual Gate mechanism requires that an adequately high concentration of FG domains be present to form a strong energy barrier. Surprisingly, deletion of up to half of the FG mass from the NPC does not cause the permeability barrier to collapse (STRAWN *et al.* 2004). Thus, either the NPC permeability barrier is highly resilient to substantial deletions or other factors can compensate for these deletions. In contrast to the energetic barrier proposed in the Brownian/Virtual Gate mechanism, two models propose a physical barrier to transport.

Reduction of Dimensionality model

The Reduction of Dimensionality model (Figure 1-6B) (PETERS 2005) proposes that FG domains form a continuous surface of potential transport binding sites with the Phe residues aligning along the inner surface of the NPC. The spacer sequences between FG repeats and other Nups are proposed to form a selectivity filter that occludes free diffusion of large molecules (PETERS 2005). Asymmetric FG repeats collect transport complexes, which then move along this Phe-surface via a two-dimensional walk, pivoting from one binding site to the next. Mathematical modeling has previously suggested that reduction of dimensionality expedites the rate at which a ligand finds its receptor (see references in (PETERS 2005)). Thus, this model predicts that deletion of FG repeats might cause gaps and disrupt the continuity of the FG surface; such gaps would re-introduce a third dimension for molecular movement through the NPC. This could be the cause of transport defects in certain FGΔ strains, including those described in Strawn (STRAWN *et*

al. 2004). In addition, the Reduction of Dimensionality model predicts that deletion of asymmetric FG repeats would diminish the efficiency of NPCs to collect transport complexes. Curiously, this is not observed in cases with the cytoplasmic filaments deleted (WALTHER *et al.* 2002) or all asymmetric FG domains removed (STRAWN *et al.* 2004). Perhaps there is functional compensation by the remaining FG repeats in these cases.

Selective Phase Partitioning model

The Selective Phase Partitioning model (Figure 1-6C) proposes that FG repeats form a meshwork (RIBBECK and GORLICH 2001). This mesh is formed by weak hydrophobic interactions between the Phe side chains of FG repeats, and the entirety of the mesh throughout the NPC resembles a hydrophobic phase or gel. The spacing between Phe-Phe contacts in the mesh is proposed to be such that small molecules can diffuse through without disturbing these contacts. Transport complexes are suggested to traverse the mesh phase by transiently binding to FG repeats and locally disrupting the meshwork. Therefore, this model predicts that FG repeats directly interact and that transport receptors can compete and transiently disrupt the Phe-Phe hydrophobic interactions. Recent experiments have demonstrated that high concentrations of FXFG domains from Nsp1 can form a gel-substance in vitro, though formation of this gel was initiated using harsh chemical conditions (FREY *et al.* 2006). Indeed, a fluorescently tagged transport receptor can partition into an FXFG gel-substance in vitro (FREY and GORLICH 2007), while a protein that cannot interact with FG repeats does not enter this gel efficiently. While it is impressive that an FXFG gel can discriminate between an inert

and an FG-interacting protein, it is not clear whether such a gel barrier could form under physiological conditions or *in vivo*. Further, mathematical modeling predicts that binding to and moving through a gel will retard the mobility of transport receptor complexes, and will decrease transport efficiency of cargo-bound receptors (*i.e.,* larger complexes) more so that free transport receptors (BICKEL and BRUINSMA 2002). Thus, the ability of this proposed FG-gel to form and support known transport rates remains controversial.

Reconciling differences between models

Recently, Patel et al. demonstrated that certain FG domains are cohesive in an *in vitro* assay designed to detect low-affinity interactions (PATEL *et al.* 2007). These assays found that the FG domain of Nup42 and the GLFG domains of Nup116, Nup100, Nup57, Nup145N, and Nup49 can all interact with each other in pair-wise tests. Curiously, though, these experiments did not detect interaction between the Nsp1 FXFG domains (PATEL *et al.* 2007), in direct contradiction with the self-interaction of these domains in the FXFG gel proposed by Frey & Gorlich (FREY and GORLICH 2007; FREY *et al.* 2006). Reconciling these discrepancies will require further refining of assays for detecting interactions and developing techniques that can test these properties *in vivo*. It is possible that a hybrid mechanism exists, such as the dually gated system proposed by Patel *et al.* with entropic barriers on either side of the NPC and a physical meshwork barrier in the center of the pore (PATEL *et al.* 2007).

Future goals are to answer key questions raised by these FG interaction experiments, including: What prevents each FG domain from forming an intramolecular network? How can a gelatinous meshwork form in a newly assembling NPC? How does

the heterogeneity of FG repeat types in the NPC or the glycosylation of vertebrate FG domains affect the stability of any gel?

As a whole, the two key differences between these models are in the nature of interactions among FG repeats and then how these interactions are altered by transport receptors. While recent work has made progress in understanding the nature of FG-FG interactions, it remains unclear how this meshwork may form and how they may be affected by their local and native environment of structural Nups and transport factors. Regardless, current evidence supports elements of both entropic repulsion and Brownian virtual gating in forming the selective yet efficient transport channel that is the NPC. Further work is required to resolve the biophysical nature of the center of the NPC translocation channel in the context of physiological conditions.

Concluding remarks

Our past and current studies have used FGΔ NPCs to examine transport functions in the genetically tractable *S. cerevisiae* model system (STRAWN *et al.* 2004; TERRY and WENTE 2007). This system has allowed us to examine and manipulate transport dynamics, and our results have direct implications for these proposed models and the current understanding of nucleocytoplasmic transport.

In this work I seek to (1) identify NPC components involved in rapid and regulated mRNA export; (2) use a yeast model to systematically define pathways for nucleocytoplasmic transport; and (3) integrate these results into current understanding of

the transport mechanism. As a whole, these studies and those that derive from this body of work will continue to refine our understanding of transport through the NPC *in vivo*.

CHAPTER 2

NUCLEAR MRNA EXPORT REQUIRES SPECIFIC FG NUCLEOPORINS FOR TRANSLOCATION THROUGH THE NUCLEAR PORE COMPLEX

Introduction

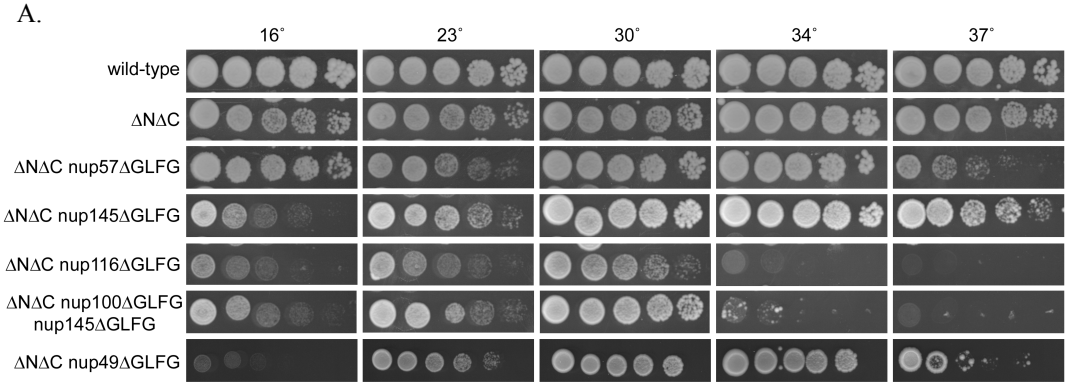
The nuclear envelope (NE) separates the contents of the nucleus and cytoplasm, and is a physical barrier for the exchange of macromolecules. The only known mechanism for nuclear import and export is via nuclear pore complexes (NPCs) (FAHRENKROG and AEBI 2003; FRIED and KUTAY 2003). Thus, the NPC is a central player in controlling gene expression and regulating nucleocytoplasmic signaling. Specifically, the NPC precludes molecules larger than ~30-40 kDa from freely diffusing through its central aqueous channel. Larger macromolecules utilize transport receptors to pass through the NPC in a signal-dependent process (PEMBERTON and PASCHAL 2005). The karyopherin (Kap) β proteins (also termed importins, exportins, and/or transportins) are a major family of transport receptors. There are 14 Kapβs in budding yeast and >20 identified in higher eukaryotes (HAREL and FORBES 2004; PEMBERTON and PASCHAL 2005). Each Kap binds a specific nuclear localization signal (NLS) or nuclear export sequence (NES) on a cargo, with Kap-cargo release and transport directionality triggered by the small GTPase Ran (FRIED and KUTAY 2003; WEIS 2003). There are non-Kapβ transport receptors for RanGDP import (Ntf2) (RIBBECK *et al.* 1998; SMITH *et al.* 1998) and for mRNA export (the heterodimer Mex67-Mtr2 [TAP/NXF1-p15/NXT1 in vertebrates]) (KATAHIRA *et al.* 1999; SANTOS-ROSA *et al.* 1998; SEGREF *et al.* 1997;

[2] This chapter adapted from "Nuclear mRNA export requires specific FG nucleoporins for translocation through the nuclear pore complex." Terry, LJ & SR Wente. J. Cell Biology.

STRASSER *et al.* 2000). With the potential for at least 16 different receptors transporting thousands of distinct cargos, the NPC is a complex machine. Indeed, it is not fully understood how such a myriad of distinct transport receptors utilize the NPC structure for presumably simultaneous translocation.

The ~40-60 MDa NPCs are formed by assembly of multiple copies of ~30 individual proteins, called nucleoporins (Nups) (CRONSHAW *et al.* 2002; FAHRENKROG and AEBI 2003; ROUT *et al.* 2000). Nups associate in discrete subcomplexes and localize in specific substructures of the NPC, including the cytoplasmic filaments, the central core structure in the pore, and a nuclear basket structure (Figure 2-1B) (CRONSHAW *et al.* 2002; FAHRENKROG and AEBI 2003; ROUT *et al.* 2000). Movement of cargo-bound Kapβs, Ntf2 or Mex67-Mtr2 through the NPC requires interactions between the given transport receptor and a specialized subset of NPC proteins, termed the phenylalanine-glycine (FG) Nups (PEMBERTON and PASCHAL 2005). The FG-Nups are defined by domains with numerous clustered repeats of the core dipeptide phenylalanine-glycine (FG) flanked by characteristic spacer sequences (ROUT and WENTE 1994). Nearly half of the Nups contain these FG-domains, each with predominant FG subtypes (FG, FXFG or GLFG), defined NPC substructural locations and corresponding orthologues across species (CRONSHAW *et al.* 2002; ROUT *et al.* 2000) reviewed in (LIM *et al.* 2006a). Some FG-Nups are exclusively on the cytoplasmic (C) NPC fibrils (in *Saccharomyces cerevisiae* Nup159, Nup42), and some are exclusively on the nuclear (N) NPC basket (in *S. cerevisiae* Nup1, Nup2, Nup60); together these are collectively defined as the asymmetric FG-Nups (Figure 2-1B). The remaining FG-Nups are distributed on both sides and through the central NPC channel and are termed the symmetric Nups (in *S.*

A.



B.

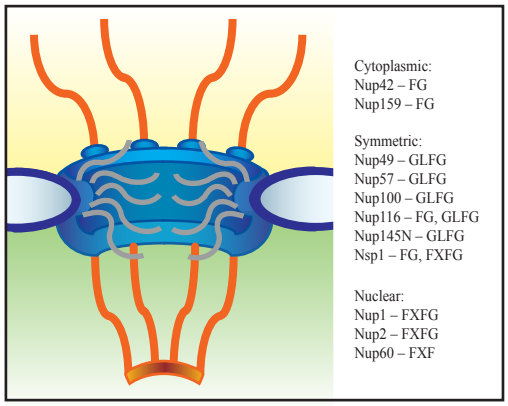


Figure 2-1. The more minimal NPC (mmp) FGΔ mutants have temperature-sensitive growth defects. (A) Wild-type, Δ*N*Δ*C*, and new mmp FGΔ yeast strains were spotted onto YPD in fivefold serial dilutions and grown at the temperatures shown. (B) Schematic representation of the distribution of FG Nups within the NPC.

cerevisiae Nup49, Nup57, Nsp1, Nup100, Nup116, Nup145) (ROUT *et al.* 2000; SUNTHARALINGAM and WENTE 2003). The physical interactions between transport receptors and FG-peptides have been structurally analyzed for Kapb1, Ntf2 and Nxt1. In these receptors, the Phe of an FG repeat is found in hydrophobic pockets on the protein surface (BAYLISS *et al.* 2000a; BAYLISS *et al.* 2002a; BAYLISS *et al.* 2000b; BAYLISS *et al.* 2002b; FRIBOURG *et al.* 2001). Indeed, transport receptor mutants with impaired FG binding are defective for NPC translocation (BAYLISS *et al.* 2002b). Thus, each transport receptor serves as a molecular bridge between FG-Nups and a signal-containing cargo. With multiple FG-repeats per FG-domain, and multiple FG-Nups in each NPC, the pore displays thousands of individual FG-repeats, each of which is a potential binding site for a transport receptor. The abundance of FG-repeats and sequence redundancies between FG-Nups have made understanding the sequence of molecular interactions between the NPC and transport receptors a formidable task.

Given their critical role in the translocation mechanism, the FG-Nups have been the focus of intense study. Models for the mechanism of NPC translocation have as their tenets the unfolded nature of the FG-domains, the huge number of FG repeats per NPC and the intrinsic binding affinities of transport receptors for FG-domains. The localization of the FG-domains in the NPC and the physiological constraints of NPC translocation rates are also key considerations. Two of the fundamental models proposed contrast the FG-domains as forming either a primarily physical or energetic barrier for selective translocation. As a physical barrier, weak interactions between FG-domains are proposed to form a hydrophobic gel into which transport receptors selectively partition due to their FG interaction capacity (FREY *et al.* 2006; RIBBECK and GORLICH 2002). The

hydrophobic gel would form a "selective-phase" and exclude macromolecules larger than the physical barrier generated by the FG interaction meshwork. As an energetic barrier, the interaction of a transport receptor with an FG-Nup(s) would allow the transport receptor to overcome an entropic threshold for diffusion through the NPC central channel (ROUT *et al.* 2003). The FG-domains would also function as repulsive bristles to entropically exclude non-transport receptor molecules (LIM *et al.* 2006b). As such, the NPC would be governed by a "virtual-gate." From the analysis of individual FG-domains *in vitro*, there is independent data to support both the selective-phase and vitual-gate models.

To analyze the requirements for FG-domains in the context of the intact NPC, we have used a large-scale genetic strategy in *S. cerevisiae* (STRAWN *et al.* 2004). By combinatorial in-frame deletions in genes encoding the FG-Nups, we showed that the asymmetric FG-domains are dispensable for facilitated transport, whereas the symmetric FG-domains are sufficient. Interestingly, although the selective-phase model predicts that the abundance or mass of FG-repeats is critical to transport function (FREY *et al.* 2006; MACARA 2001; RIBBECK and GORLICH 2001; RIBBECK and GORLICH 2002), we found that the number or mass of FG-repeats does not correlate with *in vivo* transport capacity. We also found that for a given FG deletion (designated FGΔ) mutant, only a subset of the Kapβ transport receptors were perturbed. This suggests that different transport receptors require distinct combinations of FG-domains for function (STRAWN *et al.* 2004). In support of this, biochemical studies have demonstrated that different Kaps have different relative *in vitro* binding levels for the same FG-Nup (AITCHISON *et al.* 1996; ALLEN *et al.* 2001). There is also evidence that Kap95 might use different FG binding sites than those

used by Mex67 (ALLEN *et al.* 2001; BLEVINS *et al.* 2003; STRAWN *et al.* 2001). Taken together, these studies suggest that the NPC may harbor multiple translocation pathways for different transport receptors.

To further investigate the FG dependent transport pathways through the NPC, we have now generated a new collection of FG-domain deletion mutants. We specifically compared Kapβ versus non-Kapβ translocation pathways by dissecting the requirements for Mex67-Mtr2-dependent mRNA export. Multiple laboratories have identified *nup* null or temperature-sensitive alleles that cause mRNA export defects, and overproduction of the Nup116 GLFG-domain inhibits mRNA export (COLE 2000; STRASSER and HURT 1999; STRAWN *et al.* 2001). However, our new mutants have allowed the first global analysis of specific FG-domain requirements in mRNA export. We have found striking differences in the requirements for Mex67-mediated mRNA export versus Kapβ-mediated transport. These results impact models for the *in vivo* NPC translocation mechanisms and support our hypothesis that multiple FG pathways exist for receptor-mediated translocation across the NPC.

Results

More minimal pore (*mmp*) FGΔ mutants have distinct Kap transport defects

In our prior studies, we generated a *S. cerevisiae* mutant that lacked all the asymmetric FG-domains on the N and C faces of the NPC, designated the Δ*N*Δ*C* mutant (STRAWN *et al.* 2004). The Δ*N*Δ*C* mutant has a slight rate delay in import via Kap95 and Kap104; however, it has no marked steady-state defect for any transport receptor assayed.

Thus, the asymmetric FG-domains do not serve essential functions. However, we speculated that the asymmetric FG-domains could be key to maximal transport efficiency. In addition, because the FG-domains can presumably occupy multiple topological positions in the NPC (DENNING *et al.* 2003; FAHRENKROG *et al.* 2002; LIM *et al.* 2006b), it is possible that the asymmetric FG-domains functionally compensate when individual symmetric FG-domains are deleted. We therefore selected the Δ*N*Δ*C* mutant as a foundation for studying the transport roles of individual symmetric FG-domains. In-frame, internal chromosomal deletions of the sequence encoding individual symmetric FG-domains were constructed in the Δ*N*Δ*C* background. If lethality was observed when a symmetric FG-domain was removed in the Δ*N*Δ*C* background, control complementation experiments were conducted with plasmids expressing the full length *NUP* or FGΔ mutant versions (see Materials and Methods). This generated a series of "more minimal pore" (*mmp*) FGΔ mutant strains. Specifically, the Δ*N*Δ*C* mutant was combined with individual deletions of the GLFG regions in Nup49, Nup57, Nup145, Nup100, Nup116, or the FG and FXFG regions in Nsp1. We found that all of the *mmp* FGΔ mutant strains with only one symmetric FG-domain removed were viable (Figure 2-1A and (STRAWN *et al.* 2004)). Additionally, the Δ*N*Δ*C nup100*Δ*GLFG nup145*Δ*GLFG* mutant was viable, despite having only 4 FG-Nups intact (Nsp1, Nup49, Nup57 and Nup116).

The strains in this new *mmp* FGΔ mutant collection were characterized for growth properties at a range of temperatures. As shown in Figure 2-1A, the Δ*N*Δ*C* mutant showed robust growth at all temperatures tested. In comparison, the Δ*N*Δ*C nup57*Δ*GLFG* mutant had inhibited growth at 37°C, whereas the Δ*N*Δ*C nup145*Δ*GLFG* mutant was cold sensitive at 16°C. The Δ*N*Δ*C nup49*Δ*GLFG* mutant showed both temperature sensitivity

at 37°C and cold sensitivity at 16°C. Overall, the Δ*N*Δ*C nup116*Δ*GLFG* mutant and the Δ*N*Δ*C nup100*Δ*GLFG nup145*Δ*GLFG* mutant strains had the most severe growth phenotypes with both temperature sensitivity at 34°C and cold sensitivity (Figure 2-1A). The Δ*N*Δ*C nsp1*Δ*FG*Δ*FXFG* mutant generated in our prior studies is cold sensitive at 23°C and also inhibited at 37°C (STRAWN *et al.* 2004).

We speculated that the temperature-dependent growth defects were linked to perturbations of an essential transport receptor(s). To test for defects in transport, the *mmp* FGΔ mutants were transformed with a panel of GFP-based reporters for different Kapβ transport receptors. Each transport reporter was based on a Kapβ- or Kapα-specific NLS fused to GFP or a tandem NLS-NES fused to GFP. In wild-type cells, all of the NLS-GFP reporters are predominantly nuclear whereas the NLS-NES-GFP is mostly cytoplasmic. The basic classical (c) NLS of SV40 large T antigen is imported by the Kap95-Kap60 heterodimer (CHOOK and BLOBEL 2001; SHULGA *et al.* 1996), and Nab2 and the Nab2-NLS-GFP reporter are imported by Kap104 (AITCHISON *et al.* 1996; SHULGA *et al.* 2000). The Spo12-NLS is recognized primarily by Kap121/Pse1 (CHAVES and BLOBEL 2001). The NLS-NES-GFP reporter includes a cNLS for Kap95-Kap60 import and a leucine-rich NES for Xpo1/Crm1 export (STADE *et al.* 1997). Steady-state transport assays in the wild-type and *mmp* FGΔ mutants were conducted at both the permissive temperature and after shifting to growth at 37°C for 1 hour. The results are summarized in Table 2-1. For all the mutants, no defects at steady-state were detected with either the cNLS (Kap95-Kap60) or NLS-NES-GFP (Crm1/Xpo1) reporters (Figure 2-2 and Table 2-1). However, several of the mutants showed altered Spo12-NLS-GFP (Kap121) import. This included the Δ*N*Δ*C* mutant combined with either the

Table 2-1. Summary of transport assay results.

	cNLS import	Nab2 import	Spo12NLS import	Leu-rich NES export	mRNA export
Wild-type	+[a]	+[a]	+[a]	+[a]	+
nup100ΔGLFG *nup145ΔGLFG* *nup57ΔGLFG*	+[a]	-[a]	-[a]	+[a]	-
ΔNΔC	+[a]	+[a]	+/-[a]	+[a]	+
ΔNΔC *nup57ΔGLFG*	+	+	+	+	-
ΔNΔC *nup100ΔGLFG*	+[a]	+/-[a]	-[a]	+[a]	+
ΔNΔC *nsp1ΔFGΔFXFG*	+[a]	+/-[a]	-[a]	+[a]	+
ΔNΔC *nup145ΔGLFG*	+	+	+	+	+/-
ΔNΔC *nup116ΔGLFG*	+/-	-	-	+	+
ΔNΔC *nup100ΔGLFG* *nup145ΔGLFG*	+	+	-	+	+
ΔNΔC *nup49ΔGLFG*	+	+	+	+	-

[a] Strawn et al., 2004

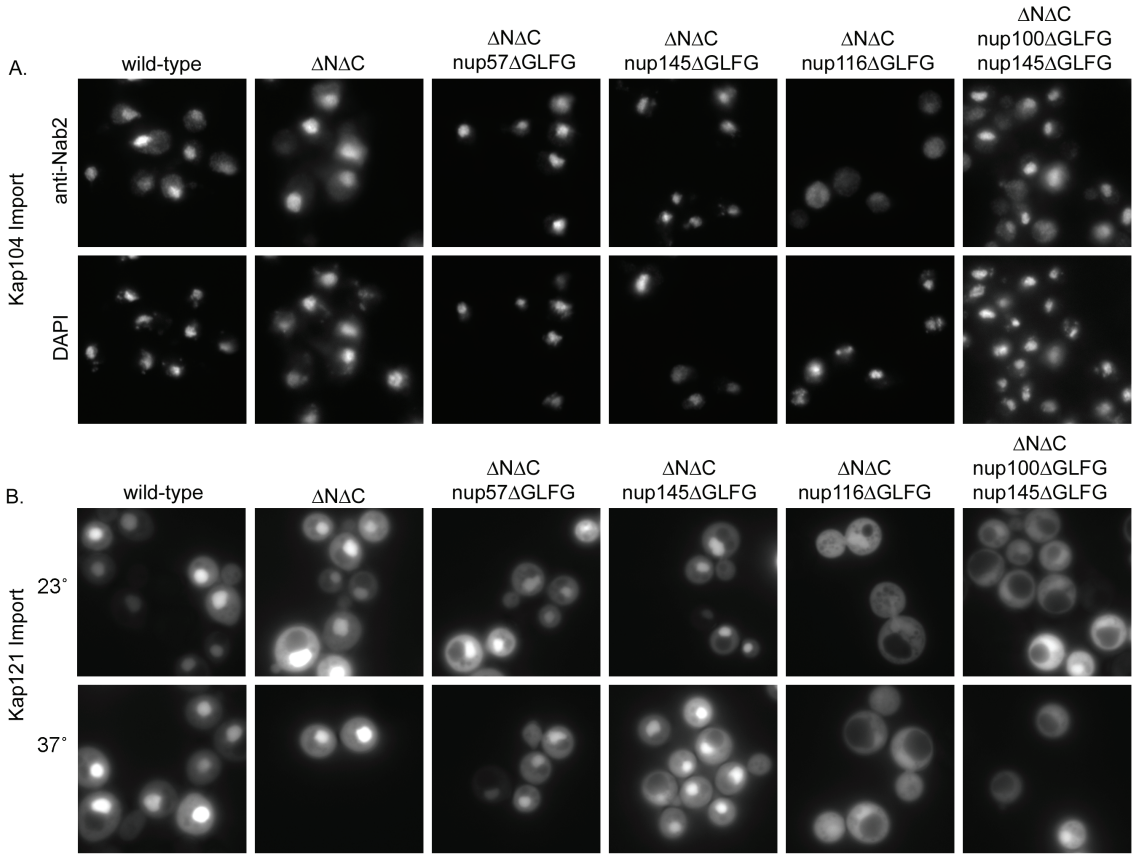


Figure 2-2. The mmp FGΔ NPC mutants have distinct defects in Kap104 and Kap121 steady-state import. (A) Indirect immunofluorescence with an anti-Nab2 antibody in yeast mmp FGΔ strains was conducted after a 1-h shift to 37°C. Nab2 localization, indicating Kap104 import, and DAPI-staining panels are shown. (B) Localization of a Spo12-NLS-GFP reporter, which is imported by Kap121, was evaluated at 23°C and after a 1-h shift to 37°C in mmp FGΔ strains.

*nup100*Δ*GLFG*, *nsp1*Δ*FG*Δ*FXFG*, *nup116*Δ*GLFG*, or *nup100*Δ*GLFG nup145*Δ*GLFG* alleles (Table 2-1, Figure 2-2, and (STRAWN *et al.* 2004)). At 37°C, the Spo12-NLS-GFP reporter showed coincident increased cytoplasmic signal and decreased nuclear intensity in the Δ*N*Δ*C nup100*Δ*GLFG nup145*Δ*GLFG* mutant and the Δ*N*Δ*C nup116*Δ*GLFG* mutant cells (Figure 2-2B). This indicated that these strains have defects in Kap121 transport.

Interestingly, only one of the *mmp* FGΔ mutant strains, Δ*N*Δ*C nup116*Δ*GLFG*, showed a strong perturbation in steady-state Nab2 import by Kap104, with diminished nuclear localization and increased cytoplasmic signal at all growth temperatures. The defect was apparent using either the Nab2-NLS-GFP reporter (data not shown) or via indirect immunofluorescence for Nab2 localization (Figure 2-2A). Steady-state transport defects for Kap104 or Kap121 were not observed in the Δ*N*Δ*C nup57*Δ*GLFG* mutant, the Δ*N*Δ*C nup49*Δ*GLFG* mutant or the Δ*N*Δ*C nup145*Δ*GLFG* mutant strains (Figure 2-2 and Table 2-1). When comparing the Kap104 and Kap121 transport defects, it was especially striking that the Δ*N*Δ*C nup100*Δ*GLFG nup145*Δ*GLFG* mutant showed differential perturbations. The Kap104 cargo Nab2 was efficiently imported (Figure 2-2A; rightmost column), whereas the Kap121 reporter accumulated in the cytoplasm at 23°C and 37°C (Figure 2-2B; rightmost column). This is the first reported *in vivo* separation of FG-domain requirements for Kap104 and Kap121 NPC translocation. Overall, the *mmp* FGΔ mutant strains showed distinct defects for transport by specific Kaps.

Symmetric FGΔ and *mmp* FGΔ mutants have poly(A)$^+$ RNA export defects

To understand the contributions of FG-domains to mRNA export, we screened a subset of our existing FGΔ mutant strains and our new *mmp* FGΔ mutant strains for mRNA export defects. This was evaluated using *in situ* hybridization with an oligo d(T) probe, which detects poly(A)$^+$ RNA. All of the viable FGΔ mutant strains with three symmetric FG-domains deleted showed nuclear accumulation of poly(A)$^+$ RNA after a 1-hour shift to 37˚C (Figure 2-3, Table 2-1, data not shown). However, the Δ*N*Δ*C* mutant cells did not show nuclear accumulation of poly(A)$^+$ RNA. We also did not observe mRNA export defects in the Δ*N*Δ*C nup100*Δ*GLFG* mutant, the Δ*N*Δ*C nsp1*Δ*FG*Δ*FXFG* mutant, the Δ*N*Δ*C nup100*Δ*GLFG nup145*Δ*GLFG* mutant or the Δ*N*Δ*C nup116*Δ*GLFG* mutant cells. For mutants that showed no nuclear poly(A)$^+$ RNA accumulation, we also used an independent assay for mRNA export capacity and analyzed the effect on heat shock protein production. Following heat shock in wild-type cells, elevated levels of Hsp104, Hsp82, Ssa4, and Ssa1 are a direct reflection of proper export and translation for the respective heat shock induced mRNAs (SAAVEDRA *et al.* 1997; STUTZ *et al.* 1997). The Δ*N*Δ*C* mutant and the Δ*N*Δ*C nup116*Δ*GLFG* mutant were competent for heat shock protein production (data not shown). We concluded that the FG-domains of the asymmetric FG-Nups (Nup159, Nup42, Nup1, Nup2 and Nup60) and three specific symmetric FG-Nups (Nup100, Nup116 and Nsp1) were not individually essential for mRNA export. In contrast, the Δ*N*Δ*C nup57*Δ*GLFG* and the Δ*N*Δ*C nup49*Δ*GLFG* mutant strains showed strong perturbations in mRNA export with marked nuclear accumulation of poly(A)$^+$ RNA (Figure 2-3 and Table 2-1). This indicated that Nup57 and/or Nup49 were preferentially required for mRNA export.

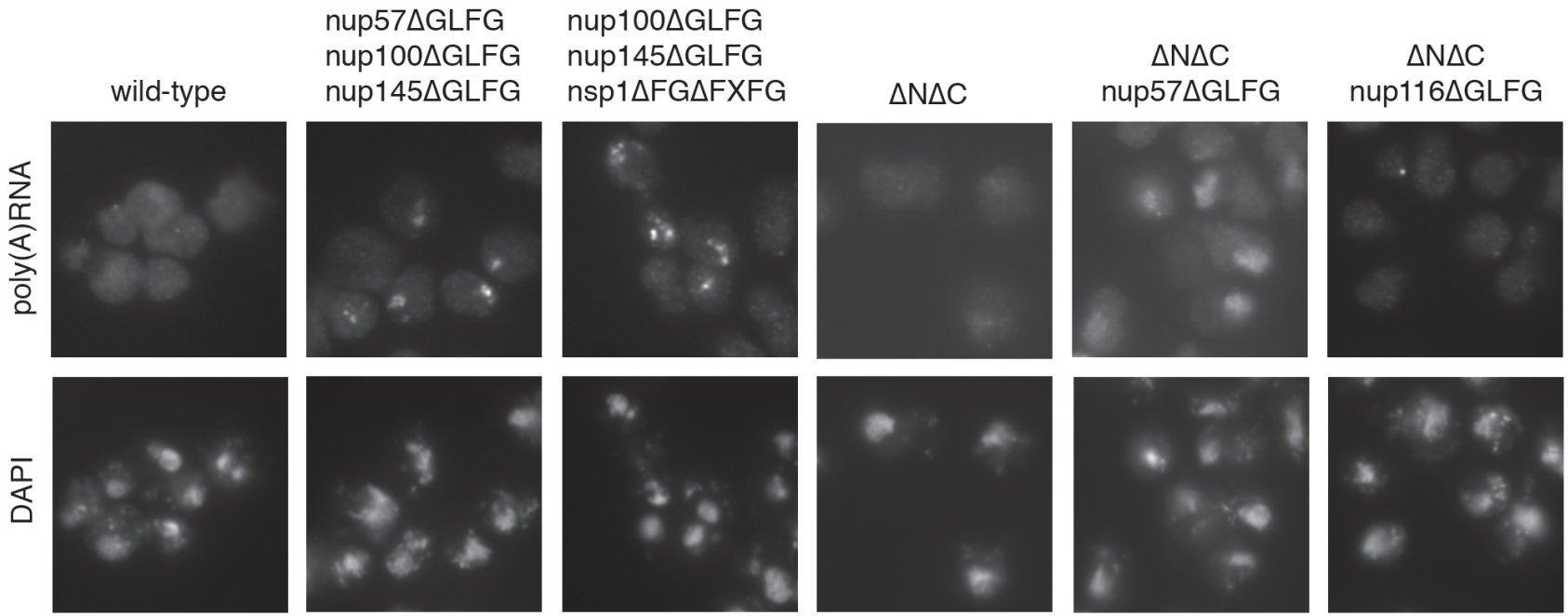


Figure 2-3. mRNA export is inhibited in the symmetric FGΔ mutants and the mmp mutant Δ*N*Δ*C nup57*Δ*GLFG*. *In situ* hybridization with an oligo d(T) probe was conducted in the FGΔ NPC mutants after a 1-h shift to 37°C. Signal for the oligo d(T) probe indicates the subcellular distribution of poly(A)$^+$ RNA in comparison with the nuclear signal (by coincident DAPI staining).

To further probe the requirements for the GLFG domains of Nup57 or Nup49, we examined a *nup57*Δ*GLFG nup49*Δ*GLFG* double mutant strain. The *nup57*Δ*GLFG nup49*Δ*GLFG* mutant was assayed for mRNA export defects as described above. Nuclear poly(A)$^+$ RNA accumulation was observed in 9.9±0.9% of the *nup57*Δ*GLFG nup49*Δ*GLFG* cells. Although this defect is significantly different from the level observed in wild-type cells (p=0.0031), it is not as penetrant as the defect in either the Δ*N*Δ*C nup49*Δ*GLFG* mutant or the Δ*N*Δ*C nup57*Δ*GLFG* mutant cells (30.3±2.5% and 26.7±6.1%, respectively). Thus, the GLFG-domains of Nup57 and Nup49 are not individually, or in combination, essential for mRNA export. This suggested that other symmetric FG-domains (Nup116, Nup100, Nup145, Nsp1) functionally compensate in the absence of the Nup57 and Nup49 GLFG-domains. However, when the asymmetric FG-domains were removed (Δ*N*Δ*C)*, the GLFG-domain of Nup57 or Nup49 was specifically required and the FG-domains from Nup116, Nup100, Nup145, and Nsp1 were not sufficient. Taken together, these results revealed a combinatorial requirement in mRNA export for specific GLFG domains with the asymmetric FG-domains. Moreover, such differential requirements for FG-domains in mRNA export were unanticipated. Prior studies have reported that Mex67 interacts *in vitro* with several of the asymmetric FG-domains (Nup159, Nup42, Nup1, Nup60) and with three symmetric FG-domains (Nup100, Nup116, Nsp1) (ALLEN *et al.* 2001; FISCHER *et al.* 2002; STRASSER *et al.* 2000; STRAWN *et al.* 2001). Although the GLFG-domains of Nup57 and Nup49 have not previously been reported to bind Mex67, these results suggested that the FG-domains of Nup57 and Nup49 are key sites *in vivo* for mRNA export.

mRNA export requires GLFG-domains of Nup57 and nuclear-face Nups

Nup57 and Nup49 are both GLFG-Nups that assemble in a heterotrimeric complex with Nsp1 (FAHRENKROG *et al.* 1998; GRANDI *et al.* 1993; SCHLAICH *et al.* 1997). Given this shared NPC localization, the common FG types (GLFG), and the growth and transport phenotypes in the *mmp* FGΔ analysis, we concluded that the Δ*N*Δ*C nup57*Δ*GLFG* mutant and the Δ*N*Δ*C nup49*Δ*GLFG* mutant strains were functionally comparable. We selected the Δ*N*Δ*C nup57*Δ*GLFG* mutant for further analysis as it was less complex genotypically (see Materials and Methods). To pinpoint which of the FG-domains in the Δ*N*Δ*C nup57*Δ*GLFG* mutant were most critical for mRNA export, we systematically generated strains with fewer FGΔ combinations. Each mutant strain was assayed for poly(A)$^+$ RNA localization by *in situ* hybridization with the oligo d(T) probe, and the percent of cells in the population showing nuclear accumulation of poly(A)$^+$ RNA was scored (Figure 2-4). The *nup57*Δ*GLFG* single mutant and the Δ*N*Δ*C* mutant did not have defects, as the percentage of cells showing nuclear poly(A)$^+$ RNA accumulation was not significantly different from wild-type. The Δ*C nup57*Δ*GLFG* mutant strain also did not have a poly(A)$^+$ RNA export defect. In contrast, Δ*N nup57*Δ*GLFG* mutant cells had a strong export defect after shifting to growth at 37˚C for 1 hour, with nearly 80% of the cells showing nuclear accumulation of poly(A)$^+$ RNA. It was striking that the defect in the Δ*N nup57*Δ*GLFG* mutant (in 79.9±9.4% of the cells at the assay time point) was more severe than that in the Δ*N*Δ*C nup57*Δ*GLFG* mutant (in 26.7±10.6% of the cells) (see Discussion).

To further dissect the Δ*N nup57*Δ*GLFG* mutant phenotype, we assayed mutants with all possible FGΔ combinations of nuclear-face FG-domains (Nup1, Nup2 and

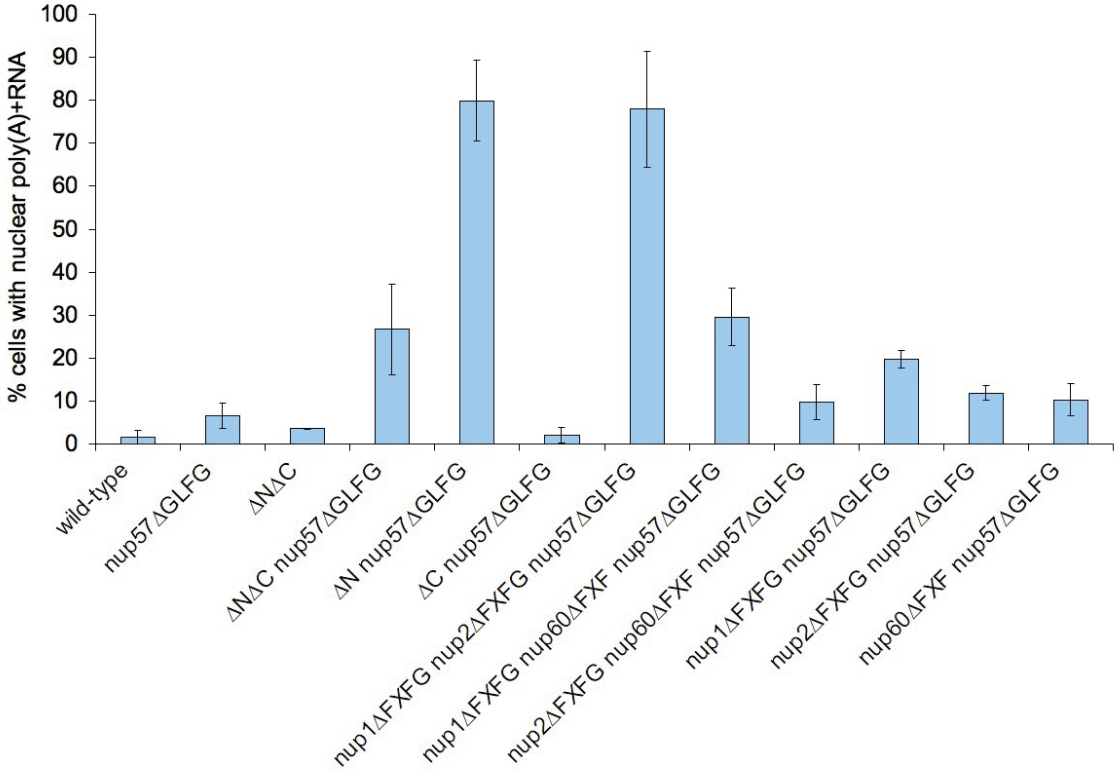


Figure 2-4. mRNA export requires the FG domains of Nup57 and nuclear face Nups. *In situ* hybridization with an oligo d(T) probe was conducted with the FGΔ strains indicated after a 1-h shift to 37°C. The percentage of cells showing the accumulation of poly(A)$^+$ RNA was calculated based on fields of >100 cells in three independent trials. Deletion of the nuclear face FG domains (*nup1ΔFXFG, nup2ΔFXFG, and nup60ΔFXF*) is abbreviated as Δ*N*. Deletion of the cytoplasmic face FG domains (*nup42ΔFG and nup159ΔFG*) is abbreviated as Δ*C*. Error bars represent SEM.

Nup60) with the *nup57*Δ*GLFG* allele. The *nup1*Δ*FXFG nup2*Δ*FXFG nup57*Δ*GLFG* triple mutant had a significant poly(A)$^+$ RNA export defect with penetrance similar to the Δ*N nup57*Δ*GLFG* mutant (Figure 2-4). This indicated that the *nup60*Δ*FXFG* allele did not contribute significantly to the Δ*N nup57*Δ*GLFG* mutant phenotype. In fact, the addition of the *nup60*Δ*FXF* mutant allele to any single or double *FG*Δ nup57ΔGLFG mutant did not result in a statistically significant difference in the level of nuclear poly(A)$^+$ RNA accumulation. The *nup1*Δ*FXFG nup57*Δ*GLFG* double mutant and the *nup2*Δ*FXFG nup57*Δ*GLFG* double mutant strains also had defects; however, the percent of cells with nuclear poly(A)$^+$ RNA accumulation was significantly less in the *nup1*Δ*FXFG nup57*Δ*GLFG* double mutant and the *nup2*Δ*FXFG nup57*Δ*GLFG* double mutant strains than in the combined *nup1*Δ*FXFG nup2*Δ*FXFG nup57*Δ*GLFG* triple mutant. Overall, these results suggested that the export of mRNA requires both a symmetric GLFG-domain (Nup57, Nup49) and the FXFG-domains on the nuclear face (Nup1, Nup2). This is the first evidence for an *in vivo* role for the specifically asymmetric FG-domains in active NPC translocation.

Mex67 binds Nup57 GLFG-domain *in vitro*

We speculated that the deletion of FG-domains critical for Mex67 docking at the NPC was the mechanistic basis for the mRNA export defects in the respective *mmp* FGΔ mutants. Specifically, the *in vivo* results suggested that Mex67 required binding sites in the FG-domains of Nup57 or Nup49 and Nup1 or Nup2. Prior studies have documented that Mex67-Mtr2 can bind representative FG, FXFG, and GLFG-domains. The FXFG-domain of Nup1 has been directly analyzed (STRASSER *et al.* 2000); however, tests of the

Nup57 GLFG region have not been reported. We conducted studies to verify this interaction biochemically with recombinant proteins and a soluble binding assay. Clarified bacterial lysates from cells expressing GST alone or GST fused with the GLFG regions of Nup57 or Nup116 (GST-GLFG-Nup57 or GST-GLFG-Nup116) were incubated with glutathione sepharose. Purified MBP-Mex67 was then applied to the resin with the respective immobilized GST fusion proteins. As shown in Figure 2-5, GST-GLFG-Nup57 bound MBP-Mex67, whereas GST alone did not bind MBP-Mex67. Binding was also detected between MBP-Mex67 and GST-GLFG-Nup116, as has previously been shown (STRAWN *et al.* 2001). Thus the GLFG-domain of Nup57 directly binds Mex67 *in vitro*.

Efficient Mex67 recruitment to NPCs requires asymmetric FG-domains and Nup57-GLFG

An mRNA export defect in a FGΔ mutant could result from either a direct effect on Mex67-NPC interactions or an indirect perturbation on Kap-mediated import of an essential mRNA export factor(s). We speculated that FGΔ mutants with primary defects in Mex67-mediated mRNA export would have decreased rates of Mex67-GFP recruitment to the NE/NPC due to the lack of critical FG binding sites. To directly examine the dynamic properties of Mex67-GFP, we developed a live-cell assay (Figure 2-6F). This strategy was based on the well-established assay for monitoring NLS-GFP import in live yeast cells (SHULGA *et al.* 1996). Wild-type parental or FGΔ-mutant cells expressing chromosomally tagged Mex67-GFP were incubated in glucose-free media in the presence of 10 mM 2-deoxy-D-glucose and 10 mM sodium azide for 45 minutes. This

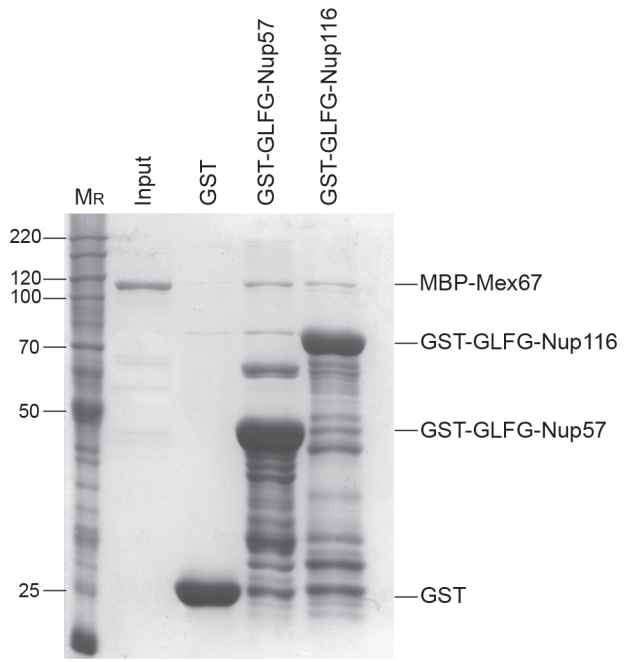


Figure 2-5. Mex67 binds the GLFG domain of Nup57. Bacterially expressed GST, GST-GLFG-NUP57, and GST-GLFG-NUP116 were each immobilized on glutathione agarose beads. Recombinant purified MBP-Mex67 was added, and the bound fraction was eluted. 10% of the input (MBP-Mex67) and the eluted fractions was resolved by SDS-PAGE and stained with Coomassie blue. Molecular mass (kilodaltons) markers are shown at the left (Mr).

treatment results in cellular energy depletion and inhibits active nuclear transport (SHULGA *et al.* 1996). The process of mRNA export is energy-dependent (PASCHAL 2002), at a minimum requiring the ATPase Dbp5 (SNAY-HODGE *et al.* 1998; TSENG *et al.* 1998). As shown in Figure 2-6, prior to energy depletion, all strains showed a strong Mex67-GFP signal at the nuclear rim. After energy depletion in all the strains, Mex67-GFP was no longer concentrated at the NE/NPC, and the cytoplasmic and nuclear signals increased. Co-expression of a dsRed-HDEL (fusion protein with amino acid signal sequence for endoplasmic reticulum (ER) retention) was used to facilitate visualization of the NE/ER. The localization of the dsRed-HDEL protein was not altered by energy depletion. As a control, we monitored the localization of two structural non-FG-Nups, GFP-Nic96 and Nup170-GFP (Figure 2-6E), and found that strong punctate NE/NPC signal was present both before and after energy depletion. Nuclear rim localization of Nup49-GFP was also not altered by energy depletion in wild type cells or in Δ*N*Δ*C* mutant cells (Figure 2-6E and data not shown, respectively). This indicated that energy depletion results in mislocalization of Mex67-GFP without a general perturbation of NE/NPC structure.

Using this assay, NE/NPC re-association kinetics were determined by fluorescence microscopic monitoring of Mex67-GFP localization. At the start of the assay, the energy-depleted cells were washed and resuspended in 23˚C glucose-containing media. The cells were then incubated until the NE/NPC signal recovered to pre-treatment levels. Individual cells (n>150) in a population were scored for normal continuous NE/NPC signal and relative levels of nucleoplasmic and cytoplasmic staining (Figure 2-6G). By plotting the percentage of cells with normal continuous NE/NPC

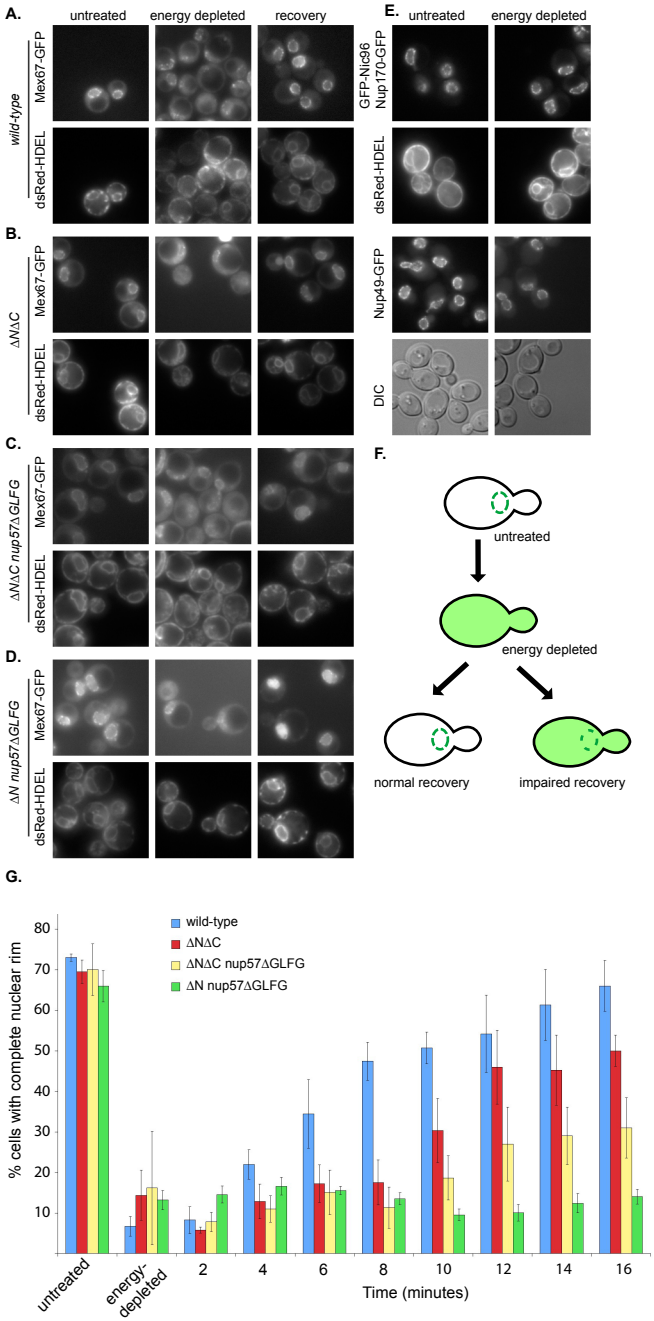


Figure 2-6. Mex67-GFP recruitment to the NE/NPC is severely inhibited in both the Δ*N*Δ *C nup57*Δ *GLFG* mutant and Δ*N nup57*Δ *GLFG* mutant. (A–D) Mex67-GFP localization in representative wild-type (A), Δ*N*Δ*C* (B), Δ*N*Δ*C nup57*Δ*GLFG* (C), and Δ*N nup57*Δ*GLFG* (D) cells before the assay (untreated; left), after energy depletion (middle), or after 5-6 min of recovery from energy depletion (right). For each, the coincident localization of the ER marker dsRed-HDEL is shown. (E) As controls, the localization of GFP-Nic96 and Nup170-GFP or Nup49-GFP under the same conditions was evaluated. (F) A schematic diagram of the energy depletion assay for Mex67-GFP localization is shown. (G) The kinetics of Mex67-GFP recovery to the nuclear rim over time after energy depletion was determined. For three independent experiments, >150 cells were scored for the subcellular distribution of GFP signal at each time point. Error bars represent SEM. DIC, differential interference contrast.

signal as a function of time, relative association rates were determined. We then compared the association kinetics wherein a single variable was changed (e.g. the FGΔ mutant background). Within six minutes after restoring energy to the system, Mex67-GFP in the wild-type cells returned to the pre-treatment phenotype with Mex67-GFP predominantly at NE/NPCs (Figure 2-6A). The Δ*N*Δ*C* mutant cells recovered more slowly than wild-type cells, and at intermediate time points an increased frequency of cells had elevated intranuclear signal relative to cytoplasmic. The recovery process in the Δ*N*Δ*C nup57*Δ*GLFG* mutant was significantly more delayed. After 15 minutes, the Δ*N*Δ*C nup57*Δ*GLFG* cells showed only minimal recovery of Mex67-GFP localization to the NE/NPC. Moreover, at the intermediate time points, the Mex67-GFP localization in the Δ*N*Δ*C nup57*Δ*GLFG* cells was mostly intranuclear with no distinct NE/NPC staining (Figure 2-6C). This phenotype was also observed in the Δ*N nup57*Δ*GLFG* mutant, where more than 50% of the cells accumulated Mex67-GFP in the nucleus and concentrated nuclear rim localization was not achieved over the time course of the assay (Figure 2-6D). Again, as in the assays of poly(A)$^+$ RNA accumulation, the rate of Mex67-GFP localization to the NE/NPC was clearly more inhibited in the Δ*N nup57*Δ*GLFG* mutant than in the Δ*N*Δ*C nup57*Δ*GLFG* mutant (see Discussion). Overall, we concluded that Mex67-GFP recruitment to the NPC in the Δ*N*Δ*C nup57*Δ*GLFG* mutant and in the Δ*N nup57*Δ*GLFG* mutant was impaired. The intranuclear localization prior to distinct NE/NPC staining might reflect efficient import of Mex67-GFP with specific mRNA export inhibition. These results correlate with our assays for poly(A)$^+$ RNA export, and suggest that the Δ*N*Δ*C nup57*Δ*GLFG* mutant and the Δ*N nup57*Δ*GLFG* mutant are

blocked for poly(A)$^+$ RNA export due to altered Mex67 recruitment to and/or translocation through the NPC.

Discussion

Many approaches have been used to study the mechanism by which transport receptors cross the NPC and the requirements for transport receptor interactions with the FG-Nups. We have used a genetic strategy in *S. cerevisiae* to generate extensive collections of mutants with specific combinations of FG-domains removed and have conducted direct tests of the *in vivo* roles of putative FG binding sites for transport receptors in the intact NPC (Strawn et al., 2004). Here we report the analysis of new "more minimal pore" *mmp* FGΔ mutants wherein the symmetric FG-domains were removed in the absence of all asymmetric FG-domains (Δ*N*Δ*C*). In some cases, the FGΔ phenotypes correlate directly with reported *in vitro* binding results. For example, previous studies have shown *in vitro* binding of Kap104 to the Nup116 GLFG region (AITCHISON *et al.* 1996; ALLEN *et al.* 2001) and indeed the Δ*N*Δ*C nup116*Δ*GLFG* mutant has defects in Kap104-mediated transport, whereas the Δ*N*Δ*C* mutant does not. This confirms that the Nup116 GLFG-domain is a critical Kap104 binding site. On the other hand, we found that not all *in vitro* binding events are essential *in vivo*. Although Mex67 interacts with the GLFG region of Nup116 *in vitro* (STRASSER *et al.* 2000; STRAWN *et al.* 2001), the Δ*N*Δ*C nup116*Δ*GLFG* mutant has no mRNA export defect. As a result, we conclude that *in vitro* binding between a transport receptor and an FG-domain does not necessarily correlate with a requirement for that FG-domain *in vivo.* Rather, the

substructural location and physiological context of each FG-domain is likely a key determinant in the organization of transport pathways through the NPC.

We have also identified binding events not previously recognized as important. We found that distinct combinations of both symmetric and asymmetric FG-domains are needed for efficient nuclear export of poly(A)$^+$ RNA and recruitment of Mex67-GFP to the NE/NPC. This includes a GLFG-domain from the symmetric Nup57 or Nup49 plus the asymmetric FXFG-domains of Nup1 and Nup2 on the nuclear NPC face. Surprisingly, import by Kaps does not require these same FG-domains. These results support a model wherein different transport receptors utilize distinct FG-domains allowing for multiple, preferred, and independent transport pathways through the NPC.

mRNA export requires combinatorial use of distinct FG-domains and non-FG binding sites

Analysis of the *mmp* FGΔ mutants reveals that at least two FG-dependent steps are required for mRNA export through the NPC. We speculate that the locations in the NPC of the respective FG-domains are key determinants for efficient mRNA export. The export cargo, a messenger ribonucleoprotein particle (mRNP), is assembled co-transcriptionally and during mRNA processing (HIERONYMUS and SILVER 2004). For such an mRNP, the first step in NPC translocation might require the nuclear-face FXFG binding sites in Nup1 and Nup2 for Mex67 recruitment to the NPC. In support of this hypothesis, the Δ*N*Δ*C* mutant alone has a defect in the rate of Mex67-GFP recruitment to the NE/NPC. This also provides the first *in vivo* evidence that asymmetric FG-domains contribute to the efficiency of mRNA export.

Second, after initial mRNP recruitment to the NPC, symmetrically localized FG-domains are needed. Specifically, a GLFG-domain from Nup57 or Nup49 in the symmetric Nsp1-Nup49-Nup57 subcomplex is required. Our results suggest that coupled interactions with the nuclear face FG-domains and with Nup57 or Nup49 are required for mRNA export. Finally, after recruitment to the FXFG-Nups on the nuclear face and translocation dependent on symmetric GLFG-Nups, a third non-FG step in mRNA export is proposed at the cytoplasmic FG face. Interestingly, the asymmetric Nup159 and Nup42 FG-domains on the cytoplasmic NPC face are not necessary for mRNA export when deleted on their own (Δ*C*, i.e. *nup159*Δ*FG nup42*Δ*FG*; data not shown) or in combination with the *nup57*Δ*GLFG* mutant (the Δ*C nup57*Δ*GLFG* mutant). However, the flanking non-FG-domains of Nup159 and Nup42 are required for mRNA export, and serve as critical docking sites for the mRNA export factors, Dbp5 and Gle1 respectively (ALCAZAR-ROMAN *et al.* 2006b; HODGE *et al.* 1999; MURPHY and WENTE 1996; SCHMITT *et al.* 1999; STRAHM *et al.* 1999; WEIRICH *et al.* 2004; WEIRICH *et al.* 2006). It is striking that in two independent assays (poly(A)$^+$ RNA export and Mex67-GFP localization) the Δ*N nup57*Δ*GLFG* mutant had a more severe phenotype than the Δ*N*Δ*C nup57*Δ*GLFG* mutant. In genetic terms, this indicates that the Δ*C* FG deletion partially suppressed the defect of the Δ*N nup57*Δ*GLFG* mutant. As such, the FG-domains of Nup159 and Nup42 might play an inhibitory role during mRNA export in the intact NPC or a role in regulating terminal mRNP release. Mex67 is a potential target of the proposed Dbp5 RNP remodeling activity (LUND and GUTHRIE 2005), and Mex67 binding to the respective Nup159 and Nup42 FG-domains might influence this mechanism.

Overall, these results support a model with three coupled steps for the efficient and regulated export of mRNPs through the NPC. Alternatively, the mRNA export and Mex67-GFP recruitment defects in the Δ*N*Δ*C nup57*Δ*GLFG* mutant and the Δ*N nup57*Δ*GLFG* mutant strains could be due to impaired mRNP assembly or disassembly rates. To date, however, only non-FG-domains have been proposed as platforms for transport complex assembly or disassembly...

Nup49/Nup57 and Nup116 define two distinct pathways through the NPC

Our finding of unique transport defects in the *mmp* FGΔ mutants provides strong evidence for the existence of multiple independent transport pathways through the NPC. For example, the Δ*N*Δ*C nup57*Δ*GLFG* mutant and the Δ*N*Δ*C nup49*Δ*GLFG* mutant strains have mRNA export defects but normal steady-state Kap104 import. In contrast, the Δ*N*Δ*C nup116*Δ*GLFG* mutant has normal mRNA export but significantly diminished steady-state Kap104 import. We propose that there are at least two distinct FG-dependent transport pathways through the NPC, defined by preferred FG binding sites for different transport receptors. The data to date pinpoint the GLFG regions of Nup49/Nup57 and Nup116 as prime determinants for the different pathways. Interestingly, comparison of the five GLFG-Nups indicates that single GLFG-domains might be required differentially by transport receptors. There are several potential explanations for what defines such functional FG differences: (1) novel spacer sequences between FG-repeats might contribute to binding of transport receptors; (2) non-FG binding sites adjacent to FG-domains might be important, such as those defined for Kap95/Kap60 (MATSUURA *et al.* 2003; PYHTILA and REXACH 2003) and mRNA export components (HODGE *et al.* 1999;

MURPHY *et al.* 1996; MURPHY and WENTE 1996; SCHMITT *et al.* 1999; STRAHM *et al.* 1999; WEIRICH *et al.* 2004); (3) the substructural location of the FG-repeat domain (LIM *et al.* 2006a) and the conformations it can assume within the NPC (FAHRENKROG *et al.* 2002; LIM *et al.* 2006b); or (4) the number of repeats in the FG-domain. Further dissection of the Nup49/Nup57 versus Nup116 GLFG-domains should pinpoint the molecular basis for such functional differences.

These studies of the *mmp* FGΔ mutants also fully corroborate our prior conclusions from analysis of asymmetric-specific versus symmetric-specific FGΔ mutants. We find no correlation between the number of FG-repeats deleted (or amount of FG-mass removed) and the severity of transport defects. For example, the Δ*N*Δ*C nup116*Δ*GLFG* mutant has 69.5% of its individual FG-repeats remaining, yet it showed more severe transport defects than the Δ*N*Δ*C nsp1*Δ*FG*Δ*FXFG* mutant, which has only 47.5% of its individual FG-repeats remaining (STRAWN *et al.* 2004). Perhaps more importantly, even small-scale FG deletions have a dramatic impact on transport. For example, the *nup1*Δ*FXFG nup2*Δ*FXFG nup57*Δ*GLFG* mutant retains 84.9% of its FG-repeats, yet has a severe mRNA export defect, whereas the Δ*N*Δ*C nup116*Δ*GLFG* mutant does not. Thus, there is no correlation between the number of FG-repeats deleted and the level of mRNA export or Kap transport defects.

We predict that the substructural distribution and location of the critical FG binding sites in the NPC is the fundamental basis for efficient transport. This conclusion is based on our findings of clear *in vivo* molecular requirements for distinct FG-domains in different transport receptor mechanisms. Export of mRNA requires the GLFG-domain of Nup57 or Nup49 in the Nic96-Nsp1-Nup49-Nup57 subcomplex. In contrast, Kap104

import requires the GLFG-domain of Nup116 in the Nup82-Nsp1-Nup116 subcomplex. In regard to the debated models for NPC translocation, these results need to be taken into account (FREY *et al.* 2006; RIBBECK and GORLICH 2002). With distinct FG requirements, each transport receptor would have its own tailored set of FG binding sites that form the basis of its given entropic barrier or selective phase for NPC entry and translocation. Overcoming an entropic or physical barrier of the NPC is thus achieved through binding to specific FG-Nup domains.

A model of multiple NPC pathways allows for competition and regulation of transport

With multiple preferred FG-domain pathways, the transport of cargo by different receptors could be regulated by NPC structural changes and influenced by transport receptor relative abundance. *Aspergillus nidulans* undergoes partial NPC disassembly during mitosis, including dissociation of several FG-Nups from the NPC (DE SOUZA *et al.* 2004; OSMANI *et al.* 2006). These changes result in altered NPC permeability and transport, and provide strong evidence that transport through the NPC can be regulated at the level of the NPC structure and FG-Nup composition. Changes in NPC composition are also observed in virally infected cells, as interferon triggers up-regulation of the FG protein Nup98, as well as Nup96 and Rae1/Gle2 (ENNINGA *et al.* 2002). Influenza virus counteracts this anti-viral response by forming an inhibitory complex with cellular mRNA export factors and by down-regulating the FG-Nup Nup98. These mechanisms impair cellular mRNA export and favor viral mRNA export, which uses an alternative transport receptor (ELTON *et al.* 2001; NEUMANN *et al.* 2000). Thus, the use of preferred

FG binding sites could allow unique mechanisms for selective regulation of different transport pathways. Our collection of FGΔ mutants fully demonstrates the range and specificity of perturbations that could be accomplished by selective NPC composition changes.

Several studies have examined the effect of a given transport receptor's concentration on its own import efficiency. Mathematical modeling has indicated that excess Kap β/importinβ can impede its own translocation (RIDDICK and MACARA 2005), but experiments in permeabilized mammalian cells suggest that increased importinβ levels improve the efficiency of nuclear import (YANG and MUSSER 2006). Recent experiments further show that modulating the levels of Kap123 in *S. cerevisiae* changes the import rate for Kap123 and its cargo in proportion to its abundance (TIMNEY *et al.* 2006). However, exactly how the concentration of each Kapβ affects the transport of other molecules and receptors has not been examined. Given our proposal for independent FG-domain requirements by different transport receptors, in a wild-type NPC direct competition for the same FG binding sites or pathways might be prevented. However, if the FG-Nup composition were to change, competition between receptors for the remaining pathway(s) and FG binding sites could impact translocation efficiency. Thus, either NPC structural changes at the level of individual FG-domains (as shown here with the *FG*Δ mutants) or receptor competition could modulate nucleocytoplasmic trafficking and allow changes in nucleocytoplasmic transport flux in response to disease or developmental state. Further analysis of the transport properties in the FGΔ mutant collection will directly allow future tests of such regulated translocation models.

Materials & Methods

Plasmids & yeast strains

Plasmids and yeast strains used in this study are listed in the Appendix, Tables 1 and 2. Plasmid cloning was carried out according to standard molecular biology strategies. Yeast strains were grown in YPD (1% yeast extract, 2% peptone, 2% glucose) or in synthetic complete (SC) media with 2% glucose and lacking appropriate amino acids. New yeast FGΔ mutants were generated using a Cre-Lox system as previously described (GULDENER *et al.* 1996; STRAWN *et al.* 2004), with the exception of the Δ*N*Δ*C nup49*Δ*GLFG* strain. Using the Cre-LoxP system, deletion of the sequence encoding amino acids 2-236 from *NUP49* was coincident with insertion of sequence for a T7 epitope tag and a LoxP site fused in-frame with the sequence encoding the C-terminal region of Nup49. The lethality of this Δ*N*Δ*C nup49*Δ*GLFG*^{LoxP} strain was rescued by transformation with a *nup49*Δ*GLFG* plasmid (pSW3261). All assays were conducted the Δ*N*Δ*C nup49*Δ*GLFG*^{LoxP} *pSW3261* strain.

Microscopy and analysis of live-cell GFP reporters

Yeast strains carrying pGAD-GFP (cNLS-GFP), pNS167 (Nab2NLS-GFP), pKW430 (NLS-NES-GFP2), or pSpo12 76-130-GFP (Spo12NLS-GFP) were grown to early-mid-log phase in SC media lacking the appropriate amino acid and supplemented with 2% glucose. Cells were examined from culture at 23˚C or after 1-hour shift to 37˚C. All images were acquired using an Olympus BX50 microscope with a UPlanF1 100x/1.30 oil immersion objective and a Photometrics Coolsnap HQ camera. Within each

experiment, all images were collected and scaled identically. Images were collected using MetaVue v4.6 and processed with Adobe Photoshop 9.0 software.

In situ hybridization & indirect immunofluorescence

Yeast cells were grown in YPD to early log phase at 23˚C, and aliquots were shifted to 37˚C for 1 or 3 hours. Cells were fixed for 10 minutes and processed as previously described (IOVINE *et al.* 1995; WENTE *et al.* 1992). For indirect immunofluorescence, cells were incubated overnight with affinity-purified rabbit anti-Nab2 antibodies (1:4000) and then detected with fluorescein-conjugated donkey anti-rabbit IgG (Jackson ImmunoResearch Laboratories, 1:200). DNA was stained with 0.1 ug/mL 4'6-diamidino-2-phenylindole (DAPI). For *in situ* hybridization, cells were incubated overnight with a digoxigenin-dUTP-labeled oligo d(T) probe and then detected with fluorescein-labeled anti-digoxigenin Fabs (Boehringer Manneheim, 1:25). Images were acquired and processed as described above.

Protein purification and GST-pulldown

GST, GST-GLFG-Nup57, GST-GLFG-Nup116 were expressed in *Escherichia coli* Rosetta (DE3) cells (EMD Biosciences). Clarified lysates of GST fusion proteins were prepared in 20mM HEPES pH 7.5, 150mM NaCl, 20% w/v glycerol . MBP-Mex67 was expressed in Rosetta cells, affinity-purified over amylose resin according to the manufacturer's protocol (New England Biolabs) and dialyzed into binding buffer of 20mM HEPES pH 7.5, 150mM NaCl, 20% w/v glycerol. Clarified GST fusion protein lysates were bound to glutathione sepharose (GE Healthcare) and washed in binding

buffer. MBP-Mex67 was applied to beads and incubated at 4˚C for 30 minutes. Samples were washed twice in binding buffer and eluted on ice for 20 minutes in binding buffer (pH 7.5) with 20mM glutathione. Equal fractions of bound protein were analyzed by SDS-PAGE and Coomassie Blue staining.

Mex67-GFP NPC recruitment assay

MEX67 was chromosomally tagged with the sequence encoding GFP in haploid wild-type and FGΔ yeast by amplification of the *GFP:HIS3MX6* region from the yeast GFP collection strain YPL169C (Invitrogen). Integrants were selected on SC-HIS and verified by PCR and by immunoblot with Rabbit anti-GFP (1:1000). To allow integration of the gene for expression of dsRED-HDEL, pKW1803 was linearized with EcoRV and transformed into yeast cells. Cells were selected on SC-TRP and integrants were verified by live-cell microscopy. For energy depletion assays, cells were grown to early log phase in YPD at 23˚C. A culture aliquot of 2.5 A_{600} units was used, and the cells were pelleted, washed, and resuspended in 1mL YP (without glucose) with 10mM NaN_3 and 10mM 2-deoxy-D-glucose. Cells were treated for 45 minutes at 23˚C, and then were pelleted, washed, and placed on ice prior to microscopy. At time=0, cells were resuspended in 23˚C YPD, mounted on a glass slide, and visualized as described above. Images of the GFP and dsRED signals were acquired every 30 seconds for 15 minutes. Cells were scored for recovery of Mex67-GFP to the nuclear rim and the relative nuclear to cytoplasmic GFP signal. Control strains SWY734 and SWY3302 were energy depleted and imaged as described above.

CHAPTER III

EXPLORATION OF MOLECULAR DETERMINANTS OF THE MULTIPLE TRAFFICKING PATHWAYS

Introduction

Cargo trafficking between the nucleus and cytoplasm requires interactions between the nuclear pore complex (NPC) and transport receptors. Transport receptors recognize and bind signal-bearing cargoes to facilitate nucleocytoplasmic transport of that cargo (PEMBERTON and PASCHAL 2005). Transport through the NPC is energy-independent, but nucleotide hydrolysis (ATP or GTP) is required to terminate export and to permit multiple rounds of transport (KOMEILI and O'SHEA 2001) (TRAN and WENTE 2006). Interactions between the transport receptor and NPC are believed to be transient and of low affinity (STEWART 2007a). The NPC proteins involved in this process are the FG-Nups, and this family of proteins has unusual properties.

FG repeats are binding sites for transport receptors

FG-Nups have clustered repeats of the di-peptide phenylalanine (F) glycine (G) with short spacer sequences between repeats of FG (ROUT and WENTE 1994). Importantly, there are three different sub-categories of FG repeats. FXFG repeats tend to have spacers that are highly charged and enriched for serine and threonine. The spacer sequences of GLFG repeats lack acidic residues and are highly enriched for serine,

threonine, asparagine and glutamine. The third repeat motif, the simple FG, may have either spacer type. Structural studies clearly show that the sidechain of a Phe residue fits into a hydrophobic binding pocket on the surface of a transport receptor (BAYLISS *et al.* 2000a; BAYLISS *et al.* 2002a; BAYLISS *et al.* 2000b; BAYLISS *et al.* 2002b; BAYLISS *et al.* 1999; FRIBOURG *et al.* 2001; GRANT *et al.* 2003; LIU and STEWART 2005). Peptide binding arrays suggest that residues adjacent to FG motifs may contribute to binding preferences of vImpβ (CUSHMAN *et al.* 2006). However, no structural study has resolved the spacer sequence residues in the context of a transport receptor-FG interaction. Given that both the repeat motif and the spacer sequences are different, we speculate that the subtle differences between FG repeats affect the binding specificity/capacity for each transport receptor.

Evidence for multiple transport pathways through the NPC

Many laboratories have demonstrated that transport receptors bind different FG domains preferentially (AITCHISON *et al.* 1996; ALLEN *et al.* 2001; ALLEN *et al.* 2002; DAMELIN and SILVER 2000; MARELLI *et al.* 1998; ROUT *et al.* 1997; SEEDORF *et al.* 1999; STRAWN *et al.* 2004; STRAWN *et al.* 2001). Even within a single FG domain, there are specific binding sites for different receptors (STRAWN *et al.* 2001), and perhaps subtle differences between spacer sequences contribute to this. In support of this, the sequence composition and length of a linker sequence in Nup1 affects Kap binding (CUSHMAN *et al.* 2006; LIU and STEWART 2005). Importantly, though, these studies rely upon *in vitro* binding and do not consider the transport event in the context of an intact NPC. *In vivo* evidence for preferred binding sites for each transport receptor comes from multiple

studies. Antibodies to vNup96 or vNup153 block only a subset of transport events (POWERS *et al.* 1997; ULLMAN *et al.* 1999), although these antibodies are not directed against FG domains. Further, Nup mutations inhibit import or export events specifically (CORBETT and SILVER 1997; SEGREF *et al.* 1997). A FRET-based assay for Kap-Nup interactions *in vivo* suggested that Kap121 and Msn5 have both overlapping and specific Nup interactions during transport. In our recent work, we have used combinatorial deletion of FG domains to directly identify FG domains required for nucleocytoplasmic shuttling of transport receptors (STRAWN *et al.* 2004; TERRY and WENTE 2007). *In vivo*, specific FG domains are required for individual transport receptor ferrying of cargoes through the NPC. Our detection of binding preferences *in vivo* indicates that there are multiple transport pathways through the NPC. We speculate that a single transport receptor moves along a preferred pathway of FG binding sites during translocation for maximal efficiency of transport.

Further evidence for the existence of multiple, preferred pathways of FG binding sites comes from considering NPC alterations during viral infection. Influenza virus selectively down-regulates or inhibits Nup98 and specific mRNA export factors (SATTERLY *et al.* 2007). Cellular mRNA export is effectively blocked, as these mRNA export factors and a binding site on Nup98 are required for mRNA export (POWERS *et al.* 1997). Export of influenza RNAs is unaffected, however, as they use a different transport receptor that does not depend on Nup98 (ELTON *et al.* 2001; NEUMANN *et al.* 2000). Similar Nup regulation strategies have been observed with other RNA viruses, including poliovirus and rhinovirus (GUSTIN 2003; GUSTIN and SARNOW 2001; GUSTIN and SARNOW 2002). Thus viruses manipulate FG transport pathways to favor their lifecycles.

The observation of multiple transport pathways through the NPC has broad impacts on our understanding of the mechanism of transport. In this chapter, I will explore the implications of having multiple NPC transport pathways and the variables that may contribute to establishment of these multiple pathways.

What are the critical determinants of each transport pathway?

Rationale for FG domain swaps

The detection of multiple NPC transport pathways raises an important question: what are the molecular determinants that distinguish each pathway? Possible factors that could influence transport receptor binding to FG repeats include: (1) the substructural location and intra-pore flexibility of each FG domain (FAHRENKROG *et al.* 2002; LIM *et al.* 2006a; LIM *et al.* 2007a; LIM *et al.* 2006b); (2) composition of the spacer sequences between FG repeats influencing/interacting with transport receptor binding (CUSHMAN *et al.* 2006); (3) non-FG binding sites adjacent to FG domains (e.g., Nup1, Nup2, Nup116, Nup42, Nup159) (GILCHRIST *et al.* 2002; GILCHRIST and REXACH 2003; LIU and STEWART 2005; MATSUURA *et al.* 2003; PYHTILA and REXACH 2003; SOLSBACHER *et al.* 2000); (4) the avidity of FG repeats. Although both mRNA export and Kap104 import are linked to GLFG domains, the specific domains involved here (of Nup57 and of Nup116) are located in different subcomplexes of the NPC, have different numbers of FG repeats, do not have identical spacer sequences, and are present in the NPC in different copy numbers (ROUT *et al.* 2000). Thus, it is possible that there are subtle functional differences between these domains in binding to transport receptors for each pathway, or

that the substructural location of these Nups is critical to their respective transport roles. None of the leading models of the transport mechanism (see Chapter 1) explicitly accounts for how multiple, preferred pathways could exist through the NPC, and exploring the determinants of these pathways will undoubtedly influence our interpretation of transport models. Other attempts at testing FG-Nup chimeras have been conducted (IOVINE *et al.* 1995; PATEL *et al.* 2007; ZEITLER and WEIS 2004). None of these found effects on specific transport pathways, although the studies by Patel *et al.* (PATEL *et al.* 2007) did observe alteration of the diffusive permeability barrier with an FG-Nup chimera. While other direct swaps of FG domains found no effects on transport, those studies also did not have a biological phenotype linked to the FG domains studied. In contrast, our experiments in Chapter 2 established direct biological functions for two specific FG domains. Therefore, we can test for specific phenotypes linked to the domains being swapped. Thus we sought to determine whether the type or location of a given FG domain is most critical for transport. We approached this by designing molecular cassettes to generate in-frame swaps of FG domains (Figure 3-1A). These constructs will specifically replace the GLFG domain of Nup57 with other FG domains. The boundaries of FG domains used are as defined in (STRAWN *et al.* 2004).

Strategy for chromosomal swap of FG domains: plans and predictions

In our prior studies, we identified a role for the GLFG domain of Nup57 in mRNA export and for the GLFG domain of Nup116 in Kap104 import (TERRY and WENTE 2007). Transport defects arose when these specific GLFG domain deletions were deleted from a Δ*N* or from a Δ*N*Δ*C* mutant strain. We designed cassettes for domain

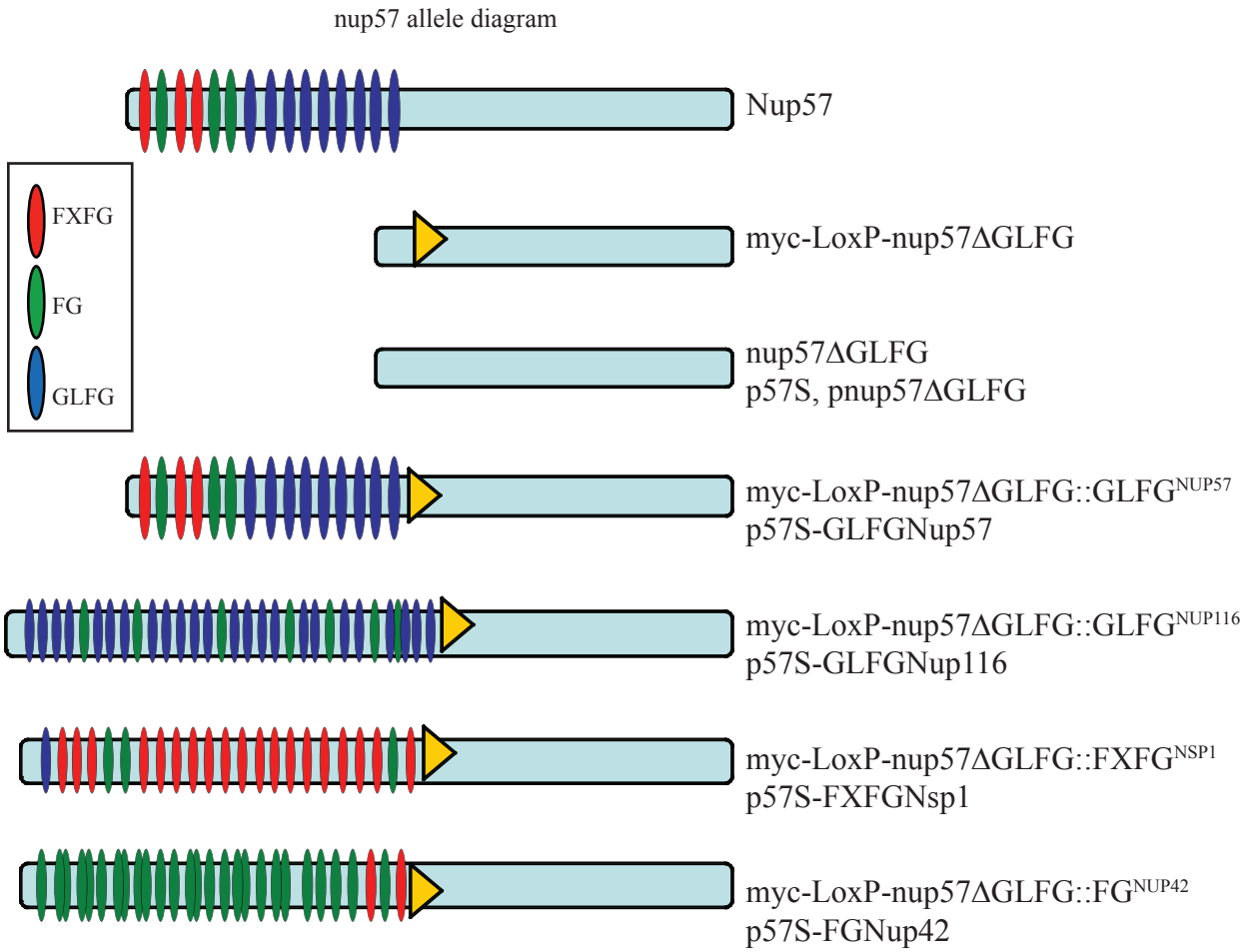


Figure 3-1. Design of nup57 FG domain swaps and logic for interpreting results.
(A) Schematic diagram of nup57 swap constructs. Each FG repeat is indicated by a filled oval (red, FXFG; green, FG; blue, GLFG) a myc-tag and LoxP site (yellow triangle) were integrated with domain swaps. Shorthand designations for each construct are designated below standard terminology name; names preceeded by "p" are name of plasmid construct used in text and figures of this Chapter.
(B) [Continued on next page] Flowchart outlining possible results and subsequent interpreations for Nup57 swap analysis.

swaps, using established Cre-LoxP technology (GULDENER *et al.* 1996; STRAWN *et al.* 2004). This enables us to make in-frame chromosomal deletions or insertions of specific sequences. Each cassette will integrate an epitope tag (to allow detection of the new fusion protein by immunoblotting), an FG domain, and the *HIS5* (*Schizzosaccharomyces pombe*) nutritional marker flanked by LoxP sites. Cre recombination loops-out the *HIS5* marker and leaves a single LoxP site in-frame between the integrated FG-domain and the remainder of the *NUP* gene sequence. Because we have specific roles for the GLFG domains of Nup57 and of Nup116, we designed cassettes to integrate either of these domains. To more broadly test the effects of different types of FG repeats, we designed cassettes to integrate the FG domain of Nup42 or the FXFG domain of Nsp1. These domains were selected because each is highly enriched for a single repeat type (either FG or FXFG) and these repeats have short, regular spacer sequences. Possible results and conclusions are summarized in Figure 3-1B. Briefly, if only location of FG repeats is critical, then swapping any of these domains into Nup57 will rescue mRNA export, but swapping the Nup57 GLFG domain onto other locations in the NPC will not rescue; mRNA export only depends on having FG domains at the Nup57 substructural NPC location. If the type of repeat (FG, FXFG, GLFG) is important, then either GLFG domain (of Nup57 or of Nup116) swapped onto Nup57 will rescue mRNA export, but the FG and/or FXFG domains will not. Finally, if there are specific binding sites of FG repeat plus linker sequence that are required, perhaps only the GLFG domain of Nup57 will suffice. The same series of logic are applied to rescue of Kap104 transport by making FG domain swaps on Nup116.

Unfortunately, after multiple attempts, we were unable to generate stable chromosomal swaps of FG domains. The reasons for failure of this approach are unclear. We were unable to confirm stable expression of the chimeric swap proteins, although our genotyping indicated that the chimeric sequences were present in the correct locus on the chromosome. It is possible that there is a molecular biology defect to the construct design (*e.g.*, reading frame shift), although this is unlikely given our thorough sequencing analysis of these plasmids. All plasmids used in the construction of swaps were sequenced, and the oligos used to amplify the integration cassettes were previously used successfully in our FGΔ constructions (STRAWN *et al.* 2004; TERRY and WENTE 2007). It is possible that there were spurious recombination events with the LoxP sequences either during the initial integration or during the subsequent Cre recombination. Swaps into the *nup57* locus were cassettes with the composition: *myc-FGdomain-LoxP-HIS5-LoxP*, and were integrated into a *myc-LoxP-nup57*Δ*GLFG* diploid. We also attempted swap construction in other mutant backgrounds, including appropriate double FGΔ mutants (*e.g.*, a *myc-LoxP-nup57*Δ*GLFG T7-LoxP-nup116*Δ*GLFG* mutant was targeted for integration of cassettes swapping FG domain sequences between *NUP57* and *NUP116* so as to minimize spurious integration into regions of FG domain homology), or a Δ*N myc-LoxP-nup57*Δ*GLFG* diploid. Because we observed failure through multiple approaches, it is likely that a common element is to blame – either a molecular design fault or a deleterious effect of the construct design (with epitope tag and LoxP flanking the swapped FG domain).

Alternative Nup57 swap construction strategy

We turned to a plasmid-based strategy as an alternative means of generating FG domain swap mutants. This approach takes advantage of the ability of yeast to stably maintain and express plasmid-borne genes and also employs the 5-FOA plasmid shuffle strategy. Briefly, in order to study the effects of swaps into Nup57, we used a disruption cassette to make a *nup57* null allele (*nup57::KAN*^{R}) in a diploid Δ*N S. cerevisiae* strain. The Δ*N* mutant was chosen for analysis of swaps into Nup57 because the Δ*N myc-LoxP-nup57*Δ*GLFG* mutant has a temperature-sensitive mRNA export defect (TERRY and WENTE 2007). At the non-permissive temperature of 37˚, this mutant accumulates poly(A)$^+$ RNA in the nucleus in ~80% of cells. As this is a substantial and readily detected transport defect, we predicted that monitoring the nuclear export of poly(A)$^+$ RNA would be a rapid assay for these FG domain swap studies. As *NUP57* is essential (GRANDI *et al.* 1995), a *URA3*/*CEN* plasmid containing the *NUP57* gene (pSW3006) was transformed into this strain. This strain was sporulated and dissected, and the progeny were screened to identify isolates with the genotype *trp1* Δ*N nup57::KAN*^{R} *pURA3-CEN-NUP57*. This strain was then transformed with FG domain swap plasmids or control plasmids. The wild-type *NUP57* expressing from the *URA3* plasmid was then removed by plasmid shuffle method over 5-FOA (Figure 3-2). As expected, an empty *pTRP1-CEN* vector (pRS314) was unable to rescue growth of Δ*N nup57::KAN*^{R} *pURA3-CEN-NUP57* on 5-FOA, while a vector with full-length *NUP57* was viable. This result confirms that *NUP57* is an essential gene and has been disrupted in our strain. Either of the two *nup57*Δ*GLFG* constructs (*pmyc-LoxP-nup57*Δ*GLFG* and *pnup57*Δ*GLFG*) were able to grow. Any of the four swap constructs tested also rescued viability. These swap

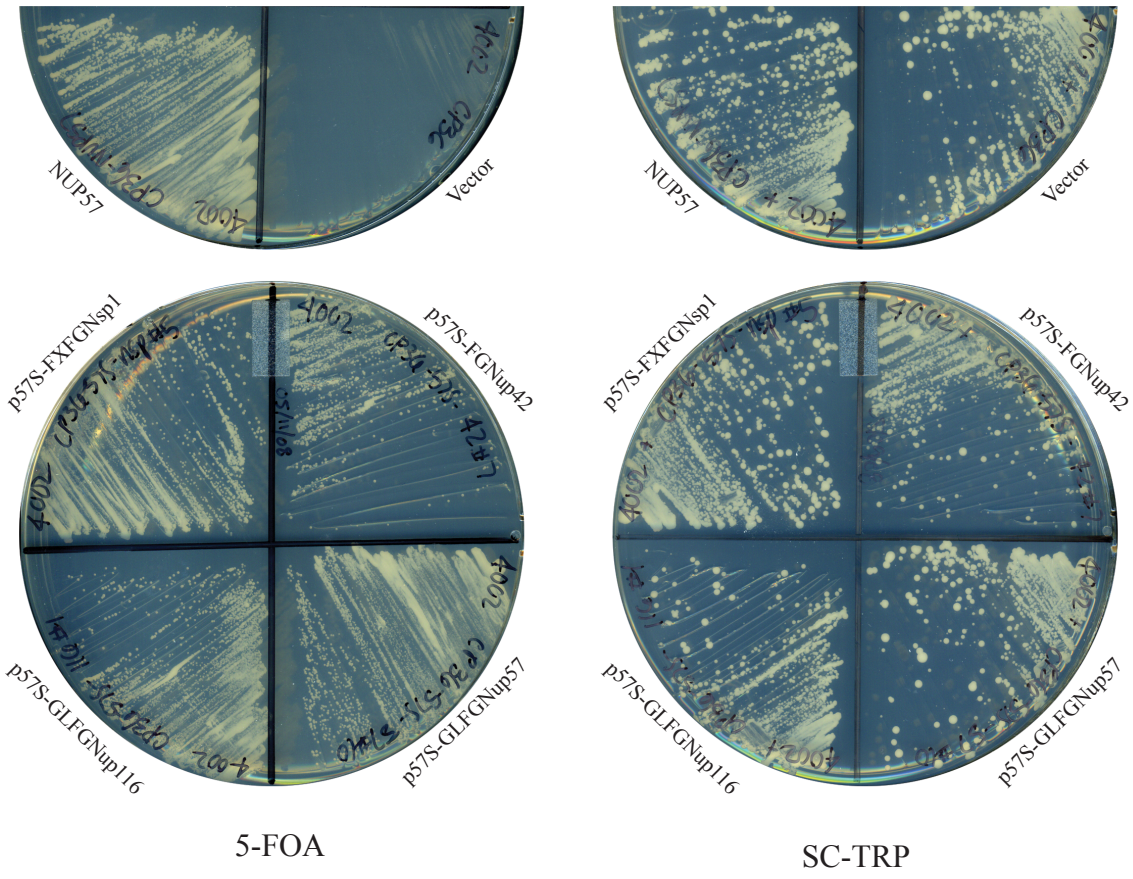


Figure 3-2. FG domain swaps into Nup57 can rescue a lethal *nup57::KAN*^{R} mutant. (A) The Δ*N nup57::KAN*^{R} p*URA3-CEN-NUP57* mutant (SWY4002) was transformed with an empty vector or with plasmids expressing various *nup57* alleles on a *TRP1* plasmid. 5-FOA plates, left, show plasmid shuffle results.

constructs replaced the GLFG domain of Nup57 with either the FG domain of Nup42, the FXFG domain of Nsp1, the GLFG domain of Nup116, or, as a control, the GLFG domain of Nup57.

Analysis of mRNA export with Nup57 swaps

We next assayed these strains for export of poly(A)$^+$RNA, as we had previously linked the GLFG domain of Nup57 to mRNA export (TERRY and WENTE 2007). The results of these assays are summarized in Figure 3-3. The NUP57 plasmid fully rescued poly(A)$^+$RNA export, while a strain expressing *myc-LoxP-nup57*Δ*GLFG* had substantial mRNA export defects in ~50% of cells. Thus, whether expressed off the chromosome (TERRY and WENTE 2007) or a plasmid, the *myc-LoxP-nup57*Δ*GLFG* deletion allele is correlated with mRNA export defects. We assayed the poly(A)$^+$RNA localization in four mutant strains with the FG domain swap plasmids. As expected, the control swap of the Nup57-GLFG domain onto the Nup57 C-terminus fully restored mRNA export (Figure 3-3A, B). Swap of Nup42-FG domain or Nsp1-FXFG domain also rescued mRNA export. Unexpectedly, cells expressing a chimeric swap of the Nup116-GLFG domain onto the Nup57 C-terminus showed subtle poly(A)$^+$RNA export defects in a majority of the cells and more intense nuclear accumulation of poly(A)$^+$ RNA in a minority of the cells. This observation raises important questions about the size of FG domains in these swaps and warrants future consideration (see Discussion). Unexpectedly, the *nup57*Δ*GLFG* plasmid (without an epitope tag) completely rescued the mRNA export defects as well. The only difference between the two *nup57*Δ*GLFG* deletion alleles is the epitope tag-LoxP site.

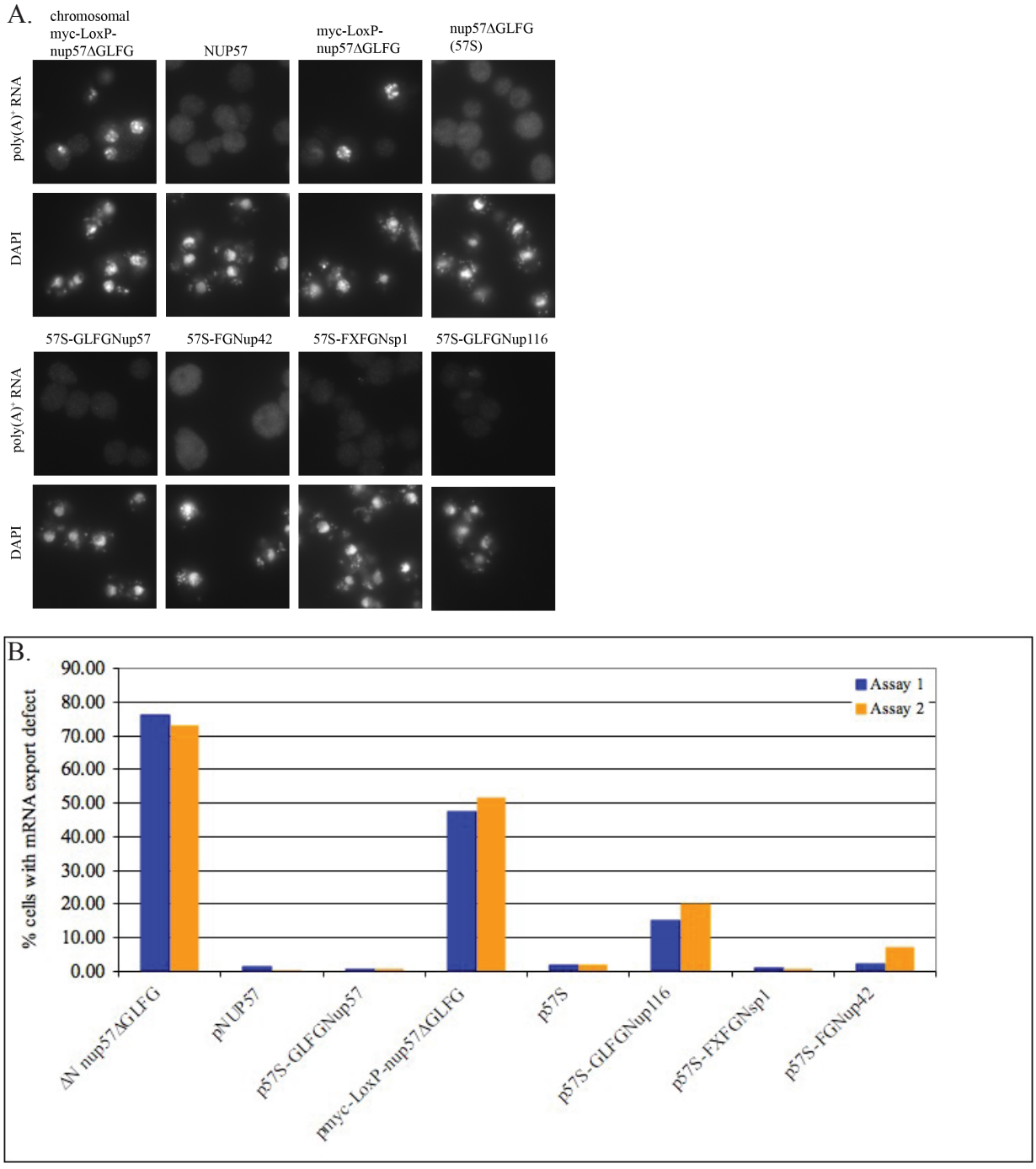


Figure 3-3. mRNA export defects in the ΔN myc-LoxP-nup57ΔGLFG mutant are attributable to the epitope tag.
(A) In situ hybridication for poly(A)$^+$ RNA in nup57 swap mutants. Genotypes are abbreviated as listed in Figure 3-1.
(B) The percentage of cells with nuclear poly(A)$^+$ RNA accumulation is scored as a percentage of total numer of cells imaged for two independent assays.

To dissect further the impact of the epitope tag-LoxP sequence, a GFP-tagging cassette was integrated to C-terminally tag Mex67 in the Δ*N nup57::KAN*^{R} *pmyc-LoxP-nup57*Δ*GLFG* and the Δ*N nup57::KAN*^{R} *pnup57*Δ*GLFG* mutants. We then performed Mex67-GFP shuttling assays, as previously described (TERRY and WENTE 2007). The Δ*N nup57::KAN*^{R} *pmyc-LoxP-nup57*Δ*GLFG* mutant had severe defects in Mex67-GFP shuttling in two independent assays, and after 15 minutes of recovery failed to recruit substantial Mex67-GFP to the nuclear rim (Figure 3-4). These results were indistinguishable from our previous assays with the ΔN *myc-LoxP-nup57*Δ*GLFG* mutant (chromosomal expression of the *myc-LoxP-nup57*Δ*GLFG* allele) (TERRY and WENTE 2007). In contrast, Mex67-GFP showed partial recovery to the nuclear rim in the Δ*N nup57::KAN*^{R} *pnup57*Δ*GLFG* mutant. The recovery phenotype in this mutant was similar to the rate of recovery of Mex67-GFP to the nuclear rim of the Δ*N*Δ*C* mutant. To date, we have not assayed Mex67-GFP shuttling in a ΔN mutant and therefore cannot conclude whether the *pnup57*Δ*GLFG* allele functions like a wild-type *NUP57* in the Δ*N* background. We can, however, conclude that there are notable differences between the contributions of the *pmyc-LoxP-nup57*Δ*GLFG* and the *pnup57*Δ*GLFG* alleles on Mex67-GFP shuttling.

Taken together, these results from both *in situ* hybridization for poly(A)$^{+}$RNA and Mex67-GFP shuttling assays suggest that the presence of the *myc-LoxP* tag, and not the deletion of the GLFG domain, causes mRNA export defects. Based on our shuttling assays, it appears that the *myc-LoxP* tag is specifically interfering with Mex67-GFP shuttling between the nuclear and cytoplasmic compartments. Immunoblotting confirmed that the *myc-LoxP-nup57*Δ*GLFG* allele is expressed from either the plasmid or

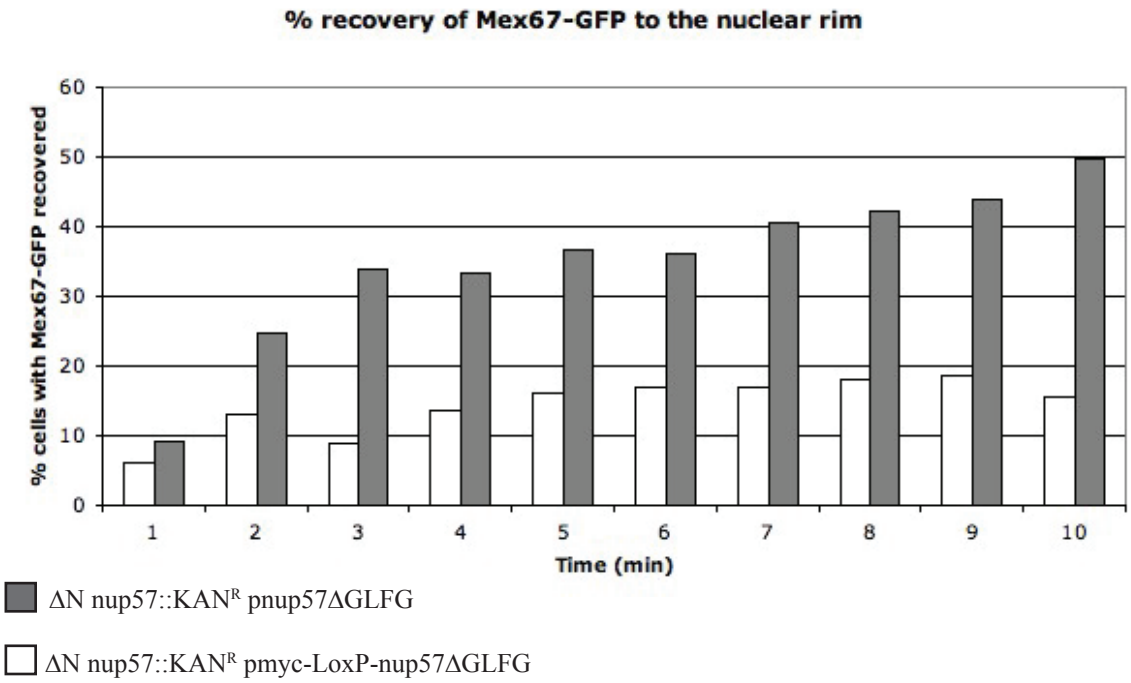


Figure 3-4. The myc-LoxP-nup57ΔGLFG allele is linked to impared recruitment of Mex67-GFP to the nuclear rim.
Mex67-GFP was expressed in Δ*N nup57::KAN*^{R} cells carrying either p*nup57*Δ*GLFG* or p*myc-LoxP-nup57*Δ*GLFG*. Cells were energy-depleted and then monitored over a time course of recovery for recruitment of Mex67-GFP to the nuclear rim. Data from two independent assays were averaged to produce this graph. For each time point, >130 cells were counted in each assay.

chromosomal constructs. To date, the reason that the myc-LoxP tag disrupts mRNA export and Mex67 shuttling is unclear.

We have previously observed that the presence of a T7 epitope tag in place of the GLFG domain in Nup49 is lethal in certain mutant backgrounds (TERRY and WENTE 2007). These epitope tag sequence elements were inserted in place of FG domains in our global chromosomal deletion strategy (STRAWN *et al.* 2004). Thus it became necessary to test for the extent of epitope tag-LoxP effects in our FGΔ mutant *S. cerevisiae* strain collection.

Epitope tags have deleterious effects on nucleocytoplasmic transport

Expression of the nup57ΔGLFG (untagged) plasmid in the Δ*N*Δ*C myc-LoxP-nup57*Δ*GLFG* (SWY3410) mutant rescued the temperature sensitivity of this strain (Figure 3-5). The *nup57*Δ*GLFG* plasmid also rescued nuclear export of 60S ribosomal subunits in two strains (Eric Shows, unpublished data). Control experiments determined that the epitope tag-*LoxP* allele is stably expressed, although it has not been determined whether this protein is efficiently incorporated into assembling NPCs. The single mutant *myc-LoxP-nup57*Δ*GLFG* does not have mRNA export defects (TERRY and WENTE 2007). Thus the effects of the *myc-LoxP* tag on the *nup57*Δ*GLFG* construct cause synthetic growth and transport defects when combined with other *FG*Δ alleles (*e.g.*, the Δ*N* mutations). As a whole, these results necessitate alternative experimental strategies for short-term and long-term studies (see below and Chapter 4) and thoughtful consideration of the interpretations of past studies with epitope tag-LoxP strains (see Conclusions, this Chapter).

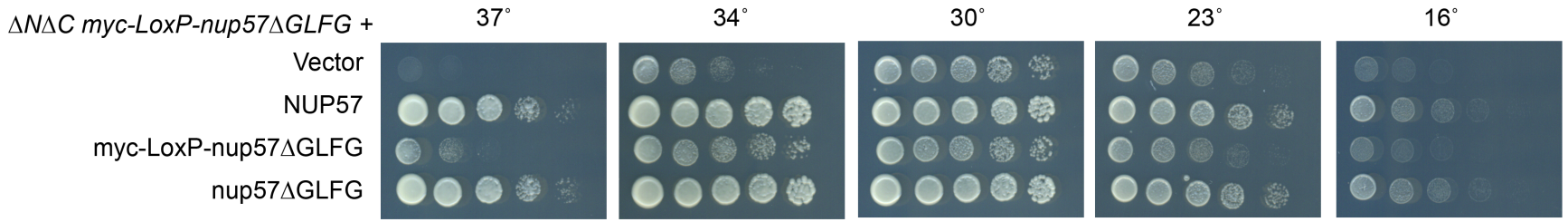


Figure 3-5. The untagged nup57GLFG construct rescues temperature sensitivity of ΔNΔC myc-LoxP-nup57ΔGLFG.
(A) The ΔNΔC myc-LoxP-nup57ΔGLFG mutant (SWY3410) was transformed with empty vector (pRS314), full-length NUP57 vector (pSW3431), myc-LoxP-nup57ΔGLFG vector (pSW3432), or nup57ΔGLFG (pSW3434; also known as p57S). Cells were grown to early log-phase in selective media and spotted as five-fold serial dilutions on appropriate media. Strains were grown at the temperature indicated for three days.

FG domain swaps into Nup49

In our prior work (TERRY and WENTE 2007), we found that a *T7-LoxP* motif in a *nup49*Δ*GLFG* allele in a Δ*N*Δ*C* mutant background was lethal. The Δ*N*Δ*C T7-LoxP-nup49*Δ*GLFG* lethal mutant was complemented by either of two different *nup49*Δ*GLFG* allele constructs. A cassette which replaces the GLFG domain with a GFP tag (*nup49GLFG::GFP*) restored viability. In addition, a plasmid *nup49*Δ*GLFG* without any epitope tags also restored viability. In both cases, the resulting strain expressed two *nup49*Δ*GLFG* alleles – the epitope tagged version and either the GFP or the untagged version. Either of these mutants also had temperature-sensitive mRNA export defects. It is possible that these defects reflect only partial complementation of *the T7-LoxP-nup49*Δ*GLFG* lethal allele. Alternatively, it is possible that the GFP or untagged *nup49*Δ*GLFG* alleles fully complement the *T7-LoxP* allele and these defects then truly represent a requirement for GLFG binding sites during mRNA export. To further differentiate between these two possibilities, and in hopes of establishing a mutant in which I could complete domain swap experiments, I generated a new mutant strain using the same paradigms as outlined for the Δ*N nup57::KAN*^{R} *pURA3-NUP57-CEN* strain (above). Plasmid shuffle and characterization experiments are presently underway. We synthesized plasmid-borne swaps of FG domains into Nup49 and will express these in a Δ*N nup49::KAN*^{R} mutant. We will apply the same logic for interpreting results as outlined in Figure 3-1B for comparable swaps into Nup57. We predict that identifying the number and type of FG repeats that must be present in Nup49 for mRNA export will provide insight into the determinants of transport and of transport pathways.

The cytoplasmic face FG domains serve to regulate mRNA export

*nup42*Δ*FG* suppresses mRNA export defects

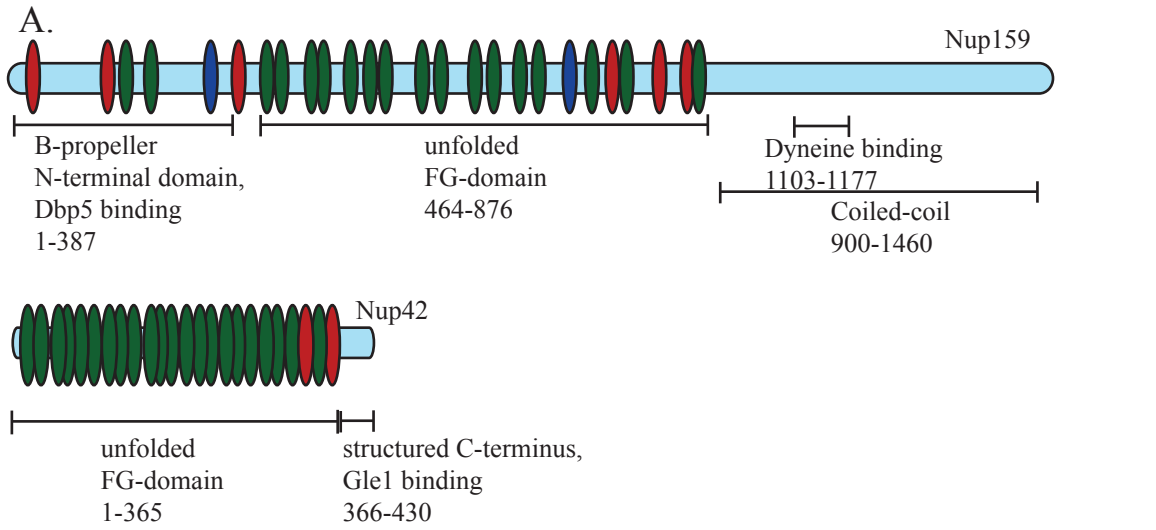
To identify FG domains required for mRNA export, we previously screened our collection of mutants with maximal FG domain deletions (TERRY and WENTE 2007). Wild-type, control, or FGΔ mutants were probed for poly(A)$^+$RNA localization by *in situ* hybridization with an oligo d(T) probe. When we identified mRNA export defects in a strain with complex FG domain deletions, we then systematically mapped which deletion(s) in that mutant were responsible for the mRNA export defect phenotype. For example, the Δ*N*Δ*C nup57*Δ*GLFG* mutant accumulates poly(A)$^+$RNA in ~30% of the cells at 37˚. To find the FG domain deletions linked to this phenotype, we then systematically generated and assayed less complex FGΔ mutants. We focused on examining the *nup57*Δ*GLFG* allele in combination with deletion of either the cytoplasmic side (ΔC) or nuclear side (ΔN) FG domains. We found a specific and robust mRNA export defect in the Δ*N nup57*Δ*GLFG* mutant, but observed no mRNA export defects in the Δ*C nup57*Δ*GLFG* mutant. Curiously, the mRNA export defect was more penetrant in the Δ*N nup57*Δ*GLFG* mutant (~80% cells) than in the Δ*N*Δ*C nup57*Δ*GLFG* mutant (~30% cells), despite the fact that the Δ*N nup57*Δ*GLFG* mutant has a more intact NPC. In other words, deletion of the cytoplasmic-face FG domains suppressed the severity of the mRNA export defect. Although the cytoplasmic FG Nups, Nup42 and Nup159, have previously been shown to have roles in mRNA export, these roles were linked to their non-FG domains (HODGE *et al.* 1999; SCHMITT *et al.* 1999; STRAHM *et al.* 1999; STUTZ *et al.* 1997). Specifically, the C-terminus of Nup42 is a binding site for the

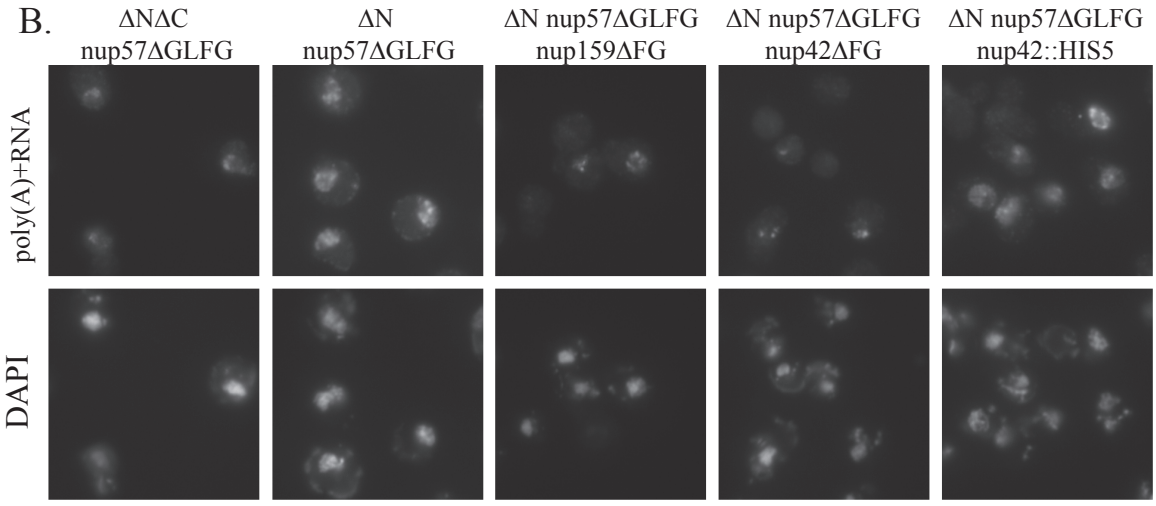
essential mRNA export factor Gle1 (MURPHY and WENTE 1996; STRAHM *et al.* 1999), and the N-terminus of Nup159 binds the essential DEAD-box helicase and mRNA export factor Dbp5 (Figure 3-6A) (HODGE *et al.* 1999; SCHMITT *et al.* 1999; WEIRICH *et al.* 2004). Interestingly, these binding sites are adjacent to FG domains that bind the mRNA export receptor Mex67-Mtr2 (STRASSER *et al.* 2000).

To identify whether the FG domain deletion from Nup42, Nup159, or both was required for this suppression, we assayed two additional mutants for poly(A)$^+$RNA export (Figure 3-6B, C). The Δ*N nup57*Δ*GLFG nup159*Δ*GLFG* mutant accumulated nuclear poly(A)$^+$RNA in ~55% of the population, a phenotype of intermediate suppression. In contrast, the Δ*N nup57*Δ*GLFG nup42*Δ*FG* mutant had mRNA export defects in ~38% of cells, demonstrating that the *nup42*Δ*FG* allele is sufficient to suppress the mRNA export defect of Δ*N nup57*Δ*GLFG*.

Juxtaposed binding sites on Nup42 contribute to mRNA export

Because the C-terminus of Nup42 provides the binding site for the essential mRNA export factor Gle1 and this site is juxtaposed to the Nup42-FG domain, we tested the importance of the Gle1 binding site in the context of the Δ*N nup57*Δ*GLFG* mutant. The mutants and assay results are summarized in Table 3-1. We transformed a *NUP42* disruption cassette into the Δ*N nup57*Δ*GLFG* mutant and selected for cells in which this cassette had undergone homologous recombination into the chromosome. This disrupts the *NUP42* locus and results in a null allele. Although Gle1 is an essential mRNA export factor, its binding site on Nup42 is not specifically required for bulk poly(A)$^+$RNA export (MURPHY and WENTE 1996; STRAHM *et al.* 1999; STUTZ *et al.* 1997). To gauge the effect





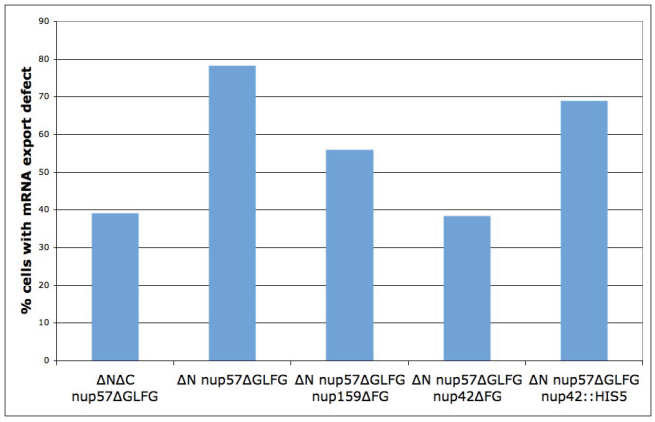


Figure 3-6. Juxtapositioning of FG and non-FG binding sites on cytoplasmic-side FG-Nups contributes to mRNA export. (A) Schematic diagram of Nup42 and Nup159, with approximate domain boundaries indicated. (B) In situ hybridization for poly(A)$^+$RNA in strains with cytoplasmic-side deletions combined with the ΔN nup57ΔGLFG mutations. (C) In situ experiments as in (B) were carried out. For each mutant, >150 cells were scored for the localization of poly(A)$^+$RNA signal. The average of two independent assays is graphed.

Table 3-1. Juxtapositioned Gle1 binding and Mex67 binding domains on Nup42 are required for mRNA export suppression.

	Dbp5 binding domain (Nup159 N-terminus)	Gle1 binding domain (Nup42 C-terminus)	Mex binding site on Nup42-FG domain	Mex binding site on Nup159-FG domain	mRNA export phenotype (percent cells with defect)
ΔNΔC nup57ΔGLFG (SWY3410)	+	+	-	-	+/- **~38%**
ΔN nup57ΔGLFG (SWY3618)	+	+	+	+	- **~78%**
ΔN nup57ΔGLFG nup159ΔFG (SWY3925)	+	+	+	-	- **~55%**
ΔN nup57ΔGLFG nup42ΔFG (SWY3927)	+	+	-	+	+/- **~37%**
ΔN nup57ΔGLFG nup42::HIS5	+	-	-	+	- **~68%**

of the *nup42::HIS3* null on mRNA export, we again performed *in situ* hybridization to detect poly(A)$^+$RNA (Figure 3-6B, C; Table 3-1). The Δ*N nup57*Δ*GLFG nup42*Δ*FG* mutant accumulated nuclear poly(A)$^+$RNA in ~38% of cells in two independent experiments, a level of defect that is comparable to the accumulation in Δ*N*Δ*C nup57*Δ*GLFG* cells. In contrast, the Δ*N nup57*Δ*GLFG nup42::HIS3* had mRNA export defects apparent in ~65% of cells, a phenotype similar to the level of defect in the Δ*N nup57*Δ*GLFG* mutant. In other words, a *nup42*Δ*FG* allele suppresses mRNA export defects, but a complete *nup42::HIS3* null does not. We therefore concluded that there is a complex regulatory effect of the adjacent Mex67-Mtr2 and Gle1 binding sites on Nup42.

Swap of FG domains into Nup42

In order to determine whether these regulatory effects were specific to the FG domain of Nup42, or instead were conferred by the simple juxtaposition of any FG domain next to the Gle1 binding site, we constructed Nup42 chimeras. The sequence encoding the GLFG domain of Nup49 or the FXFG domain of Nsp1 was fused in-frame with the C-terminus of Nup42. In this chimera, the FG domain of Nup42 was specifically replaced. We also constructed a control FG domain swap in which the sequence encoding the FG domain of *NUP42* was re-linked to the non-FG sequence of *NUP42*. These constructs were expressed as plasmids in the Δ*N nup57*Δ*GLFG nup42::HIS3* mutant strain. Preliminary results indicate that FG domain swaps into Nup42 have differential impacts on the extent of mRNA export defects when expressed in the Δ*N nup57*Δ*GLFG nup42::HIS3* mutant (data not shown).

Discussion and Conclusions

In these studies, we have explored key points regarding the nucleocytoplasmic transport mechanism by testing for differences between FG domains and examining the importance of binding sites adjacent to FG domains.

Domain swap experiments revealed unexpected effects of epitope tag-LoxP sites

To test for functional differences between FG domains, we used our defined critical FG binding sites on Nup57 and Nup116 as platforms for designing experiments to test the importance of FG repeat type or location in transport function. Unfortunately, the original chromosome-targeted swap strategy failed for reasons that are not clear. In an alternative, plasmid-based approach, we uncovered a link between epitope tags and transport defects. Replacing the Nup57 GLFG domain with a myc-LoxP motif caused defects that were greater than simply deleting that GLFG domain. The myc-LoxP tag caused synthetic mRNA export defects when combined with the ΔN FG deletion mutant. This result prompted us to conduct additional tests, which subsequently revealed that tag-LoxP sequences caused defects in multiple mutant backgrounds and affected nucleocytoplasmic transport of multiple cargoes (including mRNA and 60S ribosomal subunits). At least three different epitope tag-LoxP FGΔ Nups have defects linked to the presence of the epitope tag-LoxP sequence: *T7-LoxP-nup49ΔGLFG, flag-LoxP-nsp1ΔFGΔFXFG,* and *myc-LoxP-nup57ΔGLFG*. Two commonalities emerge from examining this list. First, these three FG-Nups are found together in a subcomplex of the

NPC (GRANDI *et al.* 1995), although Nsp1 is also found in a complex that is biased towards the cytoplasmic face of the NPC (FAHRENKROG *et al.* 1998). Perhaps the Nup49-Nup57-Nsp1 FG region of the NPC is specifically critical to the integrity of the central FG mass (and whatever structural/biophysical properties it may have). Second, there are three different epitope tags on these three ΔFG Nups, yet each has the same effect. Thus, the likely explanation is not the epitope tag (myc, T7, or flag), *per se*, but rather a common element among all of these – namely, the LoxP site or common flanking sequence (Figure 3-7).

The construct used in our studies has a short linker of 5-8 amino acid residues on either side of the LoxP site. All of the epitope tag-LoxP constructs have a high percentage of S+T (27.3-37.5%), similar to frequency with which these residues are found in FG domains (Figure 3-7) (ROUT and WENTE 1994). Each epitope tag-LoxP sequence contains at least one Cys and multiple Tyr residues, neither of which are found in *S. cerevisiae* FG domains (with the exception of one Tyr in Nup159 FG domain). In addition, the epitope tag-LoxP motifs are devoid of Pro, which are found in FG domains at low frequency (~3-8% of total FG domain residues). It is unclear whether any of the epitope tag-LoxP motifs have structural motifs or affect the folding of the remainder of the Nup. For future studies, we could consider altering the composition of the linkers on either side of the LoxP sequence or using different epitope tags, but if the problem arises from the LoxP-encoded residues itself, that cannot be remedied, as the LoxP nucleotide sequence is specifically required for recognition and efficient site-specific recombination by Cre recombinase (SAUER 1992). One possible solution may be to use different or more extensive linker sequences between the epitope tag-LoxP and the endogenous NUP

A.

epitope	Epitope-linker-LoxP-linker
HA-LoxP	YPYDVPDYATSTTLNITSYNVAYTKLLGDIRST
Myc-LoxP	EEQKLISEEDLTSTTLNITSYNVAYTKLLGDIRST
T7-LoxP	MASMTGGQQMGTSTTLNITSYNVAYTKLLGDIRST
Flag-LoxP	DYKDDDDKTSTTLNITSYNVAYTKLLGDIRST

B.

	Composition of epitope tag-LoxP motifs (percent)			
	acidic	basic	Q + N	S + T
Flag	18.75	12.5	6.25	28.13
HA	9.09	6.06	6.06	27.27
Myc	14.28	8.57	8.57	28.57
T7	2.86	5.72	11.42	31.43

C.

		Composition of yeast FG domains (percent)			
	Repeat motif(s)	acidic	basic	Q + N	S + T
Nup42	FG	0.55	3.33	17.45	29.92
Nup159	FG	11.14	7.74	6.29	29.54
Nup49	GLFG	0	2.98	21.28	24.25
Nup57	GLFG	0	2.7	19.37	27.48
Nup116	FG, GLFG	0	1.98	27.6	21.49
Nup145	GLFG	0	2.5	21.5	30.5
Nup100	GLFG	0.18	2.28	27.76	26.89
Nsp1	FG, FXFG	10.19	11.57	12.44	24.35
Nup1	FXFG	9.9	11.88	9.11	29.11
Nup2	FXFG	13.56	13.85	10.61	26.84
Nup60	FXF	15.3	14.12	14.12	20

Figure 3-7. Sequence comparison of epitope tag-LoxP motifs and FG domains.
(A) Primary amino acid sequence of epitope tag-LoxP motifs inserted in FG deletion mutants (STRAWN *et al.* 2004; TERRY and WENTE 2007). Epitope tag residues (black), linker sequences (green), LoxP sequence (orange).
(B) The relative abundance of acidic (D, E); basic (K, R, H); Q+N; and S+T in the epitope tag-LoxP motifs is given.
(C) The relative abundance of acidic (D, E); basic (K, R, H); Q+N; and S+T in each *S. cerevisiae* FG-Nup is given. For this calculation, only FG domains were analyzed, using the following boundaries (amino acid residue numbers given): Nup42-FG [4−364], Nup159-FG [464−876], Nup49-GLFG [2−236], Nup57-GLFG [2−223], Nup145-GLFG [10−209], Nup100-GLFG [2−570], Nup116-FG,GLFG [2-95, 205-715], Nsp1-FG,FXFG [13-591], Nup1-FXFG [384-888], Nup2-FXFG [189-527], Nup60-FXF [397-512].

sequence. In other *S. cerevisiae* studies, altering the composition of a linker sequence attached to a terminal myc epitope tag can ameliorate deleterious effects of the myc tag alone (SABOURIN *et al.* 2007).

How could the epitope tag-LoxP motif cause synthetic transport defects? The epitope tag-LoxP sequence might locally disrupt formation of an entropic barrier or hydrophobic meshwork, or interfere with the assembly of other NPC factors. Alternatively, the epitope tag-LoxP sequence may be an unknown high affinity binding site that sequesters some critical component of the transport machinery. This seems less probable of an explanation because not all strains with epitope tag-LoxP sequences on Nups have transport defects. For example, the Δ*N*Δ*C* mutant has no steady-state transport defects, yet has epitope tag-LoxP sequences on five different Nups. If the epitope tag-LoxP sequence locally disrupts NPC subcomplex assembly, Nups which cannot properly incorporate into the NPC would show subcellular mislocalization. We have tested a subset of strains for Nup mislocalization and have observed no defects (STRAWN *et al.* 2004). Thus, the remaining explanation – that the epitope tag-LoxP sequence disrupts the integrity or function of the NPC barrier – remains. Testing this possibility is technically beyond the scope of our current abilities.

Implications of the epitope tag/LoxP problem for our current and published research results

In light of the observation that an epitope tag-LoxP sequence contributed to non-specific transport defects, we must also re-assess the interpretation of our published results. Given that we now know that the *myc-LoxP-nup57*Δ*GLFG* allele is linked to

mRNA export defects but an untagged *nup57ΔGLFG* allele does not have the same effect, we cannot conclude that mRNA export requires the GLFG domain of Nup57. Thus it is difficult to make conclusions about *in vivo* roles for specific FG domains. We can, however, continue to support a model of multiple pathways through the NPC. The transport events affected in the *ΔNΔC myc-LoxP-nup57ΔGLFG* strain (namely, mRNA export) are different from those affected in the *ΔNΔC T7-LoxP-nup116ΔGLFG* strain (Kap121, Kap104 import) (TERRY and WENTE 2007). These defects may be caused by the FG domain deletion, the epitope tag-LoxP insertion, or the combination of both. Regardless, transport pathways are differentially affected by these altered FG-Nup sequences. Due to the non-specific effects of the tag-LoxP insertion, we have been unable to complete a thorough analysis of the importance of FG domain type versus location as determinants for each transport pathway.

Of note, not all epitope tag-LoxP insertions in place of FG domains are deleterious. A *nup42ΔFG* allele and a *HA-LoxP-nup42ΔFG* allele have the same contributions to mRNA export. Similarly, 60S ribosomal subunit export is not affected by a myc-LoxP tag *in nup159ΔFG* (Eric Shows, personal communication). Future studies will use alternative strategies to delete, mutate, and/or swap FG domains and assess the impact on transport capacity of FGΔ mutant NPCs.

FG-domains with adjacent binding sites are important in mRNA export

Binding sites adjacent to FG domains are another possible determinant influencing or establishing multiple transport pathways. There are at least three mRNA export factors with binding sites adjacent to FG domains near the cytoplasmic face of the

NPC. Motifs adjacent the FG domains of Nup1 and Nup2 on the nuclear side of the NPC contribute to terminal events in Kap95/Kap60-mediated import (GILCHRIST *et al.* 2002; GILCHRIST and REXACH 2003; LIU and STEWART 2005; MATSUURA *et al.* 2003; PYHTILA and REXACH 2003; SOLSBACHER *et al.* 2000). We have uncovered unanticipated roles for the Nup42 cytoplasmic-face FG domain in mRNA export. Why does the *nup42ΔFG* allele suppress mRNA export defects? It is possible that there are non-specific effects of this deletion on the rate of transcription or metabolism, decreased poly(A)$^+$ RNA tail length (therefore decreasing detection with our oligo d(T) probe), or increased nuclear import rate for an essential and rate-limiting mRNA export or transcription factor. Because Nup42 is a binding site for both Gle1 and Mex67-Mtr2, we speculate that the correct explanation is more direct. Perhaps the *nup42ΔFG* mutation increases the rate of export by removing a physical barrier, increasing the efficiency of mRNP remodeling and release, or increasing leakage of incompletely processed/remodeled mRNPs. If the suppression by *nup42ΔFG* is due to removing a physical barrier, then we would expect that the *nup42::HIS3* disruption would have the same effect on mRNA export; however, that was not observed. In future experiments we can differentiate between accelerated mRNP remodeling/release versus premature mRNP release (without complete remodeling) by measuring relative abundance of specific factors that cross-link to mRNPs or that are spuriously found in polysome fractions. These results have implications for regulated termination of mRNA export and will be addressed in future studies.

Materials & Methods

Plasmids & yeast strains

Plasmids and yeast strains used in this study are listed in Appendix Tables 1 and 2. Plasmid cloning was carried out according to standard molecular biology strategies. Yeast strains were grown in YPD (1% yeast extract, 2% peptone, 2% glucose) or in synthetic complete (SC) media with 2% glucose and lacking appropriate amino acids. New yeast FGΔ mutants were generated using a Cre-Lox system as previously described (GULDENER *et al.* 1996; STRAWN *et al.* 2004). Yeast transformations were carried out according to the LiAc-TE method (ITO *et al.* 1983).

Microscopy and image acquisition

All images were acquired using an Olympus BX50 microscope with a UPlanF1 100x/1.30 oil immersion objective and a Photometrics Coolsnap HQ camera. Within each experiment, all images were collected and scaled identically. Images were collected using Image Pro Express and processed with Adobe Photoshop 9.0 or higher software.

In situ hybridization & indirect immunofluorescence

Yeast cells were grown in YPD to early log phase at 23˚C, and aliquots were shifted to 37˚C for 1 or 3 hours. Cells were fixed for 10 minutes and processed as previously described (IOVINE *et al.* 1995; WENTE *et al.* 1992). For indirect immunofluorescence, cells were incubated overnight with affinity-purified rabbit anti-

Nab2 antibodies (1:4000) and then detected with fluorescein-conjugated donkey anti-rabbit IgG (Jackson ImmunoResearch Laboratories, 1:200). DNA was stained with 0.1 ug/mL 4'6-diamidino-2-phenylindole (DAPI). For *in situ* hybridization, cells were incubated overnight with a digoxigenin-dUTP-labeled oligo d(T) probe and then detected with fluorescein-labeled anti-digoxigenin Fabs (Boehringer Manneheim, 1:25). Images were acquired and processed as described above.

Mex67 shuttling assays

MEX67 was chromosomally tagged with the sequence encoding GFP in haploid wild-type and FGΔ yeast by amplification of the *GFP:HIS3MX6* region from the yeast GFP collection strain YPL169C (Invitrogen). Integrants were selected on SC-HIS and verified by PCR and by immunoblot with Rabbit anti-GFP (1:1000). To allow integration of the gene for expression of dsRED-HDEL, pKW1803 was linearized with EcoRV and transformed into yeast cells. Cells were selected on SC-TRP and integrants were verified by live-cell microscopy. For energy depletion assays, cells were grown to early log phase in YPD at 23˚C. A culture aliquot of 2.5 A_{600} units was used, and the cells were pelleted, washed, and resuspended in 1mL YP (without glucose) with 10mM NaN_3 and 10mM 2-deoxy-D-glucose. Cells were treated for 45 minutes at 23˚C, and then were pelleted, washed, and placed on ice prior to microscopy. At time=0, cells were resuspended in 23˚C YPD, mounted on a glass slide, and visualized as described above. Images of the GFP and dsRED signals were acquired every 30 seconds for 15 minutes. Cells were scored for recovery of Mex67-GFP to the nuclear rim and the relative nuclear to

cytoplasmic GFP signal. Control strains SWY734 and SWY3302 were energy depleted and imaged as described above.

CHAPTER IV

CONCLUSIONS AND FUTURE DIRECTIONS

Transport through the NPC is a dynamic process and NPCs are an essential portal allowing coordinated regulation of gene expression and signaling. Mechanisms at the level of single cargoes, transport receptors, and the NPC itself are all employed to regulate nuclear entry/exit (TERRY *et al.* 2007). As a whole, the work described here has provided new insight into the NPC as a regulatory machine. Specifically, I have demonstrated that there are multiple transport pathways through the NPC. These pathways are defined by preferred FG-Nup binding sites that are employed in nucleocytoplasmic translocation of specific transport receptors and their cargoes. In addition, I have defined FG-Nups linked to the termination of mRNA export, and have found that Kap abundance can alter transport. These discoveries and current gaps in our understanding of NPC function lay the foundation for future studies of nucleocytoplasmic transport. In this chapter, I will begin by discussing experiments and predictions to follow the current experiments presented in earlier chapters, and will then discuss potential future avenues for the field.

What are the FG-Nup binding sites for other transport receptors?

To date, we have characterized FG-Nup binding sites for less than half of known transport receptors. We have assayed for FG domains required for nuclear import via

Kap95, Kap104, Kap121 and Kap123, and nuclear export via Xpo1 (STRAWN *et al.* 2004; TERRY and WENTE 2007) (Eric Shows and Laura Terry, unpublished). Of note, we have not identified any FGΔ mutant with defects in Kap95 transport. Unlike all other known transport receptors, a complete cycle of Kap95 transport (i.e., import of the receptor-cargo complex, dissociation of the cargo, and recycling of the receptor) is energetically more expensive than transport by other karyopherins (MACARA 2001). Kap95 imports cargo in a trimeric complex consisting of Kap95, Kap60, and NLS-containing cargo (STEWART 2007a). Upon dissociation in the nucleus, Kap95 is recycled in complex with RanGTP. For the next round of import to function, Kap60 must also be recycled. Kap60 is exported by the karyopherin Cse1 in complex with RanGTP. Thus recycling of a single Kap95 molecule requires hydrolysis of one RanGTP moiety, and recycling of Kap60 via Cse1 requires a second RanGTP to be hydrolyzed. As Kap60 is essential for nuclear import of most cNLS-containing cargoes via Kap95, and we have not identified Kap95 transport defects, we must conclude that Kap95-Kap60 and Cse1 are imported properly and that recycling of Kap95-RanGTP and of Cse1-Kap60-RanGTP are also not affected by any of the FGΔ mutants assayed.

An exhaustive analysis of FG-domain requirements for all other known transport receptors is possible (pending identification of receptor-specific signal sequences and subsequent construction of reporters for each). While this approach might detect additional FG pathways, I do not consider this a high priority. There are, however, three specific transport events that would be particularly interesting to study in FGΔ mutants, including Ntf2 and large cargos (e.g., ribosome subunit export and proteasome subunit import).

Ntf2 is an interesting candidate for study in our FGΔ mutants for two reasons. First, Ntf2 functions in RanGDP import as a homodimer, and each Ntf2 monomer is predicted to have only four FG binding pockets (BAYLISS *et al.* 2002a; BAYLISS *et al.* 1999; LANE *et al.* 2000; MORRISON *et al.* 2003; QUIMBY *et al.* 2001). Does having two identical sets of FG binding pockets mean that Ntf2 binds a less diverse array of FG repeats than does, say, a Kap with many FG binding pockets? If so, Ntf2 would be more sensitive to deletion of key FG domains. Ntf2 transport cannot be completely abolished in FGΔ mutants, as not all Kaps have transport defects in our mutants (complete disruption of the RanGTP-RanGDP gradient would abolish all Kap-dependent transport). However, it is possible that Ntf2 shuttling can be impaired without collapsing Ran function or causing lethality (QUIMBY *et al.* 2001; SMITH *et al.* 2002). Second, the *ntf2-N77Y* allele is an interesting tool for study of functional differences between types of FG repeat. This mutant has increased affinity for FXFG repeats and can block transport (QUIMBY *et al.* 2001). We predict that deletion of FXFG domains would, then, remove the high-affinity binding sites for the ntf2-N77Yprotein and thus restore normal transport and ntf2-N77Y shuttling. The combination of this point mutant and our ability to delete specific FG domains provides a powerful combination for addressing the relevance of transport receptor:FG binding affinities *in vivo*.

Transport of large cargoes through the NPC is an interesting problem for study in FGΔ mutants. The narrowest portion of the central NPC has a diameter of ~45-50nm (AKEY and RADERMACHER 1993; HINSHAW *et al.* 1992; STOFFLER *et al.* 2003), and remarkably the NPC transports cargoes that approach this size limit, including 39nm NLS-coated gold particles and 32-36nm intact hepatitis B viral particles (PANTE and

KANN 2002). The eukaryotic ribosome is exported from the nucleus as separate 40S and 60S subunits, which have dimensions of up to ~25nm (NISSAN *et al.* 2004; VERSCHOOR *et al.* 1998). Experiments in permeabilized HeLa cells suggest that multiple rounds of nucleotide hydrolysis are required for transport of large cargo (LYMAN *et al.* 2002), indicating that either (a) there is GTP hydrolysis and release of receptor from cargo repeatedly during transport of these large molecules or (b) there are multiple receptors on the large cargo and each hydrolyzes a single GTP/ATP at termination of transport. In support of the latter, three different transport receptors are proposed to contribute to nuclear export of the pre-60S subunit (BRADATSCH *et al.* 2007; GADAL *et al.* 2001; HO *et al.* 2000b; HUNG and JOHNSON 2006; THOMAS and KUTAY 2003; TROTTA *et al.* 2003; YAO *et al.* 2007). In yeast, the largest substrates for nuclear import are proteasome subunits, which are imported as 19S and 20S particles (LEHMANN *et al.* 2002; WENDLER *et al.* 2004). Several proteins in the 20S proteasome have putative NLS motifs (VON MIKECZ 2006), so it is possible that multiple transport receptors are involved in transport of these relatively large particles. If, then, each transport receptor proceeds along a preferred pathway of FG binding sites, then we predict that transport of cargoes bearing multiple receptors will be more sensitive to FG deletions; deletion of FG domain(s) critical for any one of the receptors will impair transport. By identifying FGΔ mutants with defects in transport of a given large cargo, it may be possible to deduce if (and which) other transport receptors are contributing to transport of that cargo. For example, the only transport receptor identified for the 40S ribosomal subunit is yXpo1 (vCrm1) (MOY and SILVER 1999), and given the need for multiple receptors in 60S subunit export, it has been speculated that other receptors must contribute to 40S subunit export

(OEFFINGER *et al.* 2004). If we were to identify a FGΔ mutant with no Xpo1 transport defects but with 40S export defects, this would be strong evidence that another receptor must contribute to 40S export and that said receptor requires the missing FG domains for shuttling. An alternative hypothesis is that transport of large cargos is more retarded than for smaller cargoes (indeed, transport proceeds at a rate that is inversely proportional to the diameter of the cargo at hand (PAINE *et al.* 1975) and is influenced by the hydrophobicity of transporting molecules (RIBBECK and GORLICH 2002), and thus for cargoes with but a single transport receptor, increasing cargo size would exacerbate the slowness of large cargo trafficking. We could differentiate between these two possibilities by building model/reporter cargoes of varying sizes and manipulating the number of NLSs or NESs on them. This principle could also be applied to examine mRNP trafficking by building a model transcript with varying number of specific binding sites for transport receptors. These experiments will test the relationship between cargo size, receptor number and FG usage and contribute to our understanding of the NPC as a selective permeability channel.

How does competition influence transport efficiency?

Transport receptors are mediators, interacting as a molecular bridge between cargoes and the NPC to mediate exchange between the nucleus and cytoplasm. We have seen that over-expression of KAP104 or KAP121 can rescue nuclear import defects of model cargoes (Appendix A). These Kaps rescued steady-state nuclear localization of model cargoes in NPC FGΔ mutant *S. cerevisiae* strains. Because of the complex interplay between factors in nucleocytoplasmic transport, it is difficult to tease apart the

molecular basis for this rescue. Increased abundance of a Kap could favor formation of a Kap-cargo complex, a precursor step necessary prior to transport of cargoes. If this is the molecular basis of the rescue, then this rescue would be thwarted by compensatory overexpression of a cargo. A second possibility is that increased abundance of a Kap alters molecular competition for overlapping/limiting binding sites at the NPC. In this scenario, we predict that competition will be most readily apparent in FGΔ mutants wherein the number of total FG repeats is limited, thereby enhancing competition. We have identified a bank of FGΔ mutants with defects in Kap104 and/or Kap121 import, and we have successfully used live-cell microscopy to monitor the subcellular localization of model cargoes trafficked by each of these Kaps (STRAWN *et al.* 2004; TERRY and WENTE 2007). Kap104 and Kap121 are excellent choices for a thorough study of competition, as they are present at similar levels in cells (estimated 12,000 Kap104 and 18,000 Kap121 molecules per cell; (TIMNEY *et al.* 2006)) and we have cargo reporters for each that can be used in steady-state or rate assay of import. In addition, Kap104 and Kap121 have both overlapping and distinct interactions with FG-Nups (AITCHISON *et al.* 1996; ALLEN *et al.* 2001; DAMELIN and SILVER 2000; SEEDORF *et al.* 1999). We may, therefore, detect differences in Kap competition by biasing the FG composition of the NPC with deletion of binding sites for one or the other Kap. I propose to begin these studies with two groups of FGΔ mutants. In the first set, we would examine strains with deletions of multiple symmetric FG domains. We have six FGΔ mutants that remove roughly half of the central FG domains, and each of these has steady-state defects in both Kap104 and Kap121 import (STRAWN *et al.* 2004). In addition, we would examine the effects of the asymmetric or asymmetric plus symmetric mutants. Analysis of Kap

competition in the Δ*N*Δ*C nup100*Δ*GLFG nup145*Δ*GLFG* mutant is especially interesting, as this mutant has defects in Kap121 import but not in Kap104 import.

Thorough analysis of competition between Kaps will require application of a quantitative system to measure nuclear to cytoplasmic ratios for fluorescent reporters, such as the methods used by others (TIMNEY *et al.* 2006). In addition, we must use either fluorescently-tagged Kap signal intensity or immunoblotting to calibrate the degree of Kap over-expression. Ideally we will use a dual-color system in one of two ways. For looking at how Kap over-expression rescues transport of its own cargoes, monitoring the abundance of a Kap and its cargo on a single-cell resolution will allow us to calculate, in a closed system, the dependence of transport efficiency on the Kap:cargo ratio. Because plasmid copy number is variable between cells, it is necessary that we consider the effects of Kap over-expression on a cell-by-cell basis. For examining how Kap over-expression may compete with transport via another Kap, the ideal set-up would be to have, for example, a GFP-cargo for Kap121 and an RFP-cargo for Kap104 co-expressed. This would allow us to monitor nuclear-to-cytoplasmic abundance of each model cargo simultaneously, detecting differential effects of Kap over-expression on each cargo.

What are the factors that could influence how effectively a Kap competes with others for NPC binding sites? The abundance of a Kap and its cargoes affects import efficiency (HODEL *et al.* 2006). The diversity of FG binding sites with which the Kap can interact, its affinity for those binding sites, and the avidity of FG-binding hydrophobic pockets on the surface of the Kap likely also will affect transport of a receptor. These latter factors are not easily manipulatable without potentially disrupting the structure of a Kap, and are likely not feasible points to examine. However, a robust assay of varying

abundance of Kaps and substrate cargoes would provide support to our model that Kaps compete for overlapping points along multiple pathways through the NPC. This result, in turn, would force re-examination of models of transport, as no model accounts for potential differences in the ability of each Kap to overcome an entropic barrier or to differentially invade an FG gel-meshwork.

Do transport receptors use different binding sites for each direction of transport?

Maintenance of nucleocytoplasmic transport requires that a transport receptor be able to undergo bi-directional shuttling. After delivering their cargoes, import Kaps must recycle to the cytoplasm for another round of transport. And, to export cargoes, export Kaps must first be imported. This process must be further supported by shuttling of Ntf2 to continually import RanGDP. In other words, in order to import (or export) a single cargo, multiple transport events must occur: (1) Kap-cargo import; (2) Kap-RanGTP recycling; (3) Ntf2-RanGDP import; and (4) Ntf2 recycling. As the Ntf2 homodimer imports two RanGDP molecules per cycle (as visualized in (STEWART *et al.* 1998)), only one Ntf2 cycle is required per two import (or export) events. There are two exceptions to this: transport via the Kap95-Kap60 heterodimer and via Msn5. During the recycling stage following Kap95-Kap60-mediated cargo import, Kap95-RanGTP forms one recycling complex, and Kap60 is exported coupled to Cse1-RanGTP (KUNZLER and HURT 1998; KUTAY *et al.* 1997a; LEE *et al.* 2005; MATSUURA and STEWART 2004; VETTER *et al.* 1999). In contrast, Msn5 is perhaps the most efficient Kap; it is the only Kap known to both import and export cargo (MACARA 2001). How can so much

molecular traffic be coordinated – and in single NPCs, which are presumed to function for bi-directional traffic? Do Kaps bind different FGs during their import versus export phases, with or without cargo? This is certainly an attractive model – preventing the recycling form of a Kap from interfering with the cargo-bound form of a Kap. Teasing this apart in vivo is difficult. Single-molecule studies demonstrated that the movement of a Kap within the NPC is seemingly random; as these studies did not detect directed trafficking, it is unlikely that they could detect differences between transport and recycling phases of a Kap. On the other hand, a large-scale in vitro binding study detected differences in the affinity of several Kaps for FGs depending on the presence/absence of RanGTP (ALLEN *et al.* 2001). In addition, the helical pitch of Kaps is slightly shifted depending on whether they are associated with cargo and/or RanGTP (CONTI *et al.* 2006; COOK *et al.* 2007). Slight shifts of the Kap superhelix could alter the hydrophobic pockets that are most receptive to FG binding and allow Kaps to adapt to binding different cargoes. In our transport assays, we cannot differentiate between defects in the cargo-bound, recycling, or both forms of a Kap.

Perhaps one way to test would be to identify FGΔ mutant(s) with defects in Msn5 transport, such as attempted in (BELANGER *et al.* 2004). A defect in either the import or export phase likely would result in steady-state defects for all Msn5 cargoes. However, rate assays might successfully differentiate between import-specific and export-specific defects. This experiment requires establishing NLS and NES reporters for Msn5 transport that are small enough for use in energy depletion assays (i.e., can diffuse through the NPC to equilibrium). With these reporters and interesting FGΔ mutants in hand, an energy depletion assay and two-color imaging of recovery would detect import or export-

specific defects for Msn5. This assumes that Msn5 distributes equally to the nucleus and cytoplasm upon energy depletion. Because this is a rate assay, rather than steady-state, subtle differences between the import and export phases for this single Kap may be detected.

How does the NPC contribute to regulation of mRNA export?

The protein composition of mRNPs is highly dynamic, with continual changes occurring from transcription, through processing/maturation, nuclear export, and translation (IGLESIAS and STUTZ 2008). The changes in protein composition of an mRNP both mark past events (*e.g.,* splicing deposits the exon junction complex) and ready it for next steps, and mRNA maturation is coupled to changes in mRNP composition (IGLESIAS and STUTZ 2008; KOHLER and HURT 2007). Of great interest to this thesis work is understanding the mRNP composition that marks an mRNA ready for nuclear export. In addition, elucidating the quality control mechanisms that permit initiation of nuclear export (such as the Mlps in checking for splicing) is of interest – how does the NPC or NPC-associated factors function in quality control in mRNA export? For example, it is not fully understood how heat shock-induced mRNA is preferentially exported under stress conditions in *S. cerevisiae* (discussed in (ROLLENHAGEN *et al.* 2007)), though Nup42 is linked to regulation of this process. Additionally, the work presented in Chapters 2 and 3 found links between specific FG domains and mRNA export. We observed that the FG domain of Nup42, and NPC protein located in the cytoplasmic face of the pore, affects mRNA export. This FG domain is adjacent to a binding site for the mRNA export factor Gle1, and early evidence in our studies suggests that the FG domain

and the Gle1 binding domain of Nup42 together contribute to mRNA export. Although we did not identify a strong, specific role singly for the FG domain of Nup159, this FG-Nup is also at the cytoplasmic face of the NPC, and juxtaposed to the FG domain of Nup159 is a binding site for the mRNA export factor Dbp5. Terminal release of an mRNP from the NPC – the conclusion of nuclear mRNA export – is not mechanistically understood. A ratchet-like model has been proposed (STEWART 2007b). In the ratchet model, serial removal of proteins from the mRNP changes the composition of the mRNP such that it cannot retro-translocate back into the NPC. We predict that Nup42 and Nup159 – each with juxtaposed binding sites for the mRNA export receptor Mex67-Mtr2 (on FG domains) and mRNA export factors Gle1 and Dbp5 (on Nup42 and Nup159, respectively) play a critical regulatory role in mRNA export. Of note, mRNPs are relatively large cargoes. Does mRNP export require multiple transport receptors? The stoichiometry of Mex67-Mtr2 on a single transcript is unknown, although Mex67 is recruited to several different positions along the length of an mRNA (CHENG *et al.* 2006; DIEPPOIS *et al.* 2006; LE HIR *et al.* 2001). At the start of export, what marks a transcript as ready and processed for export, and what Nups or NPC-associated factors recognize this? At termination of export on the cytoplasmic face of the NPC, how do Nups or NPC-associated factors alter the mRNP composition to release it from the pore? These quality-control mechanisms on either end of the NPC are of interest to understanding regulated mRNA export.

Does the type of FG domain or the location of domains dictate transport function?

We have begun tests with a Δ*N nup49*Δ*GLFG* mutant (Chapter 3) to examine whether different FG domains can substitute for the GLFG domain of Nup49 in mRNA export. In addition to generating and testing FG domain swap constructs, we will design constructs with minimal number of FG repeats. This will allow us to test the importance of FG repeat avidity versus a specific FG repeat plus spacer sequence as the determinant of transport function. There are two methods that could be used to alter the number of FG repeats in a given FG-Nup. One option is to generate domain deletions by standard PCR and cloning methods. This strategy would delete not only FG repeat(s), but also the intervening spacer sequence(s), thus shortening the overall length of the FG domain. The alternative is to use site-directed mutagenesis to engineer sequences encoding mutated FG repeats (*e.g.*, mutate to encode Ser instead of Phe). This approach is tedious for making multiple mutations and requires judicious selection of mutations so as to change Phe residues to a residue that is commonly in FG domains. However, this also keeps intact the length of the full FG domain, which might be important for the NPC barrier or topological flexibility.

Assays of FG domain swaps and assessment of the number of FG repeats required for transport will indicate whether the type and number of FG repeats is important. Targeted swapping of FG domains will allow direct examination of whether the type of FG repeat, the FG domain size, or the location of FG repeat is most critical to transport. Consideration of *the* Δ*N*Δ*C nsp1*Δ*FG*Δ*FXFG* mutant might also give insight into this question. This mutant had five GLFG domains and one FG domain remaining; the other

three FG domains and all of the FXFG domains have been deleted. The Δ*N*Δ*C* *nsp1*Δ*FG*Δ*FXFG* mutant is temperature-sensitive and has transport defects, but is viable; in contrast, no more than three GLFG domains can be deleted (STRAWN *et al.* 2004). The results of the present study point to specific requirements for GLFG domains; thus we suspect that there are functional differences between types of repeats. In addition, the Δ*N*Δ*C* *nsp1*Δ*FG*Δ*FXFG* mutant has the least FG mass remaining of any of the mutants in our FGΔ collection. Is it the GLFG repeats specifically or the substructural location of GLFG repeats that is required for viability of this strain? This could be tested by systematically replacing each GLFG repeat with an FXFG repeat in the Δ*N*Δ*C* *nsp1*Δ*FG*Δ*FXFG* mutant. The design and execution of these experiments is complicated by our recent finding that the LoxP strategy we previously used (STRAWN *et al.* 2004) leaves an epitope tag-LoxP "scar" sequence that is deleterious to NPC function (Chapter 3). As an alternative to this method, we could construct the FG-swap coding sequences using standard molecular biology approaches and then use gene replacement techniques in *S. cerevisiae* to integrate these alleles. The major technical challenge of this is that we are unable to monitor expression and stability of the chimeric proteins, as we do not have antibodies specific to each non-FG domain. However, the non-FG domain of many FG-Nups are essential genes, and so we can rationalize that FG-swap mutants that are viable must be producing a functional fusion protein. Alternatively, we could design these swaps with an epitope tag in another location (*e.g.*, the C-terminus of the Nup with a flexible linker) or could test different epitope tags (and without the LoxP sequence). These approaches require sustained effort to build mutant strains and tools, but will provide valuable insight into the determinants of transport pathways.

How do FG-domains contribute to forming the permeability barrier?

Understanding the duality of NPC function – in permitting both free diffusion of small molecules and in trafficking large molecules/complexes – requires improved understanding of the constituents of the permeability barrier. Both FG-Nups and structural Nups have been implicated in forming this barrier (PATEL *et al.* 2007; SHULGA and GOLDFARB 2003; SHULGA *et al.* 2000). Two assays for measuring the integrity of the barrier in vivo give different results. A GFP-based assay developed by Shulga & Goldfarb (SHULGA *et al.* 2000) monitors localization of sized reporters and applied to the FGΔ mutant Δ*N*Δ*C nsp1*Δ*FG*Δ*FXFG* did not detect any impairment of the permeability barrier (STRAWN *et al.* 2004). If FG domains are the key component of the permeability barrier, then this result demonstrates that the barrier is surprisingly resilient to FGΔ; the Δ*N*Δ*C nsp1*Δ*FG*Δ*FXFG* mutant has only ~46.8% of its total FG repeats remaining (STRAWN *et al.* 2004). An alternative assay, employing a yeast one-hybrid technology, detected leakiness in the permeability barrier with deletion of certain single FG domains (PATEL *et al.* 2007). This yeast one-hybrid assay assumes that the LexA-Gal4AD reporter protein homodimerizes in the cytoplasm to form an entity that is too large for free diffusion into the nucleus. Curiously, deletion of the FXFG domain of Nup60, which removes a mere 32 FXFG repeats (4FXFG repeats in Nup60; 8 copies of Nup60 per NPC (ROUT *et al.* 2000), is a sufficient perturbation to cause apparent permeability barrier defects in this approach (PATEL *et al.* 2007). As the *nup60*Δ*FXFG* allele is one of the six deletions present in the Δ*N*Δ*C nsp1*Δ*FG*Δ*FXFG* mutant, we find it surprising that the much less substantive *nup60*Δ*FXFG* mutant alone would have permeability defects. To

fully understand the contributions of FG domains to the maintenance of the permeability barrier, the discrepancies between these two approaches must be resolved.

How are Nups regulated in disease and developmental contexts?

Alterations to the NPC, such as blocking one pathway to favor another, are a potentially rapid and dramatic strategy for changing the flux of all traffic through the NPC. The cell cycle-regulated exposure of a Kap121 binding site is an excellent example of temporal changes to the NPC that alter transport for a single receptor (MAKHNEVYCH *et al.* 2003). Likewise, the degradation of Nups by many viruses highlights the manipulatibility of the system to favor specific trafficking events (FARIA *et al.* 2005; GUSTIN 2003; GUSTIN and SARNOW 2001; GUSTIN and SARNOW 2002; SATTERLY *et al.* 2007).

Classic EM experiments have detected an increased number of NPCs in the NE of a stimulated lymphocyte (MAUL *et al.* 1971), suggesting that there are global mechanisms to regulate the total number of NPCs and to make rapid changes in NPC abundance. Are there more subtle differences in Nup expression and NPC structure/function/pathways during organism development? Tissue-specific expression of two Nups has been detected during mouse development (OLSSON *et al.* 2004; SMITHERMAN *et al.* 2000), although the molecular consequences of this altered NPC composition on signaling and trafficking is not fully understood. In *Drosophila*, expression of the structural Nup *mbo* is spatially restricted, and *mbo* has an inhibitory effect on Crm1-mediated export (UV *et al.* 2000). Further evidence for Nup roles in disease come from studies of a Nup98 knock-out mouse (WU *et al.* 2001). NUP98/NUP96 are the vertebrate homologue of the *S.*

cerevisiae Nup100/Nup116/Nup145N family of FG-Nups (RYAN and WENTE 2000). The polypeptide translated from NUP98/NUP96 transcripts is post-translationally cleaved to form two peptides: Nup98, which contains GLFG repeats, and Nup96, which is a structural protein (FONTOURA *et al.* 1999). $NUP98^{-/-}$ murine cells have defects in a subset of transport pathways (WU *et al.* 2001), and the $NUP98^{+/-}$ mice have defects in interferon responsiveness (ENNINGA *et al.* 2002). This thwarted interferon response increases susceptibility of the mice to lethal viral infection (SATTERLY *et al.* 2007), thus demonstrating the importance of functional nucleocytoplasmic transport in immune response. We predict that future analysis of gene expression patterns in varied tissues and developmental states will detect altered expression of Nups and transport components as a regulatory mechanism.

Closing

Given the hierarchical role of the NPC as the sole portal for nucleocytoplasmic exchange, understanding the NPC translocation mechanism is a priority. Our results demonstrate that there are multiple FG transport pathways through the NPC, and further studies are needed to delineate the molecular determinants of each pathway. Using mutants with limited numbers of FG repeats, we have found that the abundance of Kap directly impacts its transport efficiency. These studies set the stage for future investigation of competition between Kaps for binding the NPC. Further studies are needed to resolve the biophysical nature of the permeability barrier and explain how translocation proceeds through this barrier. The results of these experiments will also require revised models that accommodate all the documented active and diffusive

transport capacities of the NPC, as well as considerations of how interactions within and among cargoes, transport receptors and Nups impact the stability of the NPC barrier. With this in hand, the field could make rational predictions and conduct tests for how transport would be impacted by changes in Nup composition during viral infection, cell cycle transitions, signaling cascades, or cell differentiation.

APPENDIX A

Competition as a possible mechanism affecting transport

Competition at multiple levels affects transport

Competition between cargoes for binding to a Kap or between Kaps for binding to the NPC is emerging as an important consideration in studies of nucleocytoplasmic transport dynamics. Each Kap can potentially bind multiple signal-bearing cargoes. Thus, if the abundance of a Kap is limiting for a pool of cargoes, then competition between cargos potentially impacts transport efficiency. In support of this, others have suggested that Kap-cargo complex formation and disassembly, and not the actual translocation process, is the rate-limiting step in transport (GILCHRIST *et al.* 2002). Additionally, import and export cargoes can compete for binding to Kaps (MOSAMMAPARAST *et al.* 2002; OHNO *et al.* 2000). The affinity of a signal sequence (either NLS or NES) for a Kap affects the formation and disassembly of a Kap-cargo complex and thus the transport of complexes across the NPC (ENGELSMA *et al.* 2004; KUTAY and GUTTINGER 2005; TIMNEY *et al.* 2006). At the level of Kap-NPC interaction, competition for common binding sites may also impact cellular dynamics. The abundance of a Kap affects its transport rate (MOSAMMAPARAST and PEMBERTON 2004; TIMNEY *et al.* 2006; YANG and MUSSER 2006), although transport via a single Kap is saturable under conditions of excess cargo, as shown in classic microinjection experiments (BATAILLE *et al.* 1990; JARMOLOWSKI *et al.* 1994; NAKIELNY and DREYFUSS 1999; POKRYWKA and GOLDFARB 1995).

As an example of the combinatorial effects of Kap abundance and Kap-NLS affinity in nuclear import, the ribosomal protein Rpl25 is imported into the nucleus in a Ran-dependent manner, with Kap123 primarily responsible for Rpl25 import (ROUT *et al.* 1997), although as is the case with other ribosomal proteins, Kap121 can also contribute to Rpl25 import (ROUT *et al.* 1997; SYDORSKYY *et al.* 2003). In a *kap123*Δ *S. cerevisiae* mutant, a GFP reporter for Kap123-mediated import (the NLS from Rpl25 fused to GFP) is partially mislocalized to the cytoplasm, indicating that other Kaps cannot fully compensate for the loss of Kap123 (ROUT *et al.* 1997; TIMNEY *et al.* 2006). Overexpression of Kap121, but not other Kaps, in a *kap123*Δ mutant rescues nuclear import of the Rpl25NLS-GFP reporter (TIMNEY *et al.* 2006). Thus, increased abundance of an alternative Kap can bypass the requirement for Kap123. Transport is saturable when the *in vivo* concentration of Kap123 reaches ~15uM, a value estimated to be a ~300% increase over the physiological levels of Kap123 expression. Interestingly, although the endogenous expression level of Kap121 is ~6-fold lower than Kap123, overexpression of Kap121 increases Rpl25-NLS-GFP transport with similar kinetics of saturation at a Kap121 concentration of ~15uM and half-maximal transport at ~7uM. As Kap121 and Kap123 have similar affinities for the Rpl25 NLS, these results indicate that increasing Kap concentration can augment transport rates. Although Kap121 and Kap123 have different preferences for FG-Nup binding sites (Table 2, Chapter 1) (ALLEN *et al.* 2001; ALLEN *et al.* 2002; MARELLI *et al.* 1998; ROUT *et al.* 1997; SEEDORF *et al.* 1999), transport of Rpl25NLS-GFP via either Kap proceeds with similar kinetics (TIMNEY *et al.* 2006). The saturability of transport for Kap121 and Kap123 may come from increased occupancy of FG binding sites and therefore translocation becomes rate-limiting.

Alternatively, the excess of a Kap/cargo could deplete the Ran gradient (via export of RanGTP on the recycling Kap) faster than RanGDP can be re-imported. Monitoring all of these factors *in vivo* is not a trivial experiment and has not been conducted.

We have previously demonstrated that there are multiple FG pathways through the NPC, although the degree of overlapping binding site(s) between pathways remains unclear. Other studies of protein-protein interaction find that Kaps have binding preferences on specific FG-Nups (ALLEN *et al.* 2001; ALLEN *et al.* 2002; DAMELIN and SILVER 2000; STRAWN *et al.* 2004; STRAWN *et al.* 2001) (AITCHISON *et al.* 1996; ALLEN *et al.* 2001; ALLEN *et al.* 2002; DAMELIN and SILVER 2000; MARELLI *et al.* 1998; ROUT *et al.* 1997; SEEDORF *et al.* 1999; STRAWN *et al.* 2004; STRAWN *et al.* 2001). If Kaps use overlapping NPC binding sites, then competition between Kaps for these sites could impact transport dynamics.

Kap competition: FGΔ mutant NPCs are subject to competition among karyopherins

In our previous studies (STRAWN *et al.* 2004; TERRY and WENTE 2007), we have found evidence supporting multiple transport pathways through the NPC. By comparing the ability of different transport receptors to translocate model cargoes across FGΔ mutant NPCs, we found that specific FG domain deletions affected transport by only a subset of transport receptors. Previous studies have demonstrated that Kap concentrations directly impact transport efficiency of a model cargo *in vivo* (TIMNEY *et al.* 2006). To test the ability of transport receptors to compete for limited NPC binding sites, we over-expressed Kap and monitored nuclear import of a model cargo. If transport receptors

compete for overlapping binding sites, or if there are lesser-preferred, alternative pathways for a single Kap, then increased relative expression levels of a given Kap would increase its occupancy on those overlapping binding sites or force increased use of alternative pathways. The *nup49*Δ*GLFG nup100*Δ*GLFG nup145*Δ*GLFG* mutant and the *nup100*Δ*GLFG nup145*Δ*GLFG nsp1*Δ*FG*Δ*FXFG* mutant have temperature-sensitive defects in nuclear import via Kap104 ((STRAWN *et al.* 2004), Figure A-1). Kap104 import can be monitored by immunofluorescence to detect Nab2, a Kap104 cargo (AITCHISON *et al.* 1996), or by live-cell microscopy with a Nab2NLS-GFP reporter (SHULGA *et al.* 2000). Overexpression of *KAP104* does not alter Nab2 localization in wild-type cells. In both the *nup49*Δ*GLFG nup100*Δ*GLFG nup145*Δ*GLFG* mutant and the *nup100*Δ*GLFG nup145*Δ*GLFG nsp1*Δ*FG*Δ*FXFG*, a *KAP104*-2μ vector restores nuclear import of Nab2, indicating that the increased expression level of *KAP104* impacts the efficiency of nuclear import by this transport receptor. From these results, we infer that the increased levels of Kap104 result in increased nuclear localization of Nab2. This rescue of Kap104-mediated import may occur due to increased efficiency of Kap104-Nab2 complex formation (if Kap104 is rate-limiting for Nab2 nuclear import) or because the additional Kap104 can effectively compete for overlapping or alternative import FG binding sites. If Kap104 does, indeed, compete with other Kaps for FG binding sites, then overexpression of Kap104 might impair transport via another Kap. These results raise the possibility of competition for binding sites during transport, and we predict that our FGΔ mutants are biased towards detecting competition events as they have decreased number of binding sites. To directly test whether increasing the expression level of one transport receptor affected the transport dynamics of others, we examined the localization of model cargoes

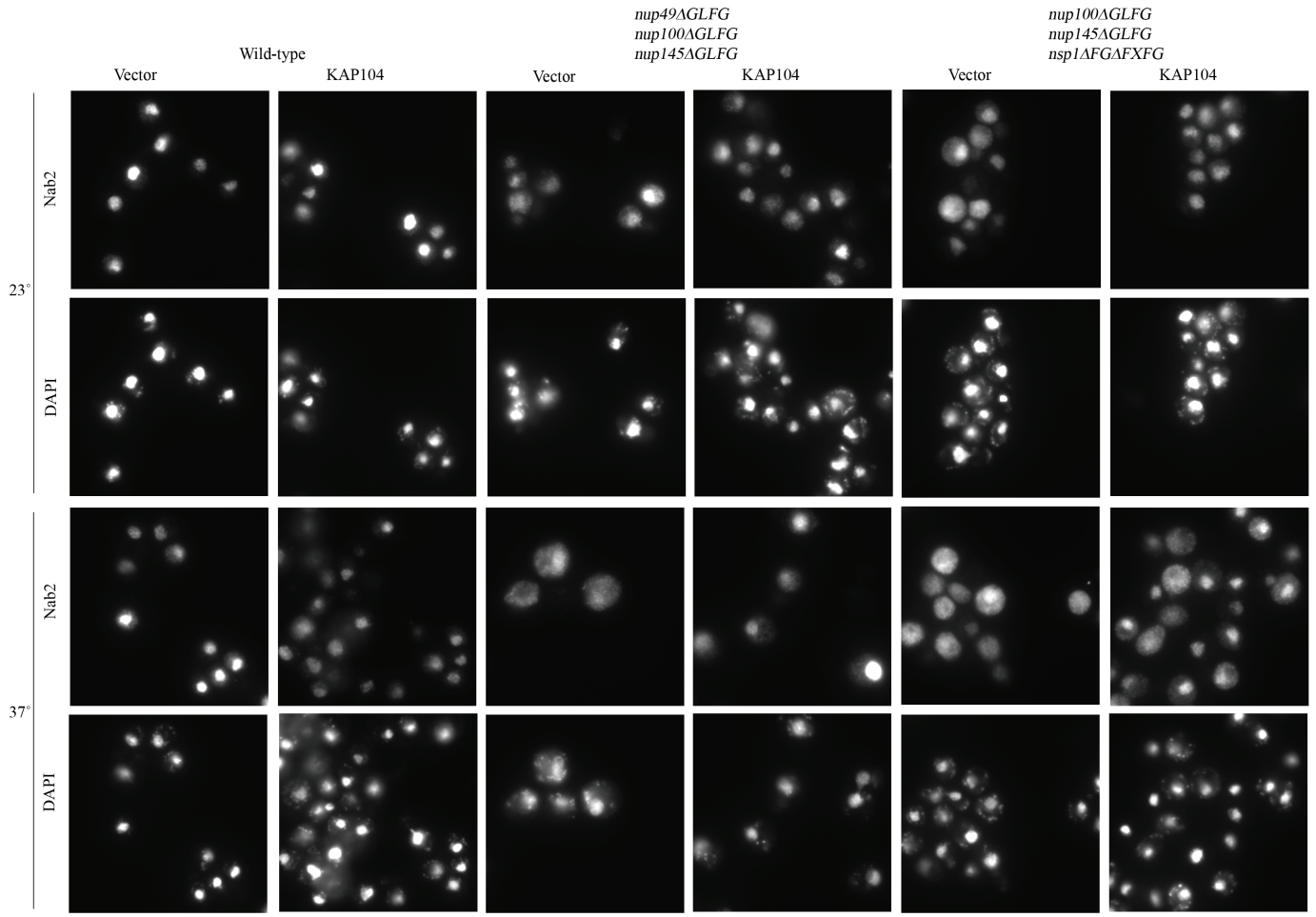


Figure A-1. Overexpression of KAP104 rescues Nab2 import defects.
Wild-type or FG mutant yeast were transformed with an empty vector or with a KAP104 2-micron overexpression vector. Cells were grown at 23˚ and/or shifted to 37˚ for 1 hour. Cells were then fixed and processed for indirect immunofluorescence against Nab2. Coincident DAPI staining detects nuclei.

in live cells with increased levels of a given Kap. In future studies we will investigate this possibility of competition using our system of Kap overexpression vectors and NLS-GFP reporters.

Kap expression levels affect transport

Third, we have tested the importance of Kap expression level using mutants where NPC binding sites are limited for a given Kap. Overexpression of *KAP104* can restore nuclear import of a model Kap104 cargo in FGΔ mutants. This observation leads us to speculate about the importance of Kap competition for limited NPC binding sites. The abundance of a Kap, therefore, influences the efficiency of cargo transport. The effect of increased Kap104 abundance is likely increasing the abundance of Kap104 activity at the NPC. Similar results have been observed with overexpression of *KAP123* (TIMNEY *et al.* 2006). The role of Kap abundance is not directly addressed by current models of the transport mechanism. If transport receptors bind and locally "dissolve" an FG meshwork, as proposed in the selective phase model, then increased transport receptor concentration would cause a net increase in the dissolution of the FG gel. Is it possible to completely dissolve the FG gel and therefore eliminate permeability? Or does the opposite happen – complete occupancy of the FG binding sites increases the barrier? Applying the tenets of the entropic barrier/virtual gating model, increased transport receptor concentration might increase occupancy of FG binding sites on either side of the NPC, thus increasing the probability/favorability of spontaneous translocation across that barrier. In any case, there is a fundamental limit to the number of transport receptors that can occupy any given pore, and this is presumably defined by the total number of

potential FG binding sites. In addition, increased transport can only be supported if terminal release mechanisms permit. Increased transport has the potential to deplete nuclear RanGTP pools if Ntf2 cannot re-import RanGDP and/or the RanGEF activity is inadequate, and maintenance of a steep RanGTP:RanGDP gradient is necessary to transport and accumulate cargo above its equilibrium (GORLICH *et al.* 2003). Mathematical modeling of transport indeed suggests that increased Kap abundance can increase transport efficiency, but only to a limited degree, as futile shuttling of the Kap can deplete the Ran gradient (RIDDICK and MACARA 2005). If increasing the concentration of one transport receptor increases its occupancy of the NPC or alters the Ran gradient, then this may have deleterious effects on shuttling of other transport receptors. We have begun *in vivo* assays in *S. cerevisiae* to examine potential negative, competitive effects of Kap overexpression using live-cell reporters for Kap transport. Disruptions to the Ran gradient might be detectable if we could adapt the technology of a FRET-based system for RanGTP vs. RanGDP localization (KALAB *et al.* 2002) for use in the yeast system. Alternatively, if not all Kaps are affected by competition, then we have *in vivo* evidence for continued functionality of the Ran system. Through these studies of Kap overexpression, we have made insights towards understanding the effects of transport receptor abundance and the potential for competition for FG binding sites at the NPC.

Materials & Methods

Kap overexpression and transport assays

Yeast strains harboring either empty vector or KAP104 over-expression vector were grown to early-mid-log phase in SC media lacking the appropriate amino acid and supplemented with 2% glucose. After culture at 23˚C or after 1-hour shift to 37˚C, cells were fixed and processed for indirect immunofluorescence against Nab2, as described above. For KAP121 over-expression analysis, yeast strains harboring either empty vector or KAP121 over-expression vector in addition to pSpo12 76-130-GFP (Spo12NLS-GFP) were grown to early-mid-log phase in SC media lacking the appropriate amino acid and supplemented with 2% glucose. Live cells were then directly imaged.

Microscopy and image acquisition

All images were acquired using an Olympus BX50 microscope with a UPlanF1 100x/1.30 oil immersion objective and a Photometrics Coolsnap HQ camera. Within each experiment, all images were collected and scaled identically. Images were collected using Image Pro Express and processed with Adobe Photoshop 9.0 or higher software.

APPENDIX B

Table A-1. List of yeast strains used in this study.

SWY#	Genotype	Source
SWY2283	ADE2 ADE3 ura3-1 his3-11,15 TRP1 leu2-3,112 lys2	(STRAWN *et al.* 2004)
SWY2284	ADE2 ADE3 ura3-1 his3-11,15 trp1-1 leu2-3,112 LYS2	(STRAWN *et al.* 2004)
SWY2285	ADE2/ADE2 ADE3/ADE3 ura3-1/ura3-1 his3-11,15/his3-11,15 trp1-1/TRP1 leu2-3,112/ leu2-3,112 LYS2/lys2	(STRAWN *et al.* 2004)
SWY2729-2731; 2737	nup2ΔFxFG	(STRAWN *et al.* 2004)
SWY2751-2754; 2757	nup57ΔGLFG	(STRAWN *et al.* 2004)
SWY2762-2765; 2766	nup100ΔGLFG	(STRAWN *et al.* 2004)
SWY2771-2774; 2775	nup60ΔFxF	(STRAWN *et al.* 2004)
SWY2783-2785; 2786	nup100ΔGLFG nup57ΔGLFG	(STRAWN *et al.* 2004)
SWY2789-2792; 2793	nup116ΔGLFG	(STRAWN *et al.* 2004)
SWY2796-2799; 2800	nsp1ΔFxFG nup2ΔFxFG	(STRAWN *et al.* 2004)
SWY2801-2802; 2803	nup1ΔFxFG	(STRAWN *et al.* 2004)
SWY2807-2809; 2809	nup159ΔFG	(STRAWN *et al.* 2004)
SWY2811-2813; 2814	nsp1ΔFG	(STRAWN *et al.* 2004)
SWY2815-2817; 2818	nup159ΔFG nsp1ΔFG	(STRAWN *et al.* 2004)
SWY2819-2821; 2822	nup116ΔGLFG nup57ΔGLFG	(STRAWN *et al.* 2004)
SWY2825-2827; 2828	nup49ΔGLFG	(STRAWN *et al.* 2004)
SWY2831-2833; 2834	nup42ΔFG	(STRAWN *et al.* 2004)
SWY2835-2837; 2838	nup100ΔGLFG nup49ΔGLFG	(STRAWN *et al.* 2004)
SWY2839-2842; 2843	nup116ΔGLFG nup49ΔGLFG	(STRAWN *et al.* 2004)
SWY2844-	nup42ΔFG nup159ΔFG	(STRAWN *et*

2846; 2847		*al.* 2004)
SWY2848-2849;	nup42ΔFG nsp1ΔFG	(STRAWN *et al.* 2004)
SWY2850-2853; 2854	nup42ΔFG nup159ΔFG nsp1ΔFG	(STRAWN *et al.* 2004)
SWY2856-2857, 3069; 2858	nup2ΔFxFG nup60ΔFxF	(STRAWN *et al.* 2004)
SWY2861-2863; 2864	nsp1ΔFxFG nup2ΔFxFG nup1ΔFxFG	(STRAWN *et al.* 2004)
SWY2867-2869; 2870	nup145ΔGLFG	(STRAWN *et al.* 2004)
SWY2871-2873; 2874	nup100ΔGLFG nup57ΔGLFG nup49ΔGLFG	(STRAWN *et al.* 2004)
SWY2882-2884; 2885	nup57ΔGLFG nup49ΔGLFG	(STRAWN *et al.* 2004)
SWY2892-2894; 2895	nup1ΔFxFG nup2ΔFxFG	(STRAWN *et al.* 2004)
SWY2896-2897; 2898	nup1ΔFxFG nup2ΔFxFG nup60ΔFxF	(STRAWN *et al.* 2004)
SWY2899-2902; 2904	nsp1ΔFxFG nup2ΔFxFG nup60ΔFxF	(STRAWN *et al.* 2004)
SWY2904-2906; 2907	nsp1ΔFxFG nup2ΔFxFG nup60ΔFxF nup1ΔFxFG	(STRAWN *et al.* 2004)
SWY2908-2910; 2955	nup42ΔFG nup159ΔFG nup1ΔFxFG	(STRAWN *et al.* 2004)
SWY2911-2912; 2956	nup42ΔFG nup159ΔFG nup2ΔFxFG	(STRAWN *et al.* 2004)
SWY2913-2914; 2957	nup42ΔFG nup159ΔFG nup60ΔFxF	(STRAWN *et al.* 2004)
SWY2915-2916;	nup116ΔGLFG nup145ΔGLFG	(STRAWN *et al.* 2004)
SWY2919-2922; 2923	nsp1ΔFGΔFxFG	(STRAWN *et al.* 2004)
SWY2924-2926; 2927	nup145ΔGLFG nup57ΔGLFG	(STRAWN *et al.* 2004)
SWY2928-2931; 2932	nsp1ΔFGΔFxFG nup49ΔGLFG	(STRAWN *et al.* 2004)
SWY2933-2935; 2936	nsp1ΔFGΔFxFG nup57ΔGLFG	(STRAWN *et al.* 2004)
SWY2937-2939; 2940	nup42ΔFG nup159ΔFG nup2ΔFxFG nup60ΔFxF	(STRAWN *et al.* 2004)
SWY2941-	nup42ΔFG nup159ΔFG nup1ΔFxFG nup60ΔFxF	(STRAWN *et*

2943; 2944		*al.* 2004)
SWY2945-2948; 2949	nup42ΔFG nup159ΔFG nup1ΔFxFG nup2ΔFxFG	(STRAWN *et al.* 2004)
SWY2950-2953; 2954	nup100ΔGLFG nup145ΔGLFG nup57ΔGLFG	(STRAWN *et al.* 2004)
SWY2958-2961; 2962	nsp1ΔFGΔFxFG nup145ΔGLFG	(STRAWN *et al.* 2004)
SWY2963-2965; 2966	nup145ΔGLFG nup49ΔGLFG	(STRAWN *et al.* 2004)
SWY2967-2969; 2970	nup100ΔGLFG nup145ΔGLFG nup49ΔGLFG	(STRAWN *et al.* 2004)
SWY2971, 3041; 3045	nup42ΔFG nup159ΔFG nup60ΔFxF nup1ΔFxFG nup2ΔFxFG	(STRAWN *et al.* 2004)
SWY2972-2973, 2982; 2974	nup100ΔGLFG nup145ΔGLFG	(STRAWN *et al.* 2004)
SWY2975-2977; 2978	nsp1ΔFGΔFxFG nup116ΔGLFG	(STRAWN *et al.* 2004)
SWY2980-2981, 2983; 3005	nsp1ΔFGΔFxFG nup100ΔGLFG nup145ΔGLFG	(STRAWN *et al.* 2004)
SWY3001	nsp1ΔFGΔFxFG nup57ΔGLFG nup49ΔGLFG	(STRAWN *et al.* 2004)
SWY3007-3009; 3010	nsp1ΔFGΔFxFG nup100ΔGLFG nup49ΔGLFG	(STRAWN *et al.* 2004)
SWY3012-3014; 3015	nsp1ΔFGΔFxFG nup100ΔGLFG nup57ΔGLFG	(STRAWN *et al.* 2004)
SWY3027-3028; 3031	nsp1ΔFxFG nup49ΔGLFG	(STRAWN *et al.* 2004)
SWY3029-3030	nsp1ΔFxFG	(STRAWN *et al.* 2004)
SWY3042-3043; 3044	nup42ΔFG nup159ΔFG nup60ΔFxF nup1ΔFxFG nup2ΔFxFG nup100ΔGLFG	(STRAWN *et al.* 2004)
SWY3062-3064, 3066; 3065	nup42ΔFG nup159ΔFG nup60ΔFxF nup1ΔFxFG nup2ΔFxFG nsp1ΔFGΔFxFG	(STRAWN *et al.* 2004)
SWY3289	trp1-1 lys2 ura3 leu2 his3 HA-loxP-nup42ΔFG myc-loxP-nup159ΔFG T7-loxP-nup1ΔFXFG myc-loxP-nup2ΔFXFG myc-loxP-nup60ΔFXF T7-loxP-nup49ΔGLFG + pSW125	This study
SWY3290	trp1-1 lys2 ura3 leu2 his3 HA-loxP-nup42ΔFG myc-loxP-nup159ΔFG T7-loxP-nup1ΔFXFG myc-loxP-nup2ΔFXFG myc-loxP-nup60ΔFXF HA-loxP-nup100ΔGLFG T7-loxP-nup49ΔGLFG + pSW125	This study

SWY3291	trp1-1 lys2 ura3 leu2 his3 HA-loxP-nup42ΔFG myc-loxP-nup159ΔFG T7-loxP-nup1ΔFXFG myc-loxP-nup2ΔFXFG myc-loxP-nup60ΔFXF HA-loxP-nup100ΔGLFG T7-loxP-nup49ΔGLFG + pSW125	This study
SWY3292	trp1-1 lys2 ura3 leu2 his3 HA-loxP-nup42ΔFG myc-loxP-nup159ΔFG T7-loxP-nup1ΔFXFG myc-loxP-nup2ΔFXFG myc-loxP-nup60ΔFXF HA-loxP-nup100ΔGLFG myc-loxP-nup145ΔGLFG	This study
SWY3304	trp1-1 lys2 ura3 leu2 his3 HA-LoxP-nup42ΔFG myc-LoxP-nup159ΔFG myc-LoxP-nup2ΔFxFG myc-LoxP-nup60ΔFxF T7-LoxP-nup1ΔFxFG myc-LoxP-nup57ΔGLFG	This study
SWY3367	trp1-1 LYS2 ura3 leu2 his3 HA-LoxP-nup42ΔFG myc-LoxP-nup159ΔFG T7-LoxP-nup1ΔFxFG myc-LoxP-nup2ΔFxFG myc-LoxP-nup60ΔFxF HA-LoxP-nup100ΔGLFG mex67-GFP:HIS5	This study
SWY3368	trp1-1 LYS2 ura3 leu2 his3 HA-LoxP-nup42ΔFG myc-LoxP-nup159ΔFG T7-LoxP-nup1ΔFxFG myc-LoxP-nup2ΔFxFG myc-LoxP-nup60ΔFxF HA-LoxP-nup100ΔGLFG mex67-GFP:HIS5	This study
SWY3369	trp1-1 lys2 ura3 leu2 his3 HA-LoxP-nup42ΔFG myc-LoxP-nup159ΔFG T7-LoxP-nup1ΔFxFG myc-LoxP-nup2ΔFxFG myc-LoxP-nup60ΔFxF HA-LoxP-nup100ΔGLFG mex67-GFP:HIS5	This study
SWY3370	trp1-1 LYS2 ura3 leu2 his3 HA-LoxP-nup42ΔFG myc-LoxP-nup159ΔFG T7-LoxP-nup1ΔFxFG myc-LoxP-nup2ΔFxFG myc-LoxP-nup60ΔFxF HA-LoxP-nup100ΔGLFG kap104-GFP:HIS5	This study
SWY3371	trp1-1 lys2 ura3 leu2 his3 HA-LoxP-nup42ΔFG myc-LoxP-nup159ΔFG T7-LoxP-nup1ΔFxFG myc-LoxP-nup2ΔFxFG myc-LoxP-nup60ΔFxF HA-LoxP-nup100ΔGLFG kap104-GFP:HIS5	This study
SWY3372	trp1-1 lys2 ura3 leu2 his3 HA-LoxP-nup42ΔFG myc-LoxP-nup159ΔFG T7-LoxP-nup1ΔFxFG myc-LoxP-nup2ΔFxFG myc-LoxP-nup60ΔFxF HA-LoxP-nup100ΔGLFG kap104-GFP:HIS5	This study
SWY3373	trp1-1 LYS2 ura3 leu2 his3 HA-LoxP-nup42ΔFG myc-LoxP-nup159ΔFG T7-LoxP-nup1ΔFxFG myc-LoxP-nup2ΔFxFG myc-LoxP-nup60ΔFxF kap104-GFP:HIS5	This study
SWY3374	trp1-1 lys2 ura3 leu2 his3 HA-LoxP-nup42ΔFG myc-LoxP-nup159ΔFG T7-LoxP-nup1ΔFxFG myc-LoxP-nup2ΔFxFG myc-LoxP-nup60ΔFxF kap104-GFP:HIS5	This study
SWY3375	trp1-1 LYS2 ura3 leu2 his3 HA-LoxP-nup42ΔFG myc-LoxP-nup159ΔFG T7-LoxP-nup1ΔFxFG myc-LoxP-nup2ΔFxFG myc-LoxP-nup60ΔFxF kap104-GFP:HIS5	This study
SWY3399	trp1-1 LYS2 ura3 leu2 his3 HA-LoxP-nup42ΔFG myc-LoxP-nup159ΔFG T7-LoxP-nup1ΔFxFG myc-LoxP-nup2ΔFxFG myc-LoxP-nup60ΔFxF myc-LoxP-nup57ΔGLFG	This study
SWY3400	trp1-1 LYS2 leu2 ura3 his3 T7-LoxP-nup1ΔFxFG flag-LoxP-nsp1ΔFGΔFxFG	This study

SWY3401	TRP1 lys2 ura3 his3 leu2 T7-LoxP-nup1ΔFxFG flag-LoxP-nsp1ΔFGΔFxFG	This study
SWY3402	TRP1 lys2 leu2 ura3 his3 myc-LoxP-nup159ΔFG flag-LoxP-nsp1ΔFGΔFxFG	This study
SWY3403	TRP1 lys2 leu2 ura3 his3 myc-LoxP-nup159ΔFG flag-LoxP-nsp1ΔFGΔFxFG	This study
SWY3404	trp1-1 LYS2 ura3 leu2 his3 myc-LoxP-nup159ΔFG flag-LoxP-nsp1ΔFGΔFxFG	This study
SWY3405	trp1-1 LYS2 leu2 ura3 his3 HA-LoxP-nup42ΔFG flag-LoxP-nsp1ΔFGΔFxFG	This study
SWY3406	TRP1 lys2 ura3 his3 leu2 HA-LoxP-nup42ΔFG flag-LoxP-nsp1ΔFGΔFxFG	This study
SWY3410	trp1-1, LYS2, leu2, ura3, his3, HA-LoxP-nup42ΔFG, myc-LoxP-nup159ΔFG, T7-LoxP-nup1ΔFxFG, myc-LoxP-nup2ΔFxFG, myc-LoxP-nup60ΔFxF, myc-LoxP-nup57ΔGLFG	This study
SWY3420	TRP/trp1-1 LYS2/lys2 leu2/leu2 ura3/ura3 his3/his3 T7-LoxP-nup1ΔFxFG/T7-LoxP-nup1ΔFxFG flag-LoxP-nsp1ΔFGΔFxFG/flag-LoxP-nsp1ΔFGΔFxFG	This study
SWY3421	TRP/trp1-1 LYS2/lys2 leu2/leu2 ura3/ura3 his3/his3 myc-LoxP-nup159ΔFG/myc-LoxP-nup159ΔFG flag-LoxP-nsp1ΔFGΔFxFG/flag-LoxP-nsp1ΔFGΔFxFG	This study
SWY3422	TRP1/trp1-1 LYS2/lys2 ura3/ura3 his3/his3 leu2/leu2 HA-LoxP-nup42ΔFG/HA-LoxP-nup42ΔFG flag-LoxP-nsp1ΔFGΔFxFG/flag-LoxP-nsp1ΔFGΔFxFG	This study
SWY3423	TRP1 lys2 ura3 his3 leu2 HA-LoxP-nup42ΔFG myc-LoxP-nup159ΔFG flag-LoxP-nsp1ΔFGΔFxFG	This study
SWY3424	TRP1 lys2 ura3 his3 leu2 HA-LoxP-nup42ΔFG myc-LoxP-nup159ΔFG flag-LoxP-nsp1ΔFGΔFxFG	This study
SWY3427	trp1-1 LYS2 ura3 his3 leu2 HA-LoxP-nup100ΔGLFG flag-LoxP-nsp1ΔFGΔFxFG	This study
SWY3428	TRP1 lys2 leu2 ura3 his3 HA-LoxP-nup100ΔGLFG flag-LoxP-nsp1ΔFGΔFxFG	This study
SWY3429	trp1-1 LYS2 leu2 ura3 his3 HA-LoxP-nup100ΔGLFG flag-LoxP-nsp1ΔFGΔFxFG	This study
SWY3442	trp1-1 LYS2 ura3 his3 leu2 T7-LoxP-nup1ΔFxFG HA-LoxP-nsp1ΔFxFG	This study
SWY3443	rp1-1 LYS2 ura3 his3 leu2 T7-LoxP-nup1ΔFxFG HA-LoxP-nsp1ΔFxFG	This study
SWY3444	trp1-1 LYS2 ura3 his3 leu2 HA-LoxP-nsp1ΔFxFG myc-LoxP-nup159ΔFG	This study
SWY3445	trp1-1 LYS2 ura3 his3 leu2 HA-LoxP-nsp1ΔFxFG myc-LoxP-nup159ΔFG	This study
SWY3446	TRP1 lys2 ura3 his3 leu2 HA-LoxP-nsp1ΔFxFG myc-LoxP-	This study

	nup159ΔFG	
SWY3447	TRP1 lys2 ura3 his3 leu2 HA-LoxP-nsp1ΔFxFG myc-LoxP-nup159ΔFG	This study
SWY3448	trp1-1 LYS2 ura3 his3 leu2 HA-LoxP-nup42ΔFG HA-LoxP-nsp1ΔFxFG	This study
SWY3449	TRP1 lys2 ura3 his3 leu2 HA-LoxP-nup42ΔFG HA-LoxP-nsp1ΔFxFG	This study
SWY3462	trp1-1 lys2 leu2 ura3 his3 HA-LoxP-nup42ΔFG myc-LoxP-nup159ΔFG T7-LoxP-nup1ΔFxFG myc-LoxP-nup2ΔFxFG myc-LoxP-nup60ΔFxF myc-LoxP-nup145ΔGLFG	This study
SWY3498	TRP1/trp1 LYS2/lys2 leu2/leu2 ura3/ura3 his3/his3 myc-LoxP-nup145ΔGLFG/myc-LoxP-nup145ΔGLFG T7-LoxP-nup116ΔGLFG/T7-LoxP-nup116ΔGLFG	This study
SWY3499	TRP1/trp1-1 LYS2/lys2 ura3/ura3 his3/his3 leu2/leu2 HA-LoxP-nup100ΔGLFG/HA-LoxP-nup100ΔGLFG flag-LoxP-nsp1ΔFGΔFxFG/flag-LoxP-nsp1ΔFGΔFxFG	This study
SWY3584	TRP1 LYS2 leu2 ura3 his3 T7-LoxP-nup1ΔFXFG myc-LoxP-nup2ΔFXFG myc-LoxP-nup57ΔGLFG	This study
SWY3585	TRP1 lys2 leu2 ura3 his3 T7-LoxP-nup1ΔFXFG myc-LoxP-nup2ΔFXFG myc-LoxP-nup57ΔGLFG	This study
SWY3586	TRP1 lys2 leu2 ura3 his3 T7-LoxP-nup1ΔFXFG myc-LoxP-nup2ΔFXFG HA-LoxP-nup100ΔGLFG	This study
SWY3587	TRP1 LYS2 leu2 ura3 his3 T7-LoxP-nup1ΔFXFG myc-LoxP-nup2ΔFXFG HA-LoxP-nup100ΔGLFG	This study
SWY3588	Trp1 LYS2 leu2 ura3 his3 T7-LoxP-nup1ΔFXFG myc-LoxP-nup2ΔFXFG T7-LoxP-nup49ΔGLFG	This study
SWY3589	TRP1 lys2 leu2 ura3 his3 T7-LoxP-nup1ΔFXFG myc-LoxP-nup2ΔFXFG myc-LoxP-nup145ΔGLFG	This study
SWY3603	trp1-1 LYS2 ura3-1 leu2-3,112 his3-11,15 HA-LoxP-nup42ΔFG myc-LoxP-nup159ΔFG T7-LoxP-nup1ΔFXFG myc-LoxP-nup2ΔFXFG myc-LoxP-nup60ΔFXF T7-LoxP-nup116ΔGLFG	This study
SWY3618	TRP1 lys2 leu2 ura3 his3 myc-LoxP-nup57ΔGLFG myc-LoxP-nup2ΔFXFG myc-LoxP-nup60ΔFXF T7-LoxP-nup1ΔFXFG	This study
SWY3619	trp1 LYS2 leu2 ura3 his3 myc-LoxP-nup57ΔGLFG myc-LoxP-nup2ΔFXFG myc-LoxP-nup60ΔFXF T7-LoxP-nup1ΔFXFG	This study
SWY3620	TRP1 lys2 leu2 ura3 his3 myc-LoxP-nup57ΔGLFG myc-LoxP-nup2ΔFXFG myc-LoxP-nup60ΔFXF T7-LoxP-nup1ΔFXFG	This study
SWY3621	TRP1 lys2 leu2 ura3 his3 myc-LoxP-nup159ΔFG HA-LoxP-nup42ΔFG myc-LoxP-nup57ΔGLFG	This study
SWY3622	trp1 LYS2 leu2 ura3 his3 myc-LoxP-nup159ΔFG HA-LoxP-nup42ΔFG myc-LoxP-nup57ΔGLFG	This study
SWY3623	TRP1 LYS2 leu2 ura3 his3 T7-LoxP-nup1ΔFXFG myc-LoxP-nup57ΔGLFG	This study

SWY3624	trp1 LYS2 leu2 ura3 his3 T7-LoxP-nup1ΔFXFG myc-LoxP-nup57ΔGLFG	This study
SWY3625	trp1 LYS2 leu2 ura3 his3 T7-LoxP-nup1ΔFXFG myc-LoxP-nup57ΔGLFG	This study
SWY3626	TRP1 LYS2 leu2 ura3 his3 T7-LoxP-nup1ΔFXFG myc-LoxP-nup57ΔGLFG	This study
SWY3627	TRP1 lys2 leu2 hura3 his3 myc-LoxP-nup2ΔFXFG myc-LoxP-nup57ΔGLFG	This study
SWY3628	trp1 LYS2 leu2 hura3 his3 myc-LoxP-nup2ΔFXFG myc-LoxP-nup57ΔGLFG	This study
SWY3629	TRP1 lys2 leu2 hura3 his3 myc-LoxP-nup2ΔFXFG myc-LoxP-nup57ΔGLFG	This study
SWY3630	trp1 LYS2 leu2 ura3 his3 myc-LoxP-nup159ΔFG HA-LoxP-nup42ΔFG T7-LoxP-nup116ΔGLFG	This study
SWY3631	trp1 LYS2 leu2 ura3 his3 myc-LoxP-nup159ΔFG HA-LoxP-nup42ΔFG T7-LoxP-nup116ΔGLFG	This study
SWY3632	TRP1 lys2 leu2 ura3 his3 myc-LoxP-nup159ΔFG HA-LoxP-nup42ΔFG T7-LoxP-nup116ΔGLFG	This study
SWY3633	TRP1 lys2 leu2 ura3 his3 myc-LoxP-nup159ΔFG HA-LoxP-nup42ΔFG T7-LoxP-nup116ΔGLFG	This study
SWY3634	TRP1 lys2 leu2 ura3 his3 T7-LoxP-nup1ΔFXFG myc-LoxP-nup2ΔFXFG myc-LoxP-nup60ΔFXF T7-LoxP-nup116ΔGLFG	This study
SWY3635	TRP1 lys2 leu2 ura3 his3 T7-LoxP-nup1ΔFXFG myc-LoxP-nup2ΔFXFG myc-LoxP-nup60ΔFXF T7-LoxP-nup116ΔGLFG	This study
SWY3636	TRP1 lys2 leu2 ura3 his3 myc-LoxP-nup2ΔFXFG myc-LoxP-nup60ΔFXF T7-LoxP-nup116ΔGLFG	This study
SWY3637	TRP1 lys2 leu2 ura3 his3 myc-LoxP-nup2ΔFXFG myc-LoxP-nup60ΔFXF T7-LoxP-nup116ΔGLFG	This study
SWY3701	ura3-1 his3-11,15 trp1-1 leu2-3 LYS2 Mex67-GFP::HIS5 trp1::dsRed-HDEL:TRP1	This study
SWY3702	trp1-1 lys2 leu2 ura3 his3 HA-LoxP-nup42ΔFG myc-LoxP-nup159ΔFG T7-LoxP-nup1ΔFXFG myc-LoxP-nup2ΔFXFG myc-LoxP-nup60ΔFXF Mex67-GFP::HIS trp1::dsred-HDEL:TRP1	This study
SWY3703	trp1-1 LYS2 leu2 ura3 his3 HA-LoxP-nup42ΔFG myc-LoxP-nup159ΔFG T7-LoxP-nup1ΔFXFG myc-LoxP-nup2ΔFXFG myc-LoxP-nup60ΔFXF myc-LoxP-nup57ΔGLFG Mex67-GFP::HIS trp1::dsred-HDEL:TRP1	This study
SWY3704	trp1-1 LYS2 leu2 ura3 his3 HA-LoxP-nup42ΔFG myc-LoxP-nup159ΔFG T7-LoxP-nup1ΔFXFG myc-LoxP-nup2ΔFXFG myc-LoxP-nup60ΔFXF T7-LoxP-nup116ΔGLFG Mex67-GFP::HIS trp1::dsred-HDEL:TRP1	This study
SWY3705	trp1 leu2 his3 ura3 lys2 HA-LoxP-nup42ΔFG myc-LoxP-nup159ΔGLFG T7-LoxP-nup1ΔFXFG myc-LoxP-nup2ΔFXFG	This study

	myc-LoxP-nup60ΔFXF T7-LoxP-nup49ΔGLFG nup49GLFG::GFP:TRP1 (pSW442 integrated)	
SWY3706	trp1 leu2 his3 ura3 lys2 HA-LoxP-nup42ΔFG myc-LoxP-nup159ΔGLFG T7-LoxP-nup1ΔFXFG myc-LoxP-nup2ΔFXFG myc-LoxP-nup60ΔFXF T7-LoxP-nup49ΔGLFG nup49GLFG::GFP:TRP1 (pSW442 integrated)	This study
SWY3708	trp1-1 LYS2 leu2 ura3 his3 myc-LoxP-nup2ΔFXFG T7-LoxP-nup1ΔFXFG nsp1FXFG::FXFGnup1-myc:HIS5 (sp)	This study
SWY3709	TRP1 lys2 leu2 ura3 his3 myc-LoxP-nup2ΔFXFG T7-LoxP-nup1ΔFXFG nsp1FXFG::FXFGnup1-myc:HIS5 (sp)	This study
SWY3710	trp1 LYS2 leu2 ura3 his3 myc-LoxP-nup2ΔFXFG T7-LoxP-nup1ΔFXFG nsp1FXFG::FXFGnup1-myc:HIS5 (sp)	This study
SWY3711	TRP1 lys2 leu2 ura3 his3 myc-LoxP-nup2ΔFXFG T7-LoxP-nup1ΔFXFG nsp1FXFG::FXFGnup1-myc:HIS5 (sp)	This study
SWY3712	TRP1 lys2 leu2 ura3 his3 myc-LoxP-nup2ΔFXFG T7-LoxP-nup1ΔFXFG nsp1FXFG::FXFGnsp1-myc:HIS5 (sp)	This study
SWY3713	trp1 LYS2 leu2 ura3 his3 myc-LoxP-nup2ΔFXFG T7-LoxP-nup1ΔFXFG nsp1FXFG::FXFGnsp1-myc:HIS5 (sp)	This study
SWY3714	TRP1 lys2 leu2 ura3 his3 myc-LoxP-nup2ΔFXFG T7-LoxP-nup1ΔFXFG nsp1FXFG::FXFGnsp1-myc:HIS5 (sp)	This study
SWY3715	trp1 LYS2 leu2 ura3 his3 myc-LoxP-nup2ΔFXFG T7-LoxP-nup1ΔFXFG nsp1FXFG::FXFGnsp1-myc:HIS5 (sp)	This study
SWY3755	trp1-1 lys2 ura3 leu2 his3 HA-loxP-nup42ΔFG myc-loxP-nup159ΔFG T7-loxP-nup1ΔFXFG myc-loxP-nup2ΔFXFG myc-loxP-nup60ΔFXF T7-loxP-nup49ΔGLFG + pSW125 + CP25	This study
SWY3756	trp1-1 lys2 ura3 leu2 his3 HA-loxP-nup42ΔFG myc-loxP-nup159ΔFG T7-loxP-nup1ΔFXFG myc-loxP-nup2ΔFXFG myc-loxP-nup60ΔFXF T7-loxP-nup49ΔGLFG + pSW125 + pSW3158 (LEU2/CEN/T7-LoxP-nup49ΔGLFG)	This study
SWY3757	trp1-1 lys2 ura3 leu2 his3 HA-loxP-nup42ΔFG myc-loxP-nup159ΔFG T7-loxP-nup1ΔFXFG myc-loxP-nup2ΔFXFG myc-loxP-nup60ΔFXF T7-loxP-nup49ΔGLFG + pSW116	This study
SWY3758	trp1-1 lys2 ura3 leu2 his3 HA-loxP-nup42ΔFG myc-loxP-nup159ΔFG T7-loxP-nup1ΔFXFG myc-loxP-nup2ΔFXFG myc-loxP-nup60ΔFXF T7-loxP-nup49ΔGLFG + pSW117	This study
SWY3759	trp1-1 lys2 ura3 leu2 his3 HA-loxP-nup42ΔFG myc-loxP-nup159ΔFG T7-loxP-nup1ΔFXFG myc-loxP-nup2ΔFXFG myc-loxP-nup60ΔFXF T7-loxP-nup49ΔGLFG + pSW242	This study
SWY3760	trp1-1 lys2 ura3 leu2 his3 HA-loxP-nup42ΔFG myc-loxP-nup159ΔFG T7-loxP-nup1ΔFXFG myc-loxP-nup2ΔFXFG myc-loxP-nup60ΔFXF T7-loxP-nup49ΔGLFG + pSW3259	This study
SWY3761	trp1-1 lys2 ura3 leu2 his3 HA-loxP-nup42ΔFG myc-loxP-nup159ΔFG T7-loxP-nup1ΔFXFG myc-loxP-nup2ΔFXFG myc-	This study

	loxP-nup60ΔFXF T7-loxP-nup49ΔGLFG + pSW3260	
SWY3762	trp1-1 lys2 ura3 leu2 his3 HA-loxP-nup42ΔFG myc-loxP-nup159ΔFG T7-loxP-nup1ΔFXFG myc-loxP-nup2ΔFXFG myc-loxP-nup60ΔFXF T7-loxP-nup49ΔGLFG + pSW3261	This study
SWY3763	trp1-1 lys2 ura3 leu2 his3 HA-loxP-nup42ΔFG myc-loxP-nup159ΔFG T7-loxP-nup1ΔFXFG myc-loxP-nup2ΔFXFG myc-loxP-nup60ΔFXF T7-loxP-nup49ΔGLFG + pSW3262	This study
SWY3764	trp1-1 lys2 ura3 leu2 his3 HA-loxP-nup42ΔFG myc-loxP-nup159ΔFG T7-loxP-nup1ΔFXFG myc-loxP-nup2ΔFXFG myc-loxP-nup60ΔFXF T7-loxP-nup49ΔGLFG + pSW3263	This study
SWY3765	trp1-1 lys2 ura3 leu2 his3 HA-loxP-nup42ΔFG myc-loxP-nup159ΔFG T7-loxP-nup1ΔFXFG myc-loxP-nup2ΔFXFG myc-loxP-nup60ΔFXF T7-loxP-nup49ΔGLFG + pSW3264	This study
SWY3854	trp1 LYS2 leu2 ura3 his3 myc-LoxP-nup57ΔGLFG myc-LoxP-nup60ΔFXF T7-LoxP-nup1ΔFXFG	This study
SWY3855	trp1 LYS2 leu2 ura3 his3 myc-LoxP-nup57ΔGLFG myc-LoxP-nup60ΔFXF T7-LoxP-nup1ΔFXFG	This study
SWY3856	TRP1 lys2 leu2 ura3 his3 myc-LoxP-nup57ΔGLFG myc-LoxP-nup60ΔFXF T7-LoxP-nup1ΔFXFG	This study
SWY3857	TRP1 lys2 leu2 ura3 his3 myc-LoxP-nup57ΔGLFG myc-LoxP-nup60ΔFXF T7-LoxP-nup1ΔFXFG	This study
SWY3858	trp1 LYS2 leu2 ura3 his3 myc-LoxP-nup57ΔGLFG myc-LoxP-nup60ΔFXF myc-LoxP-nup2ΔFXFG	This study
SWY3859	TRP1 lys2 leu2 ura3 his3 myc-LoxP-nup57ΔGLFG myc-LoxP-nup60ΔFXF myc-LoxP-nup2ΔFXFG	This study
SWY3860	trp1 LYS2 leu2 ura3 his3 myc-LoxP-nup57ΔGLFG myc-LoxP-nup60ΔFXF myc-LoxP-nup2ΔFXFG	This study
SWY3861	TRP1 lys2 leu2 ura3 his3 myc-LoxP-nup57ΔGLFG myc-LoxP-nup60ΔFXF	This study
SWY3862	trp1 LYS2 leu2 ura3 his3 myc-LoxP-nup57ΔGLFG myc-LoxP-nup60ΔFXF	This study
SWY3863	trp1 LYS2 leu2 ura3 his3 myc-LoxP-nup57ΔGLFG myc-LoxP-nup60ΔFXF	This study
SWY3888	TRP1 lys2 ura3 leu2 his3 myc-LoxP-Nup57ΔGLFG T7-LoxP-Nup49ΔGLFG pSW3261 (Leu2 nup49ΔGLFG)	This study
SWY3889	trp1 LYS2 leu2 ura3 his3 myc-LoxP-nup57ΔGLFG myc-LoxP-nup2ΔFXFG myc-LoxP-nup60ΔFXF T7-LoxP-nup1ΔFXFG mex67-GFP:HIS3 trp1::dsRED-HDEL:TRP1	This study
SWY3899	TRP1 ura3 leu2 his3 lys2 myc-LoxP-nup60ΔFXF	This study
SWY3900	trp1 ura3 leu2 his3 LYS2 myc-LoxP-nup60ΔFXF	This study
SWY3901	trp1/TRP1 ura3/ura3 leu2/leu2 his3/his3 LYS2/lys2 myc-LoxP-nup60ΔFXF/myc-LoxP-nup60ΔFXF	This study
SWY3925	TRP1 lys2 ura3 leu2 his3 T7-LoxP-nup1ΔFXFG myc-LoxP-	This study

	nup2ΔFXFG myc-LoxP-nup60ΔFXF myc-LoxP-nup57ΔGLFG myc-LoxP-nup159ΔGLFG	
SWY3926	trp1 LYS2 ura3 leu2 his3 T7-LoxP-nup1ΔFXFG myc-LoxP-nup2ΔFXFG myc-LoxP-nup60ΔFXF myc-LoxP-nup57ΔGLFG myc-LoxP-nup159ΔGLFG	This study
SWY3927	TRP1 LYS2 ura3 leu2 his3 T7-LoxP-nup1ΔFXFG myc-LoxP-nup2ΔFXFG myc-LoxP-nup60ΔFXF myc-LoxP-nup57ΔGLFG HA-LoxP-nup42ΔFG	This study
SWY3928	trp1 LYS2 ura3 leu2 his3 T7-LoxP-nup1ΔFXFG myc-LoxP-nup2ΔFXFG myc-LoxP-nup60ΔFXF myc-LoxP-nup57ΔGLFG HA-LoxP-nup42ΔFG	This study
SWY3929	trp1 lys2 ura3 leu2 his3 T7-LoxP-nup1ΔFXFG myc-LoxP-nup2ΔFXFG myc-LoxP-nup60ΔFXF myc-LoxP-nup57ΔGLFG myc-LoxP-nup159ΔGLFG HA-LoxP-nup42ΔFG	This study
SWY3930	trp1 lys2 ura3 leu2 his3 T7-LoxP-nup1ΔFXFG myc-LoxP-nup2ΔFXFG myc-LoxP-nup60ΔFXF myc-LoxP-nup57ΔGLFG myc-LoxP-nup159ΔGLFG HA-LoxP-nup42ΔFG	This study
SWY3932	ura3-1/ ura3-1 his3-11,15/his3-11,15 TRP1/trp1-1 leu2-3,112/leu2-3,112 LYS2/lys2 MTR2/mtr2::KANMX4	This study
SWY3933	trp1-1 LYS2 ura3-1 leu2-3,112 his3-11,15	This study
SWY3934	TRP1 lys2 ura3-1 leu2-3,112 his3-11,15	This study
SWY3935	trp1 lys2 ade2 ura3 his3 leu2 mex67::HIS3 CP361 (pRS316-MEX67)	This study
SWY3936	trp1 lys2 ade2 ura3 his3 leu2 mex67::HIS3 CP361 (pRS316-MEX67)	This study
SWY3937	TRP1 lys2 leu2 ura3 his3 T7-LoxP-nup1ΔFXFG myc-LoxP-nup2ΔFXFG myc-LoxP-nup60ΔFXF myc-LoxP-nup57ΔGLFG myc-LoxP-nup159ΔFG	This study
SWY3938	trp1 LYS2 leu2 ura3 his3 T7-LoxP-nup1ΔFXFG myc-LoxP-nup2ΔFXFG myc-LoxP-nup60ΔFXF myc-LoxP-nup57ΔGLFG myc-LoxP-nup159ΔFG	This study
SWY3939	trp1 LYS2 leu2 ura3 his3 T7-LoxP-nup1ΔFXFG myc-LoxP-nup2ΔFXFG myc-LoxP-nup60ΔFXF myc-LoxP-nup57ΔGLFG myc-LoxP-nup159ΔFG	This study
SWY3940	TRP1 LYS2 leu2 ura3 his3 T7-LoxP-nup1ΔFXFG myc-LoxP-nup2ΔFXFG myc-LoxP-nup60ΔFXF myc-LoxP-nup57ΔGLFG HA-LoxP-nup42ΔFG	This study
SWY3941	trp1 LYS2 leu2 ura3 his3 T7-LoxP-nup1ΔFXFG myc-LoxP-nup2ΔFXFG myc-LoxP-nup60ΔFXF myc-LoxP-nup57ΔGLFG HA-LoxP-nup42ΔFG	This study
SWY3942	TRP1 lys2 leu2 ura3 his3 T7-LoxP-nup1ΔFXFG myc-LoxP-nup2ΔFXFG myc-LoxP-nup60ΔFXF myc-LoxP-nup57ΔGLFG HA-LoxP-nup42ΔFG	This study

SWY3943	trp1 lys2 leu2 ura3 his3 T7-LoxP-nup1ΔFXFG myc-LoxP-nup2ΔFXFG myc-LoxP-nup60ΔFXF myc-LoxP-nup57ΔGLFG HA-LoxP-nup42ΔFG myc-LoxP-nup159ΔFG	This study
SWY3944	trp1 lys2 leu2 ura3 his3 T7-LoxP-nup1ΔFXFG myc-LoxP-nup2ΔFXFG myc-LoxP-nup60ΔFXF myc-LoxP-nup57ΔGLFG HA-LoxP-nup42ΔFG myc-LoxP-nup159ΔFG	This study
SWY3945	TRP1 LYS2 leu2 ura3 his3 T7-LoxP-nup1ΔFXFG myc-LoxP-nup2ΔFXFG myc-LoxP-nup60ΔFXF myc-LoxP-nup57ΔGLFG HA-LoxP-nup42ΔFG myc-LoxP-nup159ΔFG	This study
SWY3970	ura3-1 his3-11,15 trp1-1 leu2-3,112 lys2 mtr2::KAN pRS316-MTR2-(URA3-CEN)	This study
SWY3971	ura3-1 his3-11,15 trp1-1 leu2-3,112 lys2 mtr2::KAN pRS314-MTR2-(TRP1-CEN)	This study
SWY3972	ura3-1 his3-11,15 trp1-1 leu2-3,112 lys2 mtr2::KAN pRS314-mtr2-33-(TRP1-CEN)	This study
SWY3973	ura3-1 his3-11,15 trp1-1 leu2-3,112 LYS2 ADE2 gle 1-2 mtr2::KAN pRS316-MTR2-(URA3-CEN)	This study
SWY3974	ura3-1 his3-11,15 trp1-1 leu2-3,112 LYS2 ADE2 gle 1-2 mtr2::KAN pRS314-MTR2-(TRP1-CEN)	This study
SWY3975	ura3-1 his3-11,15 trp1-1 leu2-3,112 LYS2 ADE2 gle 1-2 mtr2::KAN pRS314-mtr2-33-(TRP1-CEN)	This study
SWY3976	ura3-1 his3-11,15 trp1-1 leu2-3,112 lys2 ADE2 gle 1-4 mtr2::KAN pRS316-MTR2 (URA3-CEN)	This study
SWY3977	ura3-1 his3-11,15 trp1-1 leu2-3,112 lys2 ADE2 gle 1-4 mtr2::KAN pRS314-MTR2 (TRP1-CEN)	This study
SWY3978	ura3-1 his3-11,15 trp1-1 leu2-3,112 lys2 ADE2 gle 1-4 mtr2::KAN pRS314-mtr2-33 (TRP1-CEN)	This study
SWY3979	ura3-1 his3-11,15 trp1-1 leu2-3,112 lys2 ADE2 ipk1::KAN mtr2::KAN pRS316-MTR2 (URA3-CEN)	This study
SWY3980	ura3-1 his3-11,15 trp1-1 leu2-3,112 lys2 ADE2 ipk1::KAN mtr2::KAN pRS314-MTR2 (TRP1-CEN)	This study
SWY3981	ura3-1 his3-11,15 trp1-1 leu2-3,112 lys2 ADE2 ipk1::KAN mtr2::KAN pRS314-mtr2-33 (TRP1-CEN)	This study
SWY3982	ura3-1 his3-11,15 trp1-1 leu2-3,112 lys2 ADE2 mex67::HIS3 pRS316-MEX67 (URA3-CEN)	This study
SWY4001	TRP1 leu2 lys2 ura3 his3 T7-LoxP-nup1ΔFXFG myc-LoxP-nup2ΔFXFG myc-LoxP-nup60ΔFXF nup57::KAN pSW3006 (URA3-CEN-NUP57)	This study
SWY4002	trp1 leu2 LYS2 ura3 his3 T7-LoxP-nup1ΔFXFG myc-LoxP-nup2ΔFXFG myc-LoxP-nup60ΔFXF nup57::KAN pSW3006 (URA3-CEN-NUP57)	This study
SWY4062	TRP1 lys2 leu2 ura3 his3 myc-LoxP-nup2ΔFXFG myc-LoxP-nup60ΔFXF T7-LoxP-nup1ΔFXFG myc-LoxP-nup57ΔGLFG	This study

	nup42::HIS	
SWY4063	TRP1 lys2 leu2 ura3 his3 myc-LoxP-nup2ΔFXFG myc-LoxP-nup60ΔFXF T7-LoxP-nup1ΔFXFG myc-LoxP-nup57ΔGLFG nup42::HIS	This study
SWY4064	trp1 LYS2 leu2 ura3 his3 myc-LoxP-nup2ΔFXFG myc-LoxP-nup60ΔFXF T7-LoxP-nup1ΔFXFG nup57::KAN CP36 (pCEN-TRP) pSW3006 (pURA3-CEN-NUP57)	This study
SWY4065	trp1 LYS2 leu2 ura3 his3 myc-LoxP-nup2ΔFXFG myc-LoxP-nup60ΔFXF T7-LoxP-nup1ΔFXFG nup57::KAN pSW3431 (pTRP1-CEN-NUP57)	This study
SWY4066	trp1 LYS2 leu2 ura3 his3 myc-LoxP-nup2ΔFXFG myc-LoxP-nup60ΔFXF T7-LoxP-nup1ΔFXFG nup57::KAN pSW3432 (pCEN-TRP1-myc-LoxP-nup57ΔGLFG)	This study
SWY4067	trp1 LYS2 leu2 ura3 his3 myc-LoxP-nup2ΔFXFG myc-LoxP-nup60ΔFXF T7-LoxP-nup1ΔFXFG nup57::KAN pSW3434	This study
SWY4068	trp1 LYS2 leu2 ura3 his3 myc-LoxP-nup2ΔFXFG myc-LoxP-nup60ΔFXF T7-LoxP-nup1ΔFXFG nup57::KAN pSW3435	This study
SWY4069	trp1 LYS2 leu2 ura3 his3 myc-LoxP-nup2ΔFXFG myc-LoxP-nup60ΔFXF T7-LoxP-nup1ΔFXFG nup57::KAN pSW3436	This study
SWY4070	trp1 LYS2 leu2 ura3 his3 myc-LoxP-nup2ΔFXFG myc-LoxP-nup60ΔFXF T7-LoxP-nup1ΔFXFG nup57::KAN pSW3437	This study
SWY4071	trp1 LYS2 leu2 ura3 his3 myc-LoxP-nup2ΔFXFG myc-LoxP-nup60ΔFXF T7-LoxP-nup1ΔFXFG nup57::KAN pSW3438	This study
SWY4072	SWY3001 struck to isolate yeast away from the multiple types of bacteria that contaminate the original SWY3001 perm stock.	This study
SWY4129	TRP1 lys2 leu2 ura3 his3 myc-LoxP-nup57ΔGLFG myc-LoxP-nup2ΔFXFG myc-LoxP-nup60ΔFXF T7-LoxP-nup1ΔFXFG nup42::HIS3	This study
SWY4130	TRP1 lys2 leu2 ura3 his3 myc-LoxP-nup57ΔGLFG myc-LoxP-nup2ΔFXFG myc-LoxP-nup60ΔFXF T7-LoxP-nup1ΔFXFG nup42::HIS3	This study
SWY4131	trp1 leu2 LYS2 ura3 his3 T7-LoxP-nup1ΔFXFG myc-LoxP-nup2ΔFXFG myc-LoxP-nup60ΔFXF nup57::KAN pSW3432 (myc-LoxP-nup57ΔGLFG) Mex67-GFP:HIS3	This study
SWY4132	trp1 leu2 LYS2 ura3 his3 T7-LoxP-nup1ΔFXFG myc-LoxP-nup2ΔFXFG myc-LoxP-nup60ΔFXF nup57::KAN pSW3432 (myc-LoxP-nup57ΔGLFG) Mex67-GFP:HIS5	This study
SWY4133	trp1/TRP1 leu2/leu2 LYS2/lys2 ura3/ura3 his3/his3 T7-LoxP-nup1ΔFXFG/T7-LoxP-nup1ΔFXFG myc-LoxP-nup2ΔFXFG/myc-LoxP-nup2ΔFXFG myc-LoxP-nup60ΔFXF/myc-LoxP-nup60ΔFXF nup49::ura3::KAN/NUP49	This study
SWY4134	trp1 leu2 LYS2 ura3 his3 T7-LoxP-nup1ΔFXFG myc-LoxP-	This study

	nup2ΔFXFG myc-LoxP-nup60ΔFXF nup49::ura3::KAN pSW125	
SWY734	GFP-Nup49Cterm (TRP) NUP49Δ::URA3 ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100	(BUCCI and WENTE 1997)

* Unless otherwise noted, strains carry auxotrophic markers *ura3-1; his3-11,15; leu2-3,112; trp1-1* OR *TRP1; lys2* OR *LYS2*

Table A-2. List of plasmids used in this study.

Plasmid Name	Vector/Backbone	Gene Cloned	Modification to gene	Reference
pAC1075	pRS425	KAP104		Anita Corbett, unpublished
pEB0836	pRS316	PHO4 NLS fused to three GFP	under PHO4 promoter	(KAFFMAN *et al.* 1998)
pET-Duet	pET-Duet			Novagen
pGAD-GFP	YEplac195	SV40 cNLS-GFP	under ADH promoter	(SHULGA *et al.* 1996)
pGEX-2T	pGEX-2T	GST	GST	Pharmacia
pGFP-HIS5		GFP-HIS5		J. Aitchison
pKW1803	YIplac204/TKC	dsRed-HDEL	trp1::dsRed-HDEL:TRP1 integration plasmid	(BEVIS *et al.* 2002)
pKW430	pRS426	NLS-NES-GFP	SV40 cNLS and protein kinase inhibitor NES	(STADE *et al.* 1997)
pM3927		ura3::KANR	marker swap plasmid	(VOTH *et al.* 2003)
pNS167	pGFP-N-fus	NAB2 NLS-GFP	NAB2 NLS fused to GFP under MET25 promoter	(SHULGA *et al.* 2000)
pRS313	pRS313		CEN HIS3	(SIKORSKI and HIETER 1989)
pRS314	pRS314		CEN TRP1	(SIKORSKI and HIETER 1989)
pRS315	pRS315		CEN LEU2	(SIKORSKI and HIETER 1989)
pRS316	pRS316		CEN URA3	(SIKORSKI and HIETER 1989)
pRS423	pRS423		2 micron HIS3	(CHRISTIANSON *et al.* 1992)
pRS425	pRS425		2 micron LEU2	(CHRISTIANSON *et al.* 1992)

pSH47	pRS426	Cre recombinase	GAL1 promoter	(GULDENER *et al.* 1996)
pSpo1276–130-GFP	pYX242-GFP	SPO12 NLS-GFP	SPO12 NLS fused to GFP under TPI promoter	(CHAVES and BLOBEL 2001)
pSW116	pRS315	nup49ΔGLFG	Δaa 24-239	(IOVINE *et al.* 1995)
pSW117	pRS315	nup49ΔGLFG	Δaa13-239	(IOVINE *et al.* 1995)
pSW1237	pMal-Cr1	Mex67	MBP-Mex67	(STRAWN *et al.* 2001)
pSW125	pRS316	NUP49	CEN URA3	(WENTE *et al.* 1992)
pSW1297	pGEX-2T	GLFG of *NUP116*	GST fused to GLFG region of *NUP116*	This study
pSW1308	pUG6	$HIS5_{S.pombe}$	HA-LoxP-HIS5-LoxP	(STRAWN *et al.* 2004)
pSW1309	pUG6	$HIS5_{S.pombe}$	Myc-LoxP-HIS5-LoxP	(STRAWN *et al.* 2004)
pSW131	pRS316	NUP116	CEN URA3	(WENTE *et al.* 1992)
pSW1311	pUG6	$HIS5_{S.pombe}$	T7-LoxP-HIS5-LoxP	(STRAWN *et al.* 2004)
pSW1312	pUG6	flag-LoxP-HIS5-LoxP		(STRAWN *et al.* 2004)
pSW132	pRS316	Nup100	CEN URA3	(WENTE *et al.* 1992)
pSW1379	pRS316	NSP1	CEN URA3	Lisa Strawn, unpublished
pSW190	pRS316	NUP145	CEN URA3	(WENTE *et al.* 1992)
pSW3001	pRS315	Cre	Cre recombinase under Gal promoter	This study
pSW3006	pRS316	NUP57	CEN URA3	Lisa Strawn, unpublished
pSW3158	pRS315	nup49	T7-LoxP-nup49ΔGLFG	This study
pSW3159	pRS314	Kap95, Kap104		This study
pSW3204		Nab2 GFP		This study
pSW3206		Mex67 GFP		This study
pSW3259	pSW116, pRS315	Nup49	ΔGLFG (Δaa24-239), F2A	This study
pSW3260	pSW116, pRS315	Nup49	ΔGLFG (Δaa 24-239), F2Y	This study
pSW3261	pSW117, pRS315	Nup49	ΔGLFG (Δaa 13-239), F2A	This study

pSW3261	pRS315	Nup49ΔGLFG	nup49Δaa 13-239, F2A mutation	This study
pSW3262	pSW117, pRS315	Nup49	ΔGLFG (Δaa 13-239), F2Y	This study
pSW3263	pSW242, pRS426	Nup49	F2A	This study
pSW3264	pSW242, pRS426	Nup49	F2Y	This study
pSW3265	pSW1309	Nup42	FG domain of Nup42 in LoxP vector	This study
pSW3266	pSW1309	Nup116	GLFG domain only in Lox vector	This study
pSW3267	pSW1309	Nsp1	FXFG domain	This study
pSW3268	pMAL-cR1	Nup57	GLFG domain fused to MBP	This study
pSW3269	pGEX-2T	Nup57	GLFG domain of Nup57 fused to GST	This study
pSW3269	pGEX-2T	GLFG of *NUP57*	GST fused to GLFG region of *NUP57*	This study
pSW3270	pET-14b	Nup57 GLFGS	GLFGs fused to 6xHIS	This study
pSW3303	pSW1309	Nup57	GLFG	This study
pSW3304	pSW1311	Nup57	GLFGs	This study
pSW3320	pRS423	KAP121	HIS3 2micron	Eric Shows, unpublished
pSW3417	pRS314	Nup57	ΔGLFG	This study
pSW3431	pRS314	NUP57		This study
pSW3432	pRS314	NUP57	myc-LoxP-nup57ΔGLFG	This study
pSW3433	pRS314	NUP57	ΔGLFG	This study
pSW3434	pRS314	NUP57	ΔGLFG	This study
pSW3435	pRS314	NUP57	nup57GLFG::FGNUP42	This study
pSW3436	pRS314	NUP57	nup57ΔGLFG::GLFGNUP57	This study
pSW3437	pRS314	NUp57	nup57ΔGLFG::GLFGNUP116	This study
pSW3438	pRS314	NUP57	nup57ΔGLFG::FXFGnsp1	This study
pSW3444	pRS315	NUP49		This study
pSW3445	pRS315	NUP49	ΔGLFG	This study
pSW3446	pRS315	NUP49	ΔGLFG + GLFG-NUP49	This study

pSW3447	pUG6	HIS5	LoxP	This study
pSW3448	pRS315	NUP42	ΔFG	This study
pSW3449	pRS315	NUP42	ΔFG + FG NUP42	This study
pSW3450	pRS315	NUP42	ΔFG + GLFG of NUP49	This study
pSW3451	pRS315	NUP42	ΔFG + FXFG of NSP1	This study
pSW3460	pUG6	HIS5	LoxP-HIS5-LoxP	This study
pSW3461	pRS315	Nup49	ΔGLFG, + GLFG-Nup57	This study
pSW3462	pRS315	Nup49	ΔGLFG, + GLFG-Nup116	This study
pSW3463	pRS315	Nup49	ΔGLFG, + FG-Nup42	This study
pSW3464	pRS315	Nup49	ΔGLFG, + FXFG-Nsp1	This study
pSW3465	pET-Duet	6xHis-Mtr2, Mex67	dual expression vector	This study
pSW3466	pRS314	mex67Δloop	mex67 Δbp 1225-1305	This study
pSW442	pRS304	nup49ΔGLFG-GFP:TRP1		(BUCCI and WENTE 1997)
pSW55	pBS	nup49::URA3		(WENTE *et al.* 1992)
pUG6	pUG6	KAN^R		M. Johnston

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