INOSITOL HEXAKISPHOSPHATE AND GLE1P DIRECT MRNA EXPORT BY SPATIALLY REGULATING THE DEAD-BOX PROTEIN DBP5P AT THE NUCLEAR PORE COMPLEX

By

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To my parents

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5-FOA	5-Fluoroorotic Acid
Δ	null
AAF	ATPase activating factor
ADARs	Adenosine deaminases acting on RNA
AMP	Adenine mono-phosphate
ADP	Adenine di-phosphate
ATP	Adenine tri-phosphate
cAMP	Cyclic AMP
CBC	Cap-binding complex
CDK	cyclin-dependent kinase
CTD	C-terminal domain
DAG	Diacylglyercol
DAPI	4',6-diamidino-2-phenylindole
DNA	Deoxyribonucleic acid
DNA-PK	DNA-dependent protein kinase
EJC	Exon-exon junction complex
EM	Electron microscopy
ER	Endoplasmic reticulum
FG	phenylalanine – glycine
FISH	Fluorescence *in situ* hybridization

FRET	Fluorescence resonance energy transfer
GDP	Guanine di-phosphate
GST	Glutathione S-transferase
GTP	Guanine tri-phosphate
HOG	High osmolarity glycerol response
HR	Homologous recombination
HPLC	High Pressure Liquid Chromatography
IP	Inositol Polyphosphate
IPMK	Inositol multikinase
IS_6	Inositol hexakissulphate
K	Lysine
Kd	Dissociation constant
MAP kinase	Mitogen-activated protein kinase
MBP	Maltose binding protein
mRNA	Messenger ribonucleic acid
mRNP	Messenger ribonucleoprotein particle
NES	Nuclear export signal
NHEJ	Nonhomologous end-joining
NLS	Nuclear localization signal
NPC	Nuclear pore complex
NTD	N-terminal domain
Nups	Nucleoporins

PEG	polyethylene glycol
P_i	Inorganic phosphate
PH	Pleckstrin homology
PHO	Phosphate-responsive pathway
PLC	Phospholipase
Pol II	RNA polymerase II
Q	Glutamine
R	Arginine
RanGAP	Ran-specific GTPase activating protein
RanGEF	Ran-specific guanine exchange factor
RNA	Dibonucleic acid
SAGA	Spt-Ada-Gcn5-acetyltransferase
SD	Synthetic media + glucose
s.e.m	Standard error of the mean
SRP	Signal recognition particle
TREX	Transcription/export complex
wt	Wildtype

CHAPTER I

INTRODUCTION

Intracellular transport

Transport of proteins and other macromolecules across membrane-bilayers is essential for all living organisms from bacteria to mammals. To achieve this task, cells establish membrane-bound and soluble machines that selectively take up or export cellular constituents. This process is essential to communicate and adjust to changes in environment. The compartmentalization of cellular tasks within membrane-bound intracellular bodies, characteristic of eukaryotes, adds an additional layer of complexity to the cell transport apparatus. How is the cell able to distinguish proteins and nucleic acids to be directed to distinct cellular locales while retaining speed and selectivity? The answer relies on the exquisite machines that create transport pathways or channels that recognize and translocate macromolecules, the diversity of the signals that dictate the final destination of a cargo, and the receptors that facilitate cargo-channel interactions.

Newly synthesized proteins or RNAs are directed by transport to different sites in the cytosol or within the nucleus, mitochondria, chloroplast, peroxisomes, and endoplasmic reticulum (ER). A specialized vesicle-based transport system in charge of the internalization, recycling and distribution of proteins and lipids of

the secretary pathway also exists (Grosshans et al. 2006; Lee et al. 2004a; Mellman and Warren 2000). Proteins and RNAs targeted for transport contain a signal sequence that is recognized by the discrete transport machinery of the targeted cellular compartment. These sequences are typically encoded in the primary sequence of a protein or RNA. However, distant regions of primary sequence can form a transport signal through the quaternary structure of a folded protein or the secondary structure of an RNA. Protein translocation through the ER membrane is dependent on an ER signal sequence often present at the N-terminus of proteins targeted to the membrane or lumen of the ER (reviewed in Rapoport 2007). As ribosomes initiate translation in the cytoplasm, the protruding N-terminus of the nascent polypeptide recruits a signal recognition particle (SRP). The SRP directs the ribosome to an SRP receptor in the ER membrane to allow directional translocation of the elongating polypeptide chain through the Sec61 translocator complex. Similarly, import of proteins to the mitochondrial matrix and the stroma in chloroplast depends on an N-terminal signal in the primary sequence that interacts with membrane translocators (Baker et al. 2007; Kessler and Schnell 2006). Importantly, import into the ER, mitochondria, and chloroplast requires the proteins to be unfolded and the signal sequences required for the protein import into these organelles are cleaved after the completion of transport. This is distinct from nuclear and peroxisomal transport that can translocate folded proteins and do not require signal removal. Despite its differences, intracellular protein transport studied in all organelles has been found

to require, ATP, GTP, or a favorable ion gradient to facilitate translocation (Gould and Collins 2002; Koehler 2000; Rapoport 2007; Terry et al. 2007).

Nuclear transport: The Nuclear Pore Complex

Nuclear transport is different from other types of organellar transport in that the transport machinery has to be capable of translocating huge fully folded macromolecular complexes, such as ribosomes and other ribonucleoproteins, through a double membrane barrier. This represents a formidable task, as these cargoes require a large translocation apparatus still capable of selective transport. Eukaryotic cells have overcome this problem by assembling a massive macromolecular structure to regulate nucleocytoplasmic exchange, the Nuclear Pore Complex (NPC). The NPC transverses both layers of the nuclear envelope at specific sites called nuclear pores. The NPC is composed of ~30 different proteins, called nucleoporins (Nups), and its conserved structure is organized in an 8-fold radial symmetry (Figure 1). The NPC serves as the gatekeeper of the nucleus by creating an aqueous channel less than 10 nm in diameter that allows the free diffusion of only molecules smaller than 25-40 kD (Alber et al. 2007a; Alber et al. 2007b; Daneholt 1997; Mattaj and Englmeier 1998). Larger molecules must overcome this permeability barrier by interacting directly with the NPC or through transport receptors.

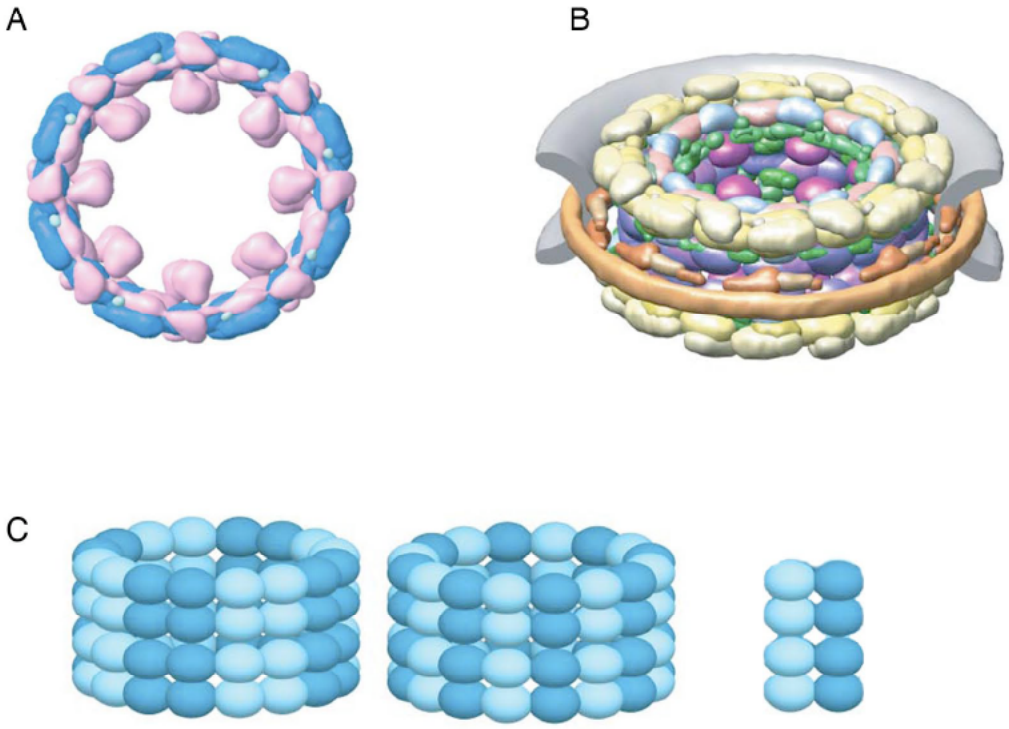


Figure 1: Architecture of the NPC (adapted from Alber *et al.* 2007b). (A) 8-fold symmetry of the NPC evident in the computational model of the inner (pink) and outer (blue) ring of the S. cerevisiae NPC. (B) Complete macromolecular structure of the NPC model showing an array of protein rings. Outer rings (yellow); membrane rings (orange); inner rings (purple); linker nucleoporins (blue and pink); and FG nucleoporins (green). Of note, FG nucleoporins face the NPC channel. (C) Structural modules of the NPC can be divided through their radial symmetry into eight spokes, each containing two columns.

The structure of the NPC has been shown to be highly conserved by three-dimensional cryo-electron microscopy (EM), and tomography (Akey and Radermacher 1993; Beck et al. 2004). In *Saccharomyces cerevisiae,* the NPC is predicted to be a ~44 MD structure (Rout et al. 2000). A recent report has modeled the yeast NPC by means of computational integration of biochemical and biophysical data (Alber et al. 2007a; Alber et al. 2007b). The resulting model (Figure 1) reveals a macromolecular assembly in which each of the eight pillars of the NPC is divided in two columns. The backbone of the NPC is thought to interact with the nuclear membrane and be the point of anchor for flexible protein extensions that stretch towards the center of the channel. These proteinaceous extensions are highly enriched in phenylalanine - glycine (FG) repeats and constitute the bulk of the NPC permeability barrier (Terry et al. 2007).

The mechanistic details of selective transport through the NPC permeability barrier is a highly debated topic (Frey et al. 2006; Lim et al. 2006; Macara 2001; Ribbeck and Gorlich 2002; Rout et al. 2003; Strawn et al. 2004; Terry and Wente 2007). It is agreed, however, that FG-domains play a critical role in establishing the selectivity of the translocation process by interacting with transport receptors. The affinity gradient model proposes that FG-domains establish a path of increasing affinity binding sites for transport receptors from one side of the NPC to the other (Ben-Efraim and Gerace 2001). This simple model accounts for directionality of transport but cannot be reconciled with observations showing that FG-domains at the cytoplasmic and nucleoplasmic

side of the NPC are not essential (Strawn et al. 2004) and can be exchanged (Zeitler and Weis 2004). The Brownian affinity gating model (Rout et al. 2000) suggests that interaction with FG-domains at the NPC increases the local concentration of transport receptors and overcomes the entropic barrier established by the narrow NPC channel. Translocation through the NPC is then mediated by Brownian random diffusion. The oily spaghetti model (Macara 2001) suggests that flexible FG-domains are pushed aside by transport receptors. Similar to the Brownian affinity gating model, transient association with FG's receptors and random motion allows translocation. Finally, the selective phase model (Frey and Gorlich 2007; Ribbeck and Gorlich 2001), based on hydrophobic interactions between FG-domains, proposes that a gel-like FG-meshwork is formed at the NPC channel. Receptor binding to the FG domains melts the meshwork locally, allowing reformation of the phase as the receptor travels through the NPC channel. This model accounts for selectivity of the transport process. However, this model is based on biophysical and biochemical observations under unphysiological conditions. The models proposed will have to take into consideration the speed and cargo capacity of the NPC, estimated to translocate up to 10 molecules simultaneously and transport ~1000 molecules per second (Ribbeck and Gorlich 2001) (Yang et al. 2004).

Interestingly, the translocation apparatus must accommodate multiple kinds of cargoes. In fact, distinct FG-domains seem to be required for specific transport receptors (Terry and Wente 2007). Similarly, it is important to

emphasize that the NPC is not an static structure. In fact, the NPC might reorganize to allow the passage of fully folded-membrane proteins to the inner nuclear membrane (Lusk et al. 2007). Additionally, metazoans must dismantle their NPCs during each cell division; a process that is only partially done in *A. nidulans*, and absent in budding yeast. How is a gel-like network made out of FG-domains disassembled and reassembled during mitosis? How is the permeability barrier affected during a closed mitosis? Strikingly, the mammalian NPCs seems to have an intrinsic ability to increase their diameter by intramolecular sliding of core NPC backbone components (Melcak et al. 2007), an attribute that could facilitate the transport of oversized cargo. To conclude, further modeling and testing *in vivo* is required to test proposed models and to tackle outstanding questions in the field regarding NPC assembly, the nature of the permeability barrier, transport of large cargoes, differential regulation of NPCs within a given cell, and establishment of directionality.

Great advances on classical protein nuclear import have been achieved, partly through the development of *in vitro* transport assays. Directional protein translocation is achieved by the asymmetric distribution of the small GTPase Ran in its GTP- and GDP-bound forms (Fried and Kutay 2003; Figure 2). The chromatin-associated Ran-specific guanine exchange factor (RanGEF) maintains a steady pool of RanGTP in the nuclear environment, whereas the Ran-specific GTPase activating protein (RanGAP) sustains GTP hydrolysis in the cytoplasm, keeping high cytoplasmic-RanGDP levels. The nuclear protein

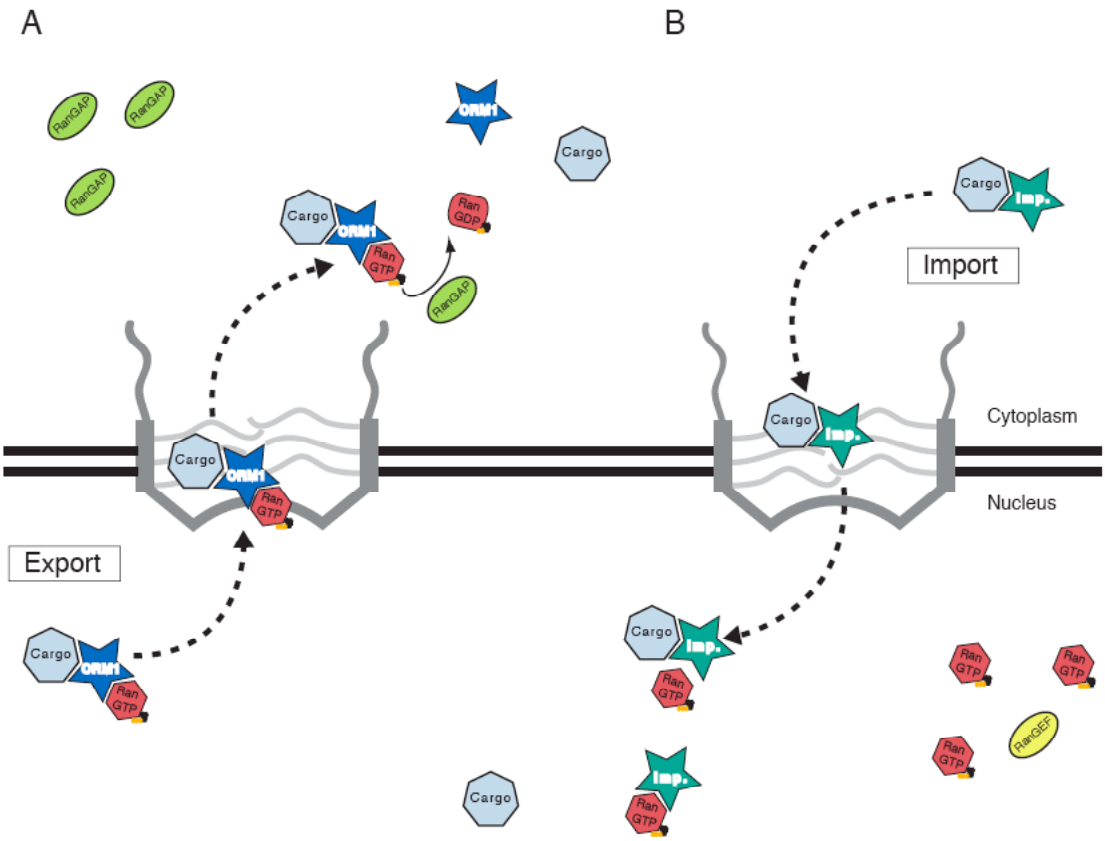


Figure 2: Nucleocytoplasmic protein transport is directed by differential localization of RanGTP and RanGDP. In the nucleus, the nuclear-localized RanGEF keeps RanGTP high. Similarly, cytoplasmic RanGAP stimulates GTP hydrolysis, resulting in high levels of RanGDP. (A) NES-containing cargoes bind nuclear export receptors (such as CRM1/Xpo1, dark blue) in a RanGTP-dependent fashion. A stable trimeric complex binds the NPC channel through CRM1 FG-binding domains. In the cytoplasm, RanGAP stimulates GTP hydrolysis resulting in GDP-bound Ran. Conformational changes in RanGDP and the export receptor results in complex dissociation. (B) Nuclear import is mediated by importins (Imp. dark green) that interact with FG-domains and cargo simultaneously. Upon arrival in the nucleus, binding to RanGTP results in cargo release.

transport machinery, composed of soluble karyopherins (also known as transportins, exportins, and importins), recognizes the RanGTP/GDP gradient, binds signal-containing cargoes, and interacts with NPC components to establish the directionality of transport. In the case of protein export, a nuclear export signal (NES) is recognized by nuclear export receptor CRM1/Xpo1 (Stade et al. 1997) in a RanGTP-dependent fashion (Fried and Kutay 2003). This complex binds and traverses the NPC. Disassembly in the cytoplasm is mediated by structural rearrangements in Ran resulting from GTP hydrolysis (Gamblin and Smerdon 1999) stimulated by cytoplasmic RanGAP. RanGDP is imported into the nucleus, where it exchanges the GDP for a GTP. In the case of nuclear protein import, a nuclear localization signal (NLS) is recognized by an importin. Importin-cargo complexes bind FG-domain and translocate through the NPC. Once in the nucleus, RanGTP disrupts importin-cargo interactions to finalize the import process (Fried and Kutay 2003). In this fashion, Ran plays the role of a molecular switch controlled by local regulation of GTP exchange and hydrolysis. tRNA, microRNA, small nuclear RNA, and ribosomal RNA export are exported utilizing a RanGTP/GDP gradient (Kohler and Hurt 2007). A new paradigm, however, seems to drive mRNA export, and the work presented here seeks to illuminate this novel mechanism. First, an overview of mRNA metabolism and the body knowledge acquired by studies in yeast and metazoans is presented.

mRNA: From birth to export

The nuclear export of mRNAs to the cytoplasm is tightly linked to transcription, processing, quality control, translation and degradation (Figure 3). Each of the steps in the life of a mRNA is regulated by a host of protein and RNA factors that facilitate intra- or intermolecular reactions. Consequently, it is crucial to picture mRNAs in the context of a dynamic messenger ribonucleoprotein particle (mRNP). The mRNP is born at the doorsteps of polymerase II (Pol II)-driven transcription, where the protruding mRNA recruits proteins as soon as ~20 nucleotide have been transcribed (Bentley 2005; Gu and Lima 2005). From that point onward, the mRNP experiences dramatic changes recruiting proteins required for capping, splicing, mRNA export, translation, and degradation (Buratowski 2005; Fasken and Corbett 2005; Jensen et al. 2003; Vinciguerra and Stutz 2004)

pre-mRNAs are transcribed by Pol II-driven transcription. The forming pre-mRNA is extruded through the Pol II exit channel at the rate of 10-30 bases per second (Bentley 2005). However, the role of Pol II is not limited to regulation of transcription. The C-terminal domain (CTD) of Pol II recruits a number or mRNA processing factors that bind the mRNA co-transcriptionally, facilitating mRNP formation. Interestingly, these same factors seem to have a positive impact on the transcription process.

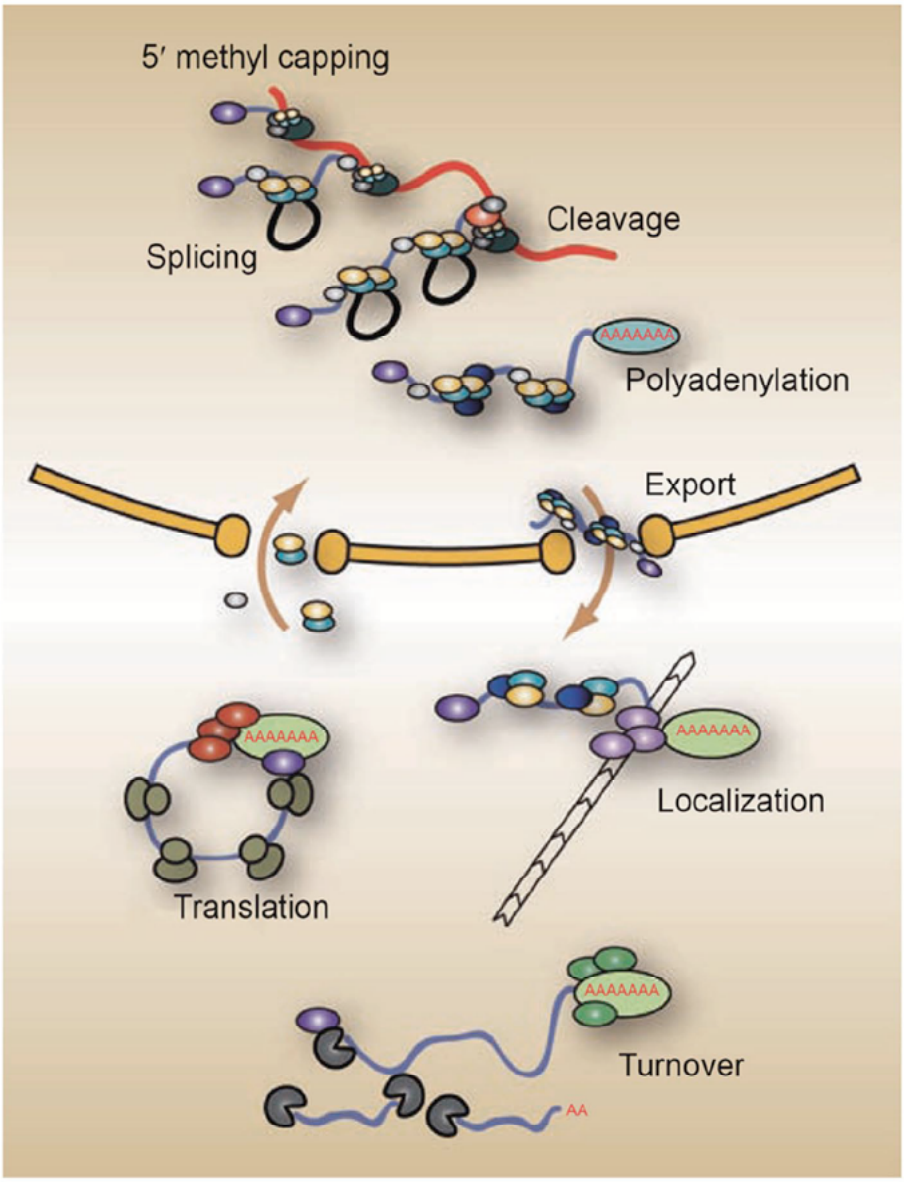


Figure 3: Coupling of mRNA export with capping, splicing, cleavage, polyadenylation, translation, localization, and turnover (reprinted from McKee and Silver 2007).

The first step in mRNA processing is the capping of the 5' end. In capping, the 5' terminal phosphate is cleaved and a GMP nucleotide is linked by a 5'-5' triphosphate bridge to produce GpppN. Subsequently, the G is methylated at the N7 position, and this m^7G constitutes the cap (Shatkin 1976). These reactions take place as the Pol II pauses and the capping enzymes bound to the CTD of Pol II interact with the 5' end of the protruding message (Sims et al. 2004). The 5' cap then recruits a cap-binding complex (CBC) that is able to interact with multiple other factors important for gene expression. Capping of the mRNA is thought to stabilize the mRNA and have roles in 3' end formation, and translation (Cheng et al. 2006; Lewis and Izaurralde 1997).

Next, the mRNA undergoes splicing, a process in which introns are removed and exons are joined in the primary sequence of a mRNA. Splicing sites are marked by three sequences in exons and introns that direct the splicing reaction and define the branch site, splicing donor and splicing acceptor. These sequences also determine splicing efficiencies that can give rise to alternative splicing. The splicing machinery is deposited at splice sites co-transcriptionally in every intron, however, intron excision can occur either co- or post-transcriptionally within the same transcript (Wetterberg et al. 1996; Wetterberg et al. 2001). Splicing radically alters the fate of a mRNA molecule. In addition to the changes in protein coding information, the splicing machinery deposits a protein complex in the vicinity of exon-exon junctions (Isken and Maquat 2007). The exon-exon junction complexes (EJCs) mark the sites of intron excision, a label

that is thought to be critical to avoid the mRNA degradation machinery that targets unspliced and aberrant messages.

Transcription of the 3' end of a pre-mRNA is followed by 3' end cleavage and poly(A) addition to the newly formed 3' hydroxyl group. Protein complexes that recognize primary sequences upstream and downstream of the cleavage site are first associated with the CTD and then transferred to the mRNA as it emerges from Pol II (Gilmartin 2005). Once synthesized, poly(A) tails are recognized by the poly(A) binding proteins. In the yeast nucleus, Nab2 and Pab1 are thought to be the potential poly(A) binding proteins (Dunn et al. 2005; Green et al. 2002; Marfatia et al. 2003). Addition of poly(A) tails is thought to play important roles in mRNP release from sites of transcription, mRNA export, translation, and stability (Gilmartin 2005). In fact, protein-protein interactions have been detected between poly(A) binding proteins and the CBC, the mRNA export machinery, and the cytoplasmic mRNA decay systems.

By the end of mRNA processing, a mature mRNP will have a 5' cap, followed by the 5' UTR, coding sequence marked with EJCs, an in-frame stop codon, a 3' UTR, and a poly(A) tail. Additionally, a mRNA will be decorated with a host of proteins recruited during processing like the CBC, EJC, and poly(A) binding proteins. Together this cohort of proteins and mRNA constitute the export competent mRNP. Some of these proteins, important in export and translation, will be discussed in the next section. Importantly, the cell employs several quality control or surveillance mechanisms to ensure that only properly processed

mRNPs are utilized by the translation machinery. Some of these mechanisms reside in the nucleus whereas others work in the cytoplasm.

Nuclear degradation of mRNA can go from 5'-3' or vice versa. 3'-5' degradation is carried out by the nuclear exosome, whereas the 5'-3' is mediated by the Rat1 exoribonuclease (Libri et al. 2002; Rosonina et al. 2006). For degradation to proceed, caps and poly(A) tails must be removed. If a message is not capped, the exposed 5' end is recognized by Rat1 and the message is degraded (Kim et al. 2004). In yeast, splicing mutants give rise to an increase of pre-mRNAs. These mRNAs are stabilized by mutations in genes encoding proteins of the nuclear exosome (Bousquet-Antonelli et al. 2000), suggesting that the cell is able to target these messages for degradation through the nuclear exosome. An additional surveillance mechanism is provided by the NPC-associated Mlp1 and Mlp2 proteins that retain unspliced messages at the NPC (Galy et al. 2004). Since these proteins mark the gate of the NPC, it is argued that the NPC scrutinizes the mRNPs allowing only the exit of properly processed messages.

Nuclear export of mRNA

In essence, mRNA export follows the classical paradigm of intracellular transport. Namely, the mRNA is recognized by an export receptor that interacts with the transport channel (the NPC) to facilitate translocation. However, the interconnectivity between transcription, processing, and export makes it

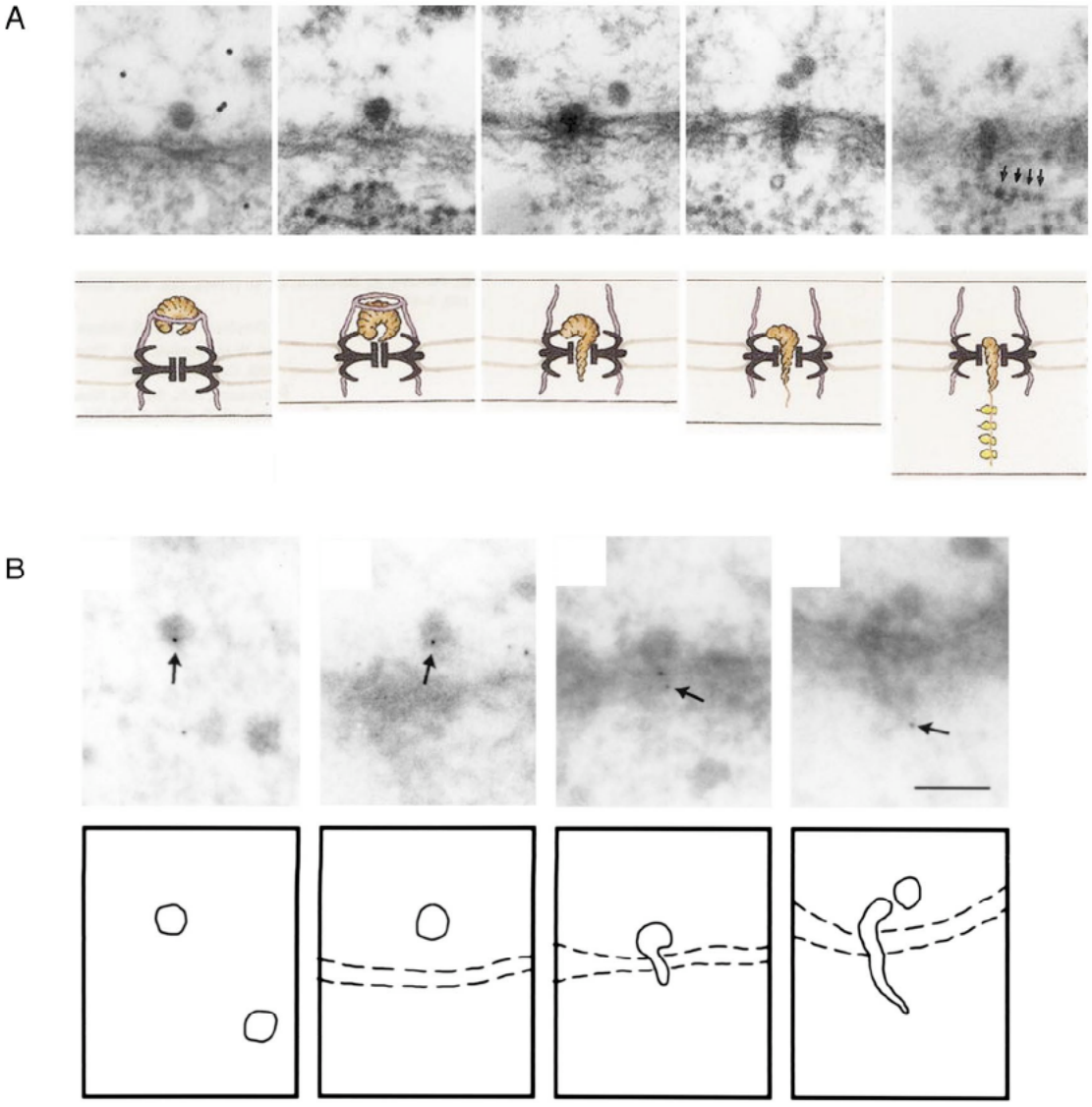


Figure 4: Nuclear export of the giant Balbiani ring mRNP (adapted from Daneholt 2001a; Daneholt 2001b). (A) The Balbiani ring, an electron dense particle (also depicted in cartoon), approaches the NPC (electron dense structure on the nuclear membrane). Conformational changes of the Balbiani ring are evident during NPC translocation. In the cytoplasm, ribosomes (arrows) associate with the exported Balbiani ring mRNP. (B) Export of the Balbiani ring mRNP proceeds in a 5' to 3' direction. Localization of the Balbiani ring CBC by immuno-EM detection of CBP20 localizes the 5' end of the mRNA. Gold particles are marked by arrows and indicate the localization of the 5' during the export of the Balbiani ring mRNP. Schematic representations are placed below each figure in (A) and (B).

impossible to isolate mRNA export as a single simple mechanism. Additionally, translocation of large macromolecular complexes, such as mRNPs, through the NPC requires dynamic changes in the composition and structure of the cargo. For instance, EM studies of the giant insect mRNP known as the Balbiani ring revealed a dramatic relaxation in mRNP structure upon NPC docking (Mehlin et al. 1992). Finally, mRNA export feeds directly into translation, and recent data suggest a tight communication between these two processes. How is the mRNP recruited to the NPC? What are the biochemical requirements for mRNP remodeling? How is the mRNP released in the cytoplasm for translation?

One of the most informative observations in the mRNA export field have come form direct visualization of the electron dense Balbiani ring by EM (Daneholt 2001a, b; Mehlin et al. 1992). These observations suggest that remodeling events allow the straightening and correct orientation of the mRNP, which is extruded in a 5' to 3' direction (Figure 4 A-B). Furthermore, whereas some proteins are recruited co-transcriptionally and stay associated with the mRNP during specific processing steps and translation, others are only transiently associated with the message.

Export of mRNA is mediated by the conserved mRNA export receptor Mex67 (Figure 5). The yeast Mex67 functions with Mtr2 as a heterodimer (TAP/NXF1-p15/NXT1 in metazoans) and represents a novel class of export receptors distinctive from the karyopherin family in sequence and structure (Gruter et al. 1998; Segref et al. 1997). Additionally, Mex67-driven export is not

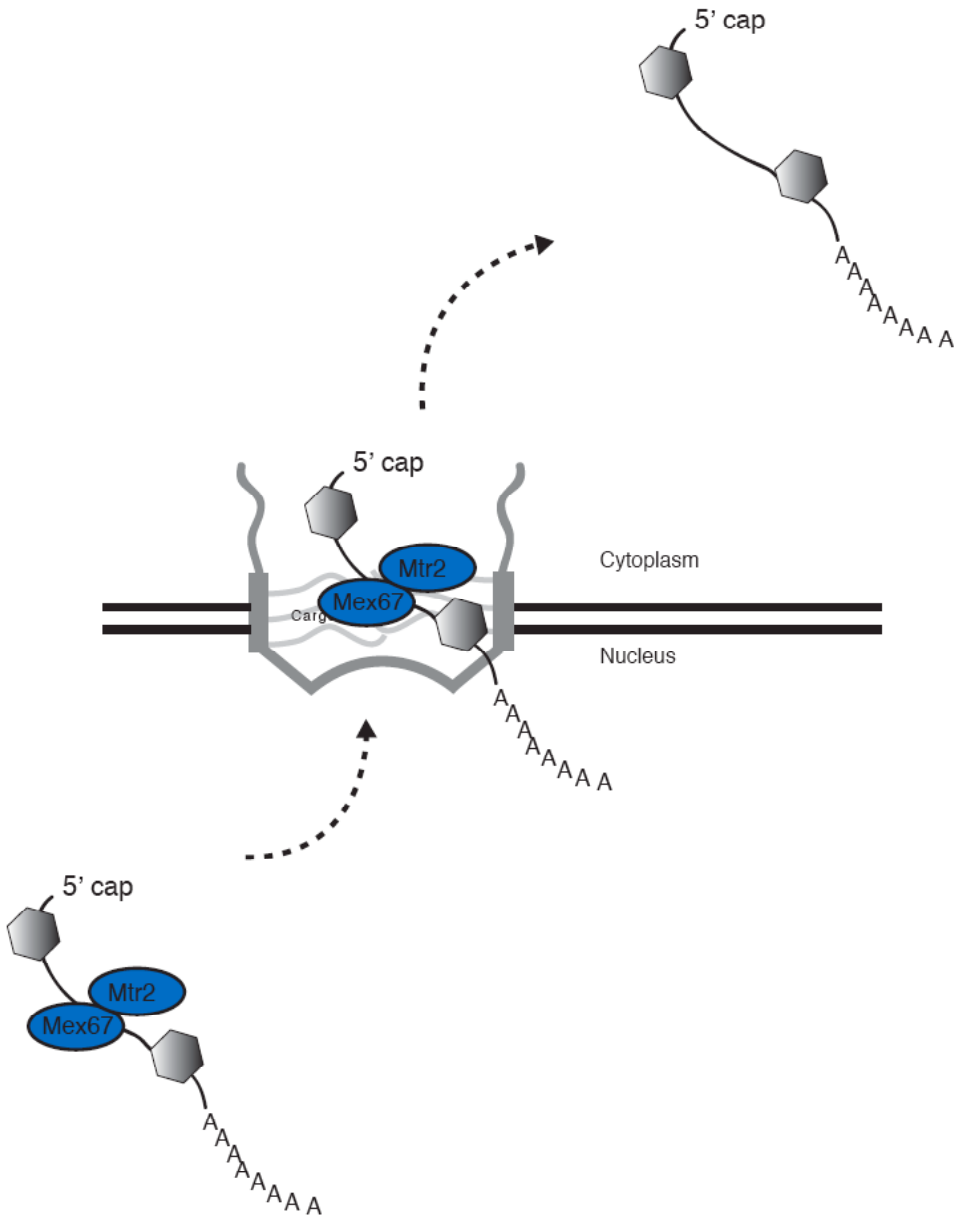


Figure 5: The mRNA export receptor Mex67. Mex67 and Mtr2 work as a heterodimer that is loaded to mRNP cotranscriptionally. Mex67 binds FG-domains in the aqueous channel of the NPC in order to facilitate translocation. mRNPs are released in the cytoplasmic side of the NPC, and Mex67/Mtr2 is cycled back into the nucleus for further rounds of export.

regulated by Ran cycles of GTP hydrolysis. Nevertheless, Mex67 shares many of the properties of nuclear exporters of the karyopherin class. This includes interaction with FG domains, ability to overcome the NPC permeability barrier, and recycling back to the nucleus for subsequent rounds of export.

Association of Mex67 with the mRNP is considered to be an essential step during the mRNA export process. Accordingly, the eukaryotic cell has created several mechanisms to guarantee this step. Mex67 is recruited to the mRNA cotranscriptionally by its physical association to the yeast SR protein Npl3 (Gilbert and Guthrie 2004). Initially, Npl3 associates with the nascent mRNA in a phosphorylated state (Gilbert et al. 2001). Npl3 dephosphorylation stimulates the recruitment of Mex67, facilitating mRNP export. Once in the cytoplasm, re-phosphorylation of Npl3 destabilizes its interaction with Mex67 (Gilbert et al. 2001). In mammals, TAP/NXF1 recruitment is also regulated by phosphorylation cycles of SR-proteins (Huang et al. 2003). However, unlike its yeast counterparts, mammalian SR-proteins regulate splicing, and load TAP/NXF1 during this step. An alternative or complementary route for Mex67 recruitment is provided by Yra1 (ALY/REF in metazoans), which is itself, recruited to activated genes (Lei et al. 2001). In fact, Yra1 is part of the transcription/export (TREX) complex, which has multiple roles during transcription and mRNP formation (Strasser et al. 2002; Zenklusen et al. 2001).

To sum up, the multiple roles of mRNP proteins in transcription, processing, and export are conveniently intertwined to facilitate the flow of the

mRNP maturation process and provide communication between these multiple steps. An export competent mRNP containing Mex67 is targeted for translocation through the NPC. FG-domains within the NPC channel are able to bind Mex67 and facilitate mRNP translocation. Interestingly recent studies *in vivo* suggest that Mex67-containing mRNPs interact with distinct FG-nucleoporins (Terry and Wente 2007), suggesting specialized pathways within the NPC for mRNA export.

Gle1 and Dbp5: Conserved mRNA export factors

Release of the mRNP in the cytoplasm is thought to be mediated by Dbp5, a member of the conserved DEAD-box helicase family (Cordin et al. 2006; Linder 2006). Moreover, Dbp5 is thought to remodel the mRNP by dissociating a subset of RNA binding proteins from the mRNP (Lund and Guthrie 2005; Tran 2007). Since Nup159 provides a Dbp5 binding site at the cytoplasmic side of the NPC (Figure 6) (Schmitt et al. 1999; Weirich et al. 2004), Dbp5 has been suggested to remodel the mRNP for regulation of later stages of mRNA export.Consistent with this model, mutations in *DBP5* result in strong poly(A)$^+$ mRNA accumulation in the nucleus at non-permissive temperatures (Snay-Hodge et al. 1998; Tseng et al. 1998). These stalled mRNPs have been found to contain Mex67 (Lund and Guthrie 2005). Importantly, a mutation in *MEX67* is able to rescue both the Mex67-5 accumulation and growth defect of a *dbp5* strain; suggesting Mex67

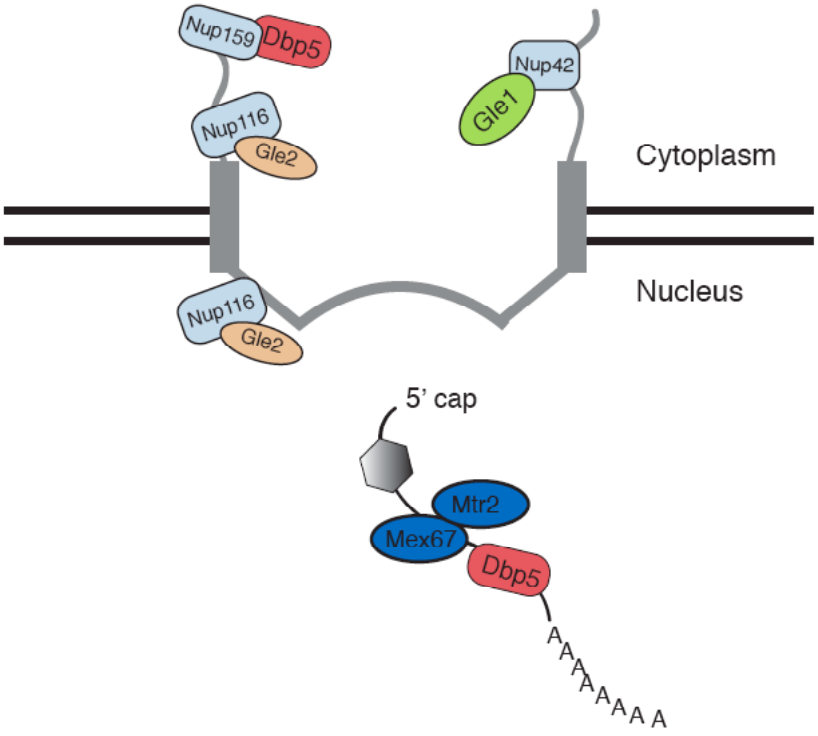


Figure 6: mRNA export factors and their relationship to the NPC. mRNPs recruit RNA export factors during transcription and processing. A mature mRNP associates with the NPC (gray) through the export receptor Mex67/Mtr2. Nup42 and Nup159 provide binding sites for the mRNA export factors Gle1 and Dbp5 at the cytoplasmic side of the NPC. Additional nups (such as Nup116) play important roles in export and is localized at both sides of the NPC.

dissociation is facilitated by Dbp5. Importantly, a recent study has reported a role for Dbp5 in translation (Gross et al. 2007). This exciting finding expands our view of interconnected regulation of gene expression, revealing yet another mechanism with intimate ties to the mRNA export machinery.

Regulation of the final steps in mRNA export has also been attributed to the novel and conserved mRNA export factor Gle1 (human (h)Gle1A or B in mammals) (Murphy and Wente 1996; Watkins et al. 1998; Figure 6). Gle1 was originally isolated in a *NUP100* synthetic lethal screen. The resulting *gle1* temperature sensitive alleles show a strong mRNA accumulation in the nucleus. Additional studies have identified interactions between Gle1 and mRNA export components. Pull downs utilizing *in vitro* transcription translation and yeast-two-hybrid studies have demonstrated that Gle1 binds Dbp5 (Hodge et al. 1999; Schmitt et al. 1999; Strahm et al. 1999). Additionally, Gle1 has been found to interact with Gfd1 (Hodge et al. 1999; Strahm et al. 1999; Suntharalingam et al. 2004), a factor with physical and genetic interactions with Dbp5 and Nab2. Nup42, a nucleoporin localized at the cytoplasmic face of the NPC, provides a binding site for Gle1 (Murphy and Wente 1996; Figure 6), an interaction conserved in metazoans (Kendirgi et al. 2005). The genetic and physical data gathered strongly suggest a role for Gle1 in the later stages of mRNA export and release. Consistent with this hypothesis, immuno-EM studies have shown that Gle1 localizes more predominantly at cytoplasmic side of the NPC (Miller 2004; Rout et al. 2000). Interestingly, in humans a splice variant results in the

expression of two Gle1 isoforms, hGle1A and hGle1B. hGle1B is the most abundant isoform in the cell. Importantly, it contains an additional 43-amino acid yeast-Gle1 (yGle1)-like domain at its C-terminus that mediates NPC binding through hCG1 (the human homologue of Nup42). hGle1 has also been found to interact with hNup155 (Rayala et al. 2004), but this interaction is not sufficient for NPC recruitment. Additionally, hGle1 contains a shuttling domain not conserved in yGle1 that plays a role for mRNA export (Kendirgi et al. 2003). It is unclear whether both hGle1B and hGle1A have complementary roles in mRNA export and whether yGle1 can shuttle like its human homologue. Due to the high sequence similarity between yeast and human Gle1 proteins, they are expected to have similar roles in mRNA export.

Genetic studies in yeast have identified components of the inositol signaling pathway that gives rise to the production of inositol hexakisphosphate (IP_6) as important players in the Gle1-driven step in mRNA export (York et al. 1999). In wildtype (wt) cells, production of soluble inositols is not essential. However, cells harboring temperature sensitive mutations in *gle1* or *dbp5* are unable to survive if IP_6 levels are reduced (Miller 2004; York et al. 1999). Additionally, lack of IP_6 production has detrimental effects on growth of cells with mutations in *NUP42, NUP159, NUP116,* and *GLE2,* genes encoding proteins localized at the cytoplasmic side of the NPC. Importantly, yeast cells unable to produce IP_6 have a weak poly(A)-mRNA accumulation at 37 ˚C. What is the role of IP_6 in mRNA export? What is its biochemical target?

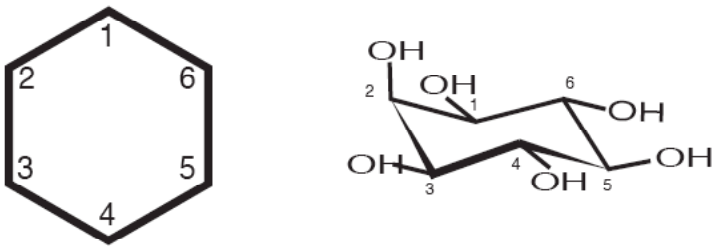
The field has gained important insights into the mechanism of mRNA export. Of great importance is the discovery of a physical link between the transcription and processing machineries with export receptors and the NPC itself, a phenomenon called gene gating (Blobel, 1985). However, little is known about the later stages of mRNA export at the cytoplasmic face of the NPC. Furthermore, a recent report providing a link between mRNA export and translation has extended the field and suggest a role for Dbp5 in both processes. The work presented here seeks to elucidate the mechanisms of mRNA export at the cytoplasmic face of the NPC. Specifically, it seeks to unveil the precise roles of Gle1 and IP_6 at the later stages of mRNA export.

It has been known that soluble inositols represent versatile intracellular second messengers with multiple roles in gene expression (see below). However, the understanding of how they regulate cellular physiology is still in its infancy. In order to understand IP_6, and its potential regulation of mRNA export, it is important to review the roles of other inositols polyphosphates (IPs) in cell physiology. Recent advances in the inositol-signaling field have underlined the importance of these molecules and are reviewed below.

The IP signaling pathway

Inositol was first identified more than a century and a half ago by the German chemist Josef Scherer (Scherer 1850, 1851) who isolated it from muscle tissue and coined the new molecule inos (Greek for muscle). We now find that

A



B

Name	Common name	Structure
$PI(4,5)P_2$	PIP_2	
$I(1,4,5)P_3$	IP_3	
$I(1,2,3,4,5,6,)P_6$ Inositol hexakisphosphate Phytic Acid	IP_6	
$5\text{-}PP\text{-}I(1,3,4,6)P_4$	$PP\text{-}IP_4$	
$5,6\text{-}PP\text{-}I(1,2,3,4)P_4$	IP_8	

Figure 7: Structure and nomenclature of *myo*-D-inositol derivatives. (A) *myo*-Inositol is shown as a simplified ring structure omitting the hydroxyl groups (left). The more thermodynamically stable chair-conformation represents the most likely physiological structure of *myo*-inositol. The D-numbering system is shown placing the axial phosphate in the D-2 position. (B) Examples of common inositides and their nomenclatures. PIP_2, IP_3, IP_6, $PP\text{-}IP_4$, and IP_8 are shown in simplified and chair conformations. Of note, the common names do not indicate the positions of phosphates. Red balls indicate phosphates in IPs, whereas blue is used for IPPs.

the study of soluble and membrane-bound inositols, referred to as inositides, has intersected almost every aspect of cellular biology. Of the nine possible isomers of inositol, *myo*-inositol is the most abundant in nature (Kersting et al. 2003) with the six-carbon ring harboring one axial hydroxyl at the D-2 position and five equatorial hydroxyl groups (Figure 7). Building on this structure, a great diversity of inositol derivatives is achieved with multiple combinations of mono- and pyro-phosphate groups attached to each of the six hydroxyls moieties. Additional molecular complexity is accomplished by the potential incorporation of these derivatives in lipid head groups. To date, more than 37 distinct inositides have been identified in biological systems (Irvine 2005; York 2006), and it is likely that future work will discover additional inositol molecules with important physiological roles. The cytoplasmic functions of the lipid-anchored inositides, referred to as inositol lipids, phosphoinositides, or phosphatidylinositols (PIPs), have been reviewed in depth (Strahl and Thorner 2007) and include essential structural and signaling roles in vesicular trafficking, actin cytoskeleton rearrangements, and Akt signaling. On the other hand, soluble phosphoinositides, referred to as IPs have been found to have roles in nuclear processes especially in gene expression. Many of these physiological breakthroughs have been reported in the context of the budding yeast *Saccharomyces cerevisiae* model system. Importantly, recent key advances have identified long-sought kinases and elucidated molecular targets of IPs.

The metabolic machinery for soluble IPs

Inositol is essential for the life of a eukaryotic cell. However, its origin does not seem to be eukaryotic because *myo*-inositol derivatives have been found in archaea and some prokaryotes (Krings et al. 2006; Michell 2007). It is tempting then to speculate that inositol-generating enzymes evolved before the appearance of eukaryotes (reviewed in (Irvine 2005; Michell 2007), with the entire armada of kinases, phosphatases, and other inositol-metabolizing enzymes emerging thereafter. *S. cerevisiae* requires one of two mechanisms to acquire inositol; uptake of inositol molecules from the extracellular media by inositol-specific transporters or conversion of glucose-6-phosphate (G-6-P) to inositol-3-phosphate through the enzymatic activity of inositol-l-phosphate synthase (Ino1). Itr1 and Itr2 are yeast inositol permeases with high homology to the sugar transporter superfamily (Nikawa et al. 1991). Inositol uptake is completely abolished in yeast cells that lack both the Itr1 and Itr2 transporters, suggesting that they represent the only route for inositol uptake into the cell. Not surprisingly, yeast cells maintain inositol homeostasis by altering transcription, localization, and stability of Itr1, Itr2, and Ino1 during differential growth demands and availability of extracellular inositol (Lai and McGraw 1994; Nikawa et al. 1993) (Robinson et al. 1996). In mammalian cells, de novo production of inositol also relies on G-6-P conversion to inositol-3-phosphate by the highly conserved INO/MIP synthase (Guan et al. 2003; Ju et al. 2004). Additionally, inositol is transported via H^+ *myo*-inositol cotransporter and sodium–*myo*-inositol

cotransporter 1/2, respectively (Coady et al. 2002; Kwon et al. 1992; Uldry et al. 2001), which uptake inositol from serum levels that range from 30 to 70 μM (Dolhofer and Wieland 1987; Kouzuma et al. 2001; MacGregor and Matschinsky 1984). Once in the cytoplasm, inositol is available for incorporation into lipids and subject to multiple rounds of phosphorylation and subsequent dephosphorylation. This process results in a diverse collection of lipid-anchored PIPs.

IP signaling depends entirely on the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) by members of the phospholipase-C (PLC) family. In budding yeast, this reaction is carried out by Plc1 (Flick and Thorner 1993; Payne and Fitzgerald-Hayes 1993; Yoko-o et al. 1993). Such hydrolysis results in the generation of membrane-bound diacylglyercol (DAG) and the release of inositol 1,4,5-trisphosphate (IP_3). This IP_3 serves as the backbone for the formation of all other IPs in yeast and metazoans (Figure 8).

The conversion of IP_3 to all other IPs is mediated by specific inositol kinases and phosphatases. A simplified chart depicting the main mammalian and fungal inositol metabolic pathways is presented in Figure 8. In budding yeast, Plc1-mediated release of IP_3 is the rate-limiting step for the production of inositol hexakisphosphate (IP_6), the fully monophosphorylated inositol species. The framework for the kinase pathway was built on the discovery of the genes encoding the IP kinases Ipk1 and Ipk2 in a budding yeast genetic screen

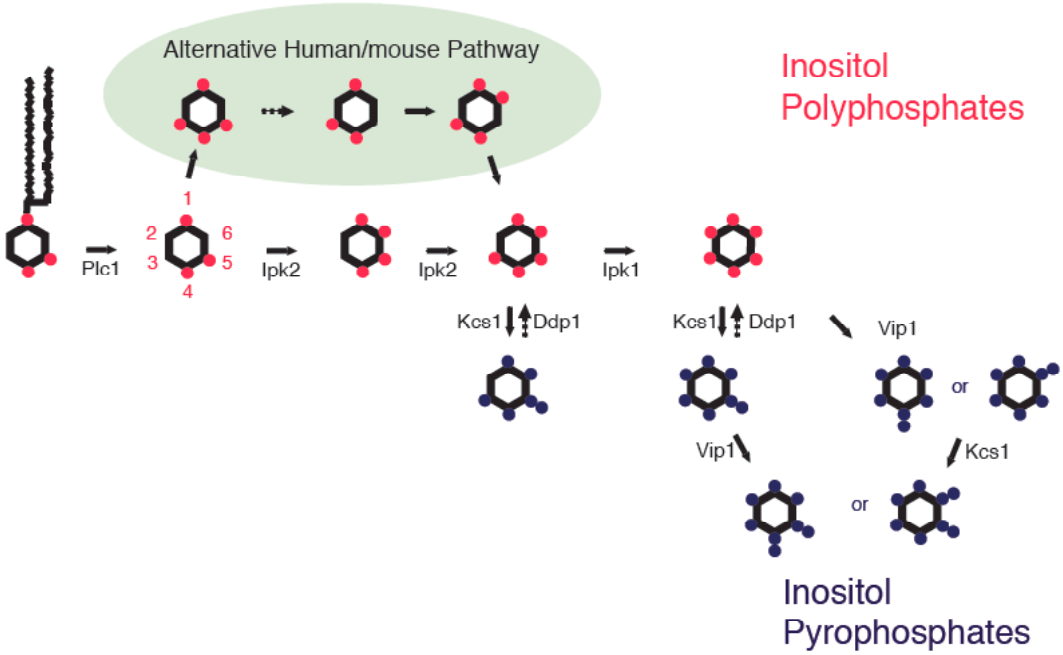


Figure 8: Inositol polyphosphate-signaling pathway. A simplified chart of the major Plc1-driven IPs signaling pathway in yeast and humans (conserved in *Drosophila melanogaster, Rattus norvegicus,* and *Mus musculus*) is presented. Hydrolysis of membrane-bound PIP_2 by Plc1 releases IP_3 (phosphates labeled) and initiates IP signaling. The IP_3 is sequentially phosphorylated to IP_5 by the dual-specificity kinase Ipk2. On an alternative pathway (green shadow), the human pathway is also able to isomerize IP_3 converting $I(1,4,5)P_3$ to $I(1,3,4)P_3$. This isoform of IP_3 is then phosphorylated by 6- and 5-kinases to produce IP_5. Both pathways utilize phosphorylation of the $I(1,3,4,5,6)P_5$ isoform by the 2-kinase Ipk1 to produce the fully monophosphorylated IP_6. Additionally, IP_5 and IP_6 are substrates for IPPs kinases Kcs1 and Vip1. Red balls indicate phosphates in IPs, whereas blue is used for IPPs. Solid arrows indicate Plc1-driven hydrolysis and phosphorylation events. Dashed arrows indicate dephosphorylation.

studying mRNA export (York et al. 1999). The IP_6-5-kinase Kcs1 (Saiardi et al. 1999) and the recently identified IP_6-4/6 kinase Vip1 (Mulugu et al. 2007) are responsible for the production of a number of pyrophosphorylated IP isoforms, designated inositol pyrophosphates (IPPs). An alternative low-abundance IP pathway has been reported relying on the IP_3 3-kinase (ITPK) activities of yeast Ipk2 and mammalian ITPKs Itpka, Itpkb, Itpkc and inositol phosphate multikinase IPMK (omitted in Figure 8 for simplicity; (Frederick et al. 2005; Leyman et al. 2007; Seeds et al. 2005). In mammals, the inositol metabolic pathway is more complex and involves both a yeast-like pathway driven by Ipk2/inositol multikinase (IPMK) and an alternative pathway driven by isomerization of $I(1,4,5)P_3$ to $I(1,3,4)P_3$ and two subsequent rounds of phosphorylation by $I(1,3,4)P_3$ 5/6-kinase (Verbsky et al. 2005b) to form inositol (1,3,4,5,6) pentakisphosphate (IP_5). Strikingly, both yeast and mammals utilize the same IP_5 isomer and conserved Ipk1 enzymes for the formation of IP_6, the linchpin for multiple higher pyrophosphorylated IPs.

Cellular logistics of the IPs

The molecular diversity of the *myo*-inositol derivatives and their ubiquitous presence is equally matched in scope by the range of their cellular abundances across species and cell types. IP_6, is the major form of phosphorus in plant seeds and accounts for ~1% of a typical seed's dry weight (Raboy 1997). Similarly, in the budding yeast, IP_6 levels are at much higher concentrations than IP_3, inositol

(1,3,4,5) tetrakisphosphate (IP_4), and IP_5 (York et al. 1999). Mammalian IP_6 levels range from 10 to 100 μM (Bunce et al. 1993; French et al. 1991; Pittet et al. 1989; Szwergold et al. 1987), and in many cases, cells accumulate IP_5 to similar levels as IP_6 (Bunce et al. 1993; Feng et al. 2001; Frederick et al. 2005; Glennon and Shears 1993; Verbsky et al. 2005a). In contrast, in zebrafish embryos, IP_3 is the predominant steady-state isoform with relative IP_6 levels below detection (Sarmah et al. 2005). This suggests different functional roles for the IPs in distinct species and suggests that there are potentially novel regulatory mechanisms that control the respective IP levels.

Many biological processes are regulated not only by the production and abundance of its constituents but also by spatial–temporal constraints. The inositol-signaling pathway likely follows this paradigm. A classical view of Plc1 places it on the plasma membrane, restricting PIP_2 hydrolysis activity in the cytoplasm. It is now evident that PIP_2 and Plc1 are present both at the plasma membrane and in the nucleus in yeast and mammals (Audhya and Emr 2003; Cocco et al. 1998; Martelli et al. 1992; Strahl and Thorner 2007). In fact, Plc1 shuttles between the nucleus and cytoplasm (Strahl and Thorner 2007). With both substrate and enzyme at both locales, it is possible that Plc1 localization and activity is regulated and that the distinct pools of IP_3 are released in the nucleus and cytoplasm with different fates and physiological roles.

A number of IP-metabolizing enzymes localize primarily to the nucleus or nuclear periphery in budding yeast cells (Table 1). This observation complements

Table 1: Subcellular localization of the inositide metabolic machinery leading to nuclear IPs in *S. cerevisiae*

Protein	Function	Products	Localization	References
Itr1	Inositol transport	-	Plasma membrane	Miyashita et al. 2003
Itr2	Inositol transport	-	Plasma membrane	Miyashita et al. 2003
Ino1	IP synthase	IP	Cytoplasmic	Culbertson and Henry 1975; Huh et al. 2003
Inm1	Inositol phosphatase	I	Nucleus/cytoplasm	Huh et al. 2003; Murray and Greenberg 1997
Pis1	PI synthase	PI	ER	Huh et al. 2003; Nikawa and Yamashita 1982
Pik1	PI kinase	PIP	Nucleus/cytoplasm	Flanagan and Thorner 1992; Strahl et al. 2005
Mss4	PIP kinase	PIP_2	Nucleus/cytoplasm	Audhya and Emr 2003
Plc1	PIP_2 phospholipase	IP_3	Nucleus/cytoplasm	Strahl and Thorner 2007
Ipk2	IP_3/IP_4 kinase	IP_4; IP_5	Nucleus	El Bakkoury et al. 2000; Odom et al. 2000
Ipk1	IP_5 Kinase	IP_6	Nuclear Periphery	York et al. 1999
Kcs1	$IP_5/IP_6/IP_7$ 5-kinase	PP-IP_4; IP_7; IP_8	Nucleus/cytoplasm	Luo et al. 2002
Vip1	IP_6/IP_7 4/6-kinase	IP_7; IP_8	Nucleus/cytoplasm	Huh et al. 2003; Mulugu et al. 2007
Ddp1*	IPP phosphatases	IP_5; IP_6	Nucleus/cytoplasm	Huh et al. 2003; Safrany et al. 1999

the increasing diversity of IP roles reported in nuclear processes (see below). Table 1 summarizes the subcellular localizations of reported yeast IPs transporters, kinases, and phosphatases leading to nuclear IPs. Despite the wealth of information available on the localization of inositol-metabolizing enzymes, one fundamental question of inositol physiology has remained unanswered: Where are the IPs themselves localized? In mammalian cells, it is known that IP_3 released from the plasma membrane can diffuse through the cytoplasm and activate IP_3 receptors localized in the endoplasmic reticulum membrane (Foskett et al. 2007). Studies in HL60 promyeloid cells suggest that IPs have free access to the cytoplasm as plasma membrane-permeabilized cells do not retain their IPs (Stuart et al. 1994). Similarly, at least some yeast IPs can diffuse from their sites of production. The yeast Ipk1 enzyme, responsible for the generation of IP_6, is localized at the nuclear periphery (York et al. 1999); however, when Ipk1 is artificially anchored to the plasma membrane, IP_6 is generated and able to reach its target at the NPC and facilitate mRNA export (Miller 2004). Thus, free exchange and diffusion through the cytoplasm might be a universal characteristic of IPs. A more fascinating alternative would be that some IPs are restricted to specific cellular structures or regions, having access to only a subset of targets. Plants seeds, for example, compartmentalize IP_6 in membrane-bound structures called protein storage vacuoles, albeit, as salts of mineral cations (Otegui et al. 2002). Additionally, IP_6 has been suggested to bind membranes utilizing Ca^{2+}or Mg^{2+} ions possibly through interactions with

phospholipids (Poyner et al. 1993). Direct observation of the dynamic IP localization *in vivo* through the development of fluorescent dyes or protein probes would undoubtedly help resolve this issue and should be a goal for future studies.

IP protein recognition domains

The great variety of IPs generated by the metabolic machinery, taken together with their diverse roles, suggests the presence of an exquisite system that differentiates between mono- and pyro-phosphorylated forms of IPs in their different stereoisomeric forms. Recent structural data reveal that no single general fold is employed by IP-binding proteins. The reported IP-bound structures are grouped in two categories: IP-kinases and IP-binding proteins.

IP kinases

Based on the high-resolution protein structures available and sequence homologies, the characterized IP kinases can be subgrouped into three main families: the inositol 5/6-kinases, the inositol 2-kinases, and the IPKs. The IPK group can be further divided into 3-kinases, IPMKs, and IP_6 kinases (Holmes and Jogl 2006; Miller et al. 2005). To date, high-resolution structures have been reported for the mammalian $I(1,4,5)P_3$ 3-kinase (Chamberlain et al. 2005; Gonzalez et al. 2004; Miller and Hurley 2004), the Entamoeba histolytica (Miller et al. 2005) and mammalian(Chamberlain et al. 2005) $I(1,3,4)P_3$ 5/6-kinases, (Miller et al. 2005) and the budding yeast $I(1,4,5)P_3$ 3/6-kinase Ipk2 (Holmes and

Jogl 2006). A structure for an IP_5 2-kinases family member has not been completed.

Analysis of both the $I(1,4,5)P_3$ 3-kinase and $I(1,3,4)P_3$ 5/6-kinases reveals structurally distinct conformations. The overall fold of the $I(1,4,5)P_3$ 3-kinase is very similar to lipid and protein kinases, especially in the catalytic amino and carboxyl domains (Gonzalez et al. 2004). On the other hand, the $I(1,3,4)P_3$ 5/6-kinase shows striking similarity to the ATP-grasp fold proteins (Cheek et al. 2002; Miller et al. 2005). For both the $I(1,4,5)P_3$ 3-kinase and $I(1,3,4)P_3$ 5/6-kinase, predominant lysine, arginine, and histidine residues form a positively charged pocket wherein the respective IP binds. IP recognition is achieved by direct binding to the phosphate rather than the hydroxyl groups. It is interesting to note that constrictions in the active site of both structures suggest specific physical determinants that elucidate the absence of activity on lipid phosphatidylinositides.

The recently solved X-ray crystallography structure for the yeast Ipk2 shows similarity to the $I(1,4,5)P_3$ 3-kinase, confirming a conservation of folds in the IPK family (Holmes and Jogl 2006). It is interesting to note that Ipk2 recognizes and phosphorylates both IP_3 and IP_4 (Odom et al. 2000); (Saiardi et al. 1999). The Ipk2 structure reveals an active site that presumably would allow IPs in several possible orientations. This suggests a potential mechanism by which multiple IP substrates are accommodated (Holmes and Jogl 2006), a prominent characteristic for some IPKs *in vitro* and *in vivo* (Miller and Hurley 2004). Importantly, rat IPMK has been shown to be a highly specific $PI(4,5)P_2$ 3-

kinase *in vitro*, and its overexpression increases $PI(3,4,5)P_3$ levels *in vivo* (Resnick et al. 2005). This dual lipid and soluble inositol kinases capacity might be attributed to key differences in the inositol-binding domains of $I(1,4,5)P_3$ 3-kinases and Ipk2, the latter containing two of the five alpha-helices present in $I(1,4,5)P_3$ 3-kinases (Holmes and Jogl 2006).

IP-binding proteins

Several IP-binding proteins have also been recently cocrystallized with their respective IPs (Bosanac et al. 2002; Macbeth et al. 2005) (Milano et al. 2006; Tan et al. 2007). In all reported cases, the IP-binding site is formed by the clustered localization of basic amino acid residues in a three-dimensional pocket. This is similar to the IP kinases. However, in contrast, these basic lysine and arginine residues do not fall into a defined motif. Rather, they are dispersed through a ~200–300-amino acid region of the particular protein's primary sequence. Importantly, the solved IP-binding targets also fold in completely unrelated domains that lack sequence or structural similarities. For these reasons, an easily identifiable IP-binding motif has not been resolved, and there is currently no bioinformatic approach available to the search for novel IP-binding proteins. However, the basic residues are presumably critical for IP binding as shown in mutant studies of the tetrameric IP_3 receptor (Yoshikawa et al. 1996). Overall, the protein targets clearly recognize the specific phosphorylation state of the inositol ring and different isomeric forms. In the future, new structures of IP-

binding proteins will hopefully provide additional insights to allow general rules for governing IP binding and their impact in protein activity and function.

Molecular mechanisms for IP regulation of biological activities

The molecular means by which IPs modulate protein function are speculated to include (1) serving as an essential structural cofactor, (2) triggering allosteric or induced fit structural changes, or (3) by direct antagonistic substrate competition.

Two recent reports suggest that IP_6 functions as a required cofactor for proper protein folding. The human RNA-editing enzyme human adenosine deaminases acting on RNA (ADAR)2 and the F-box protein subunit of the ubiquitin–ligase complex SCF (Tir1) were both serendipitously cocrystallized with IP_6 that remained tightly associated throughout rounds of protein purification (Macbeth et al. 2005; Tan et al. 2007). Heterologous expression of hADAR2 in budding yeast showed that the production of IP_6 is essential for hADAR2 stability and activity (Macbeth et al. 2005). For Tir1, IP_6 interacts with several structurally important elements of the tertiary structure (Tan et al. 2007); however, an essential role for IP_6 in SCF–ubiquitin activity has not been directly demonstrated.

The allosteric potential for IPs in protein function has been elegantly demonstrated by classical studies with the tetrameric IP_3 receptor (Foskett et al. 2007), wherein IP_3 binding results in structural changes required for channel relaxation and endoplasmic Ca^{2+} release. It has also been shown that IP_3 and IP_7

function as antagonistic competitors of PIP binding by pleckstrin homology (PH) domain-containing proteins, in mammals and Dictyostelium, respectively (Hirose et al. 1999; Luo et al. 2003). This IP-PIP competition is not surprising given the obvious structural similarities between the IPs and the head groups of their lipid counterparts. Strikingly, others have shown that IP_4 has a positive impact on the plasma membrane recruitment of several PH domain-containing proteins (Huang et al. 2007). The authors suggest that this reflects an induced fit model by which IP_4 binding functions as a priming event to induce allosteric changes in PH domains and results in higher affinity-binding pockets for PIP_3. Undoubtedly, physiological and structural data on the modulation of IP targets such as Ku70/80 (Hanakahi and West 2002), and the Pho80–Pho85 CDK complex (Lee et al. 2007) will reveal additional clues on IP-mediated protein regulation.

Concluding remarks

mRNA export is a dynamic process that ties many steps in pre-RNA synthesis in the nucleus with protein translation in the cytoplasm. Understanding how this machinery operates is of great importance to human physiology and disease. Moreover, regulation of this machinery could efficiently be employed during cell signaling, growth, and adaptation; accelerating or inhibiting the export of specific messages as the cell demands. This work embarks upon the understanding of the conserved mechanism of mRNA export in the budding yeast *Saccharomyces cerevisiae.* It gives particular interest to the molecular

mechanisms by which the mRNA export factor Gle1 and the small molecule IP_6 regulate mRNA export. These studies conclude that Gle1 and IP_6 regulate the DEAD-box protein Dbp5 at the NPC. Furthermore, the molecular determinants in Gle1 that are required for IP_6-mediated Dbp5 activation *in vivo* and *in vitro* are identified. Local activation of Dbp5 by Gle1 and IP_6 is thought to remodel the mRNP to facilitate its directional transport and release in the cytoplasm. This study expands our understanding of nuclear mRNA export, allows us to draw striking comparisons to the protein export machinery, and provides a platform for understanding cellular physiology and disease.

CHAPTER II

IP_6 AND Gle1 REGULATE THE DEAD-BOX PROTEIN Dbp5 DURING mRNA EXPORT

Introduction

It is well established that the directionality of the import and export of nuclear proteins through the NPC is controlled by interactions between karyopherin transport factors and the GTPase Ran (Pemberton and Paschal 2005). In contrast, the export of most cellular mRNAs requires a novel transport factor Mex67 (also known as TAP and NXF1); reviewed in (Rodriguez 2004). Mex67 does not require RanGTP for function, but does bind mRNPs and a Mex67–Mtr2 heterodimer interacts with Nups. Thus, Mex67 may act as a bridge between the mRNP and the NPC. The directionality of mRNA transport is elegantly linked to controlled changes in the protein composition of the mRNP complexes. One set of factors involved in cotranscriptional mRNP assembly and mRNA processing is not present in the cytoplasm, and its specific removal from the nuclear mRNP is considered a prerequisite for export (Moore 2005). Other mRNA binding proteins are removed during the export process and are subsequently recycled back into the nucleus for future export events. It is likely that members of the DEAD-box helicase family mediate these mRNP protein remodelling steps at different stages along the mRNA biosynthetic and export

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This chapter is adapted from "Inositol hexakisphosphate and Gle1 activate the DEAD-box protein Dbp5 for nuclear mRNA export. Alcázar-Román AR, Tran EJ, Guo S, Wente SR. Nat. Cell. Biol. *2006* 8(7): 711-6"

pathway (Rocak and Linder 2004). The DEAD-box protein Dbp5 (also known as Rat8) shuttles between the nucleus and cytoplasm (Hodge et al. 1999) and is recruited to mRNA during transcription by interaction with components of the transcription elongation factor TFIIH (Estruch and Cole 2003). Immuno-EM studies of the Balbiani ring-particle mRNP export have also shown that Dbp5 stays associated with exporting transcripts from synthesis through to NPC translocation (Zhao et al. 2002). As Dbp5 interacts with Nup159 at the cytoplasmic fibrils of the NPC (Hodge et al. 1999; Schmitt et al. 1999; Weirich et al. 2004), it is predicted to act in mRNP remodelling specifically during NPC extrusion but the mechanism has not been defined.

We have focused our efforts on analysing the role of NPC-associated Gle1 (Kendirgi et al. 2003; Murphy and Wente 1996), a highly conserved, essential mRNA export factor. During our studies of Gle1 function, the enzymes responsible for the metabolic pathway that results in the production of soluble IP_6 by cleavage of membrane-anchored $PI(4,5)P_2$ and sequential inositide phosphorylation (Odom et al. 2000; York et al. 1999) in budding yeast were identified. This pathway requires three enzymes: Plc1, Ipk2 and Ipk1. IP_6 can also be converted to diphosphorylated inositols (such as diphosphoryl inositol 1,3,4,5,6 pentakisphosphate; PP-IP_5) by the enzyme Kcs1 (York et al. 2005). IPs are key regulatory molecules in many aspects of cell physiology and have been implicated in multiple protein–nucleic acid activities including chromatin remodelling, telomere length regulation and RNA editing (Macbeth et al. 2005;

Odom et al. 2000; Steger et al. 2003; York et al. 2005). The efficient export of mRNA specifically requires IP_6 production by the IP_5 2-kinase activity of Ipk1 (Moore 2005). To define the mechanism by which IP_6 production influences mRNA export, a systematic analysis of genetic interactions between the *ipk1* null (Δ) mutant and a panel of mutant genes encoding known Nups and nucleocytoplasmic transport factors was conducted (Miller 2004). A specific subset of mutants was identified, all of which are directly linked to Gle1 and Dbp5 function and physically localized to a NPC substructure on the cytoplasmic fibrils. Here we identify a role for Gle1 and IP_6 in the regulation of Dbp5 for mRNA export.

Materials and methods

Yeast media and manipulations

Strains were grown in YPD (1% yeast extract, 2% peptone, 2% glucose) at 23 ˚C unless otherwise noted. Synthetic media lacking appropriate amino acids was supplemented with 2% Glucose (SD). Media containing drugs for plasmid loss or drug resistance were supplemented as follows: 5-Fluoroorotic acid (5-FOA; US Biological) was used at 1.0 mg/ml; G418 (US Biological) was used at 200 μg/ml. Yeast cells were transformed using a lithium acetate method (Ito et al. 1983). Genetic manipulations including yeast matings, dissections, and selections of desired yeast strains were done following standard techniques (Sherman et al. 1986).

Yeast strains

A list of strains used in this study in is Table 2. For strain SWY3547, the *DHH1* gene was deleted by amplifying the kanamycin resistance gene using oligonucleotide primers that included sequence complementary to the flanking regions of *DHH1*. The PCR product was transformed into a wild-type diploid strain and kanamycin resistant colonies were sporulated and dissected. The *dhh1Δ* spores were confirmed by PCR-based analysis and rescue of temperature sensitivity by transformation with a *DHH1* harboring plasmid (pSW3177). Double mutants used were generated by mating, sporulation and dissection of tetrads.

Multicopy suppresor screen

For the high copy suppression screen, the *S. cerevisiae* strain SWY2114 (Miller 2004) was transformed with a genomic library in vector Yep13 (*LEU2/2μ*) (Nasmyth and Tatchell 1980). The *ipk1Δ nup42Δ* cells transformed with a yeast genomic library were plated on selective media, grown at 30 °C for 2 hours and then at 37 °C for seven days. Approximately 21,000 transformants were screened and the 48 resulting colonies were retested for growth and plasmid dependence. Plasmids from positive isolates were analysed by DNA sequencing. The strongest specific isolate contained a genomic fragment from chromosome IV spanning 7.2 kb that harbored *DHH1* (pSW3175). Plasmids with individual open reading frames (ORFs) were generated and direct tests were conducted by transforming the *ipkΔ nup42Δ* strain and assaying for growth rescue.

Plasmids

The plasmids used in this study are detailed in Table 2. The *pIPK1/URA3* (pSW1272) was constructed by PCR amplification of *IPK1* from a library plasmid isolate using oligonucleotides complementary to pRS316 flanking sequences. The PCR product was cloned by gap-repair into the BamHI and SalI sites in a pRS316 vector containing *ADE3* (pSW1157). The plasmid for bacterial expression of GST-Dbp5 (pSW1319) was constructed by PCR amplification of the *DBP5* coding region from genomic yeast DNA with gene-specific oligonucleotide primers flanked by BamHI restriction sites. The resulting PCR product was subcloned into the BamHI site of pGEX-5X-3 (GE Healthcare). Full length wildtype *DHH1 2μ* (pSW3177) and *PDE2 2μ* (pSW3181) plasmids were constructed by PCR amplification from library plasmid isolate with oligonucleotides primers flanked with BamHI and Sal1 sites. The PCR product was subcloned into pRS425 (Christianson et al. 1992). The *dhh1-E196Q 2μ* plasmid (pSW3180) was constructed by oligonucleotide-directed *in vitro* mutagenesis of pSW3177. Expression of Dhh1-E196Q protein was confirmed by testing for rescue of temperature sensitive *dhh1Δ* (SWY3547) mutant cells at 30 ˚C.

Protein expression and purification and IP_6

Recombinant maltose binding protein (MBP)–Gle1 (pSW449) and GST–Dbp5 (pSW1319) fusion proteins were expressed in *Escherichia coli* Rosetta (DE3) cells (EMD Biosciences). MBP–Gle1 was purified by affinity chromatography using amylose resin (New England Biolabs) according to manufacturers' instructions followed by SP FastFlow (Sigma) ion exchange chromatography. Purified MBP–Gle1 was dialysed against Buffer B (20 mM HEPES at pH 7.5, 150 mM NaCl, 20% w/v glycerol). GST–Dbp5 was affinity purified using glutathione resin (GE Healthcare). GST-tag removal was performed by cleavage with Factor Xa (New England Biolabs) during dialysis against Buffer B and untagged Dbp5 was isolated by a second passage over glutathione resin. IP_6 was prepared by resuspending phytic (Sigma) acid in 50 mM Hepes-HCL pH 7.5 and adjusting pH with 10 N NaOH to reach ~pH 7.5. pH was tested by placing a drop of solution on pH paper indicator (Litmus) and comparing to standards. IP_6 solution was stored at 4 ˚C and prepared fresh every 3 months.

ATPase assays and kinetic analysis

Dbp5 ATPase assays were conducted in a 10 μl total volume of Buffer A (16 mM HEPES at pH 7.5, 120 mM NaCl, 3 mM MgCl2, 16% w/v glycerol, 1 mg ml-1 BSA) supplemented with 1 U/μl SUPERasin (Ambion) and 1 mM DTT.

Recombinant proteins, IP_6 (Sigma) and either a 25 mer poly(A)$^+$ RNA oligonucleotide (Dharmacon, Layfayette, CO) or a 31 mer ssRNA (5'-AUGUUGUUAUAGUAUCCCACCUACCCUGAUG-3') (kind gift from Ron Emeson) were used as indicated, followed by addition of unlabelled ATP and 1 Ci -^{32}P-ATP (3000 Ci mmol-1; PerkinElmer LAS, Wellesley, MA). The amounts of recombinant proteins used, and the incubation times, were varied to maintain substrate conversion within a linear range. Reactions were incubated for 10 min at 30 °C, unless otherwise noted, and stopped with 2μl 50 mM Tris at pH 7.4, 5 mM EDTA, 1.5% SDS and 2 mg ml-1 proteinase K (Ambion). After 30 min at 37 °C, sample fractions were spotted onto PEI-cellulose thin-layer chromatography sheets (Mallinckrodt Baker) and separation of ADP and ATP was achieved by incubation in 0.6 M potassium phosphate at pH 3.4. Radioactivity was detected using a Typhoon 9200 Imager and quantified using Image Quant v. 5.2 (Molecular Dynamics, Inc.). Apparent kinetic parameters and EC-50 values were calculated using GraphPad Prism v.4 software. For EC-50 determination, data was fit using a dose-response curve with variable slope and setting top and bottom constraints at 100% and 0% activation, respectively. Dbp5 catalytic rates were obtained by considering the initial reaction rates and fitting the data to the Michaelis-Menten equation saturation plot. All kinetic and EC-50 measurements were calculated using a least square approximation for data fitting.

IP_6 binding and stability assays

A modified polyethylene glycol (PEG)-precipitation assay was used to measure IP_6 binding (Shears 1997). In brief, 30 μl binding reactions were conducted with 10 nM ^{3}H-IP_6 (21.4 Ci mmol-1; PerkinElmer LAS) in Buffer A and recombinant purified MBP–Gle1 and/or Dbp5. As indicated, reactions were performed in the presence of RNA (25 mer poly(A)$^+$ RNA oligonucleotide; Dharmacon), ATP γ-S (Roche, Indianapolis, IN) and competing unlabelled inositides — IP_6 (Sigma), IP_5 (Matreya Inc., Pleasant Gap, PA) and IS_6 (Sigma). Samples were incubated for 30 min at 30 °C and proteins were precipitated by adding 21 μl of 30% PEG 3350 (Sigma), followed by incubation on ice for 10 min. Precipitated proteins with bound ^{3}H-IP_6 were separated by centrifugation for 20 min at 35,000*g* at 4 °C. The supernatant was aspirated and the pellets were solubilized with 300 μl 1% SDS. Radioactivity was measured by scintillation counting. To analyse IP_6 stability during Dbp5 ATP hydrolysis, ATPase reactions were conducted (30 °C, 2 h, 500 nM Dbp5, 250 nM Gle1, 1 μM ^{3}H-IP_6 (3 nCi pmol-1)) and stopped by addition of 100 μl 0.5 M HCl. The ^{3}H-IP_6 levels were analysed by HPLC with a Partisphere strong-anion exchange column as previously described (York et al. 1999).

Analysis of steady-state GFP import cargoes and mRNA export defects

Wild-type and mutant strains containing reporters pGAD-GFP (cNLS-GFP)

or pSpo1276-130-GFP (Spo12-NLS-GFP) were grown to mid-log phase at 23°C or 30°C. Cultures were shifted to 36°C and processed as previously described (Strawn et al. 2004). The localization of poly(A)$^+$ RNA was analyzed by growing strains in rich or synthetic medium at 23°C prior to shifting to 36°C for one hour. *In situ* hybridizations were performed using an digoxigenin-oligo (dT)30 probe as previously described (Iovine et al. 1995). Nuclei were visualized by 4',6-diamidino-2-phenylindole (DAPI) staining. Cells were observed using a fluorescent microscope (model BX50; Olympus, Lake Success, NY) using an Uplan 100x/1.3 objective. Images were taken using a digital camera (Photometrics Cool Snap HQ) and process using MetaVue software (Universal Imaging) and Adobe Photoshop 7.0.

Results

IP_6 and not IP_7, is specifically required for proper mRNA export *in vivo*

Genetic interactions between *gle1* or *dbp5* mutants and the *kcs1Δ* mutant, which lacks production of PP-IP_5 from IP_6, were examined (Figure 9 A). In contrast with the lethality of *ipk1Δ gle1-2* and *ipk1Δ dbp5-2* double mutants, *kcs1Δ gle1-2* and *kcs1Δ dbp5-2* mutants were viable. This supports our hypothesis that IP_6 is directly required for mRNA export. In addition, there are no genetic interactions between the *ipk1Δ* mutant and *mex67* mutants (Miller 2004), indicating that IP_6 and Mex67 execute two separate steps in the mRNA export pathway. A recent

report has linked IP_6 production with the activity of the tRNA editing enzyme Tad1 in budding yeast (Macbeth et al. 2005). We found there is no enhanced sickness interaction between *tad1Δ* and *gle1-2* mutations suggesting that IP_6 works independently in mRNA export and editing (data not shown).

ipk1Δ nup42Δ mutant cells have temperature sensitive growth and mRNA export defects

To gain further insight into the function of IP_6 in mRNA export, the conditional *ipk1Δ nup42Δ* double mutant strain was characterized. At the non-permissive growth temperature, mRNA export was defective (Figure 9 B). Interestingly, these defects were most prominent in rich media when compared to synthetic media (Figure 9 D). These results could suggest a potential regulation of mRNA export in different growth conditions. Such adaptations could have evolved to allow yeast colonies adjust growth and metabolic rates when found in less ideal environmental conditions in nature. In fact, bulk poly(A)$^+$ mRNA is retained in the nucleus through an unknown mechanism during heat shock (Saavedra et al. 1997) and the mRNA export factor Dbp5 undergoes reversible changes in its localization during ethanol stress (Takemura et al. 2004), a condition in which cells also retain mRNA in the nucleus. We further characterized the *ipk1Δ nup42Δ* double mutant and found no perturbations in the steady-state localization of Gle1, Dbp5, or Mex67, or the nuclear import of protein cargoes by the nuclear transport factors, Kap95 and Kap121 (data not shown).

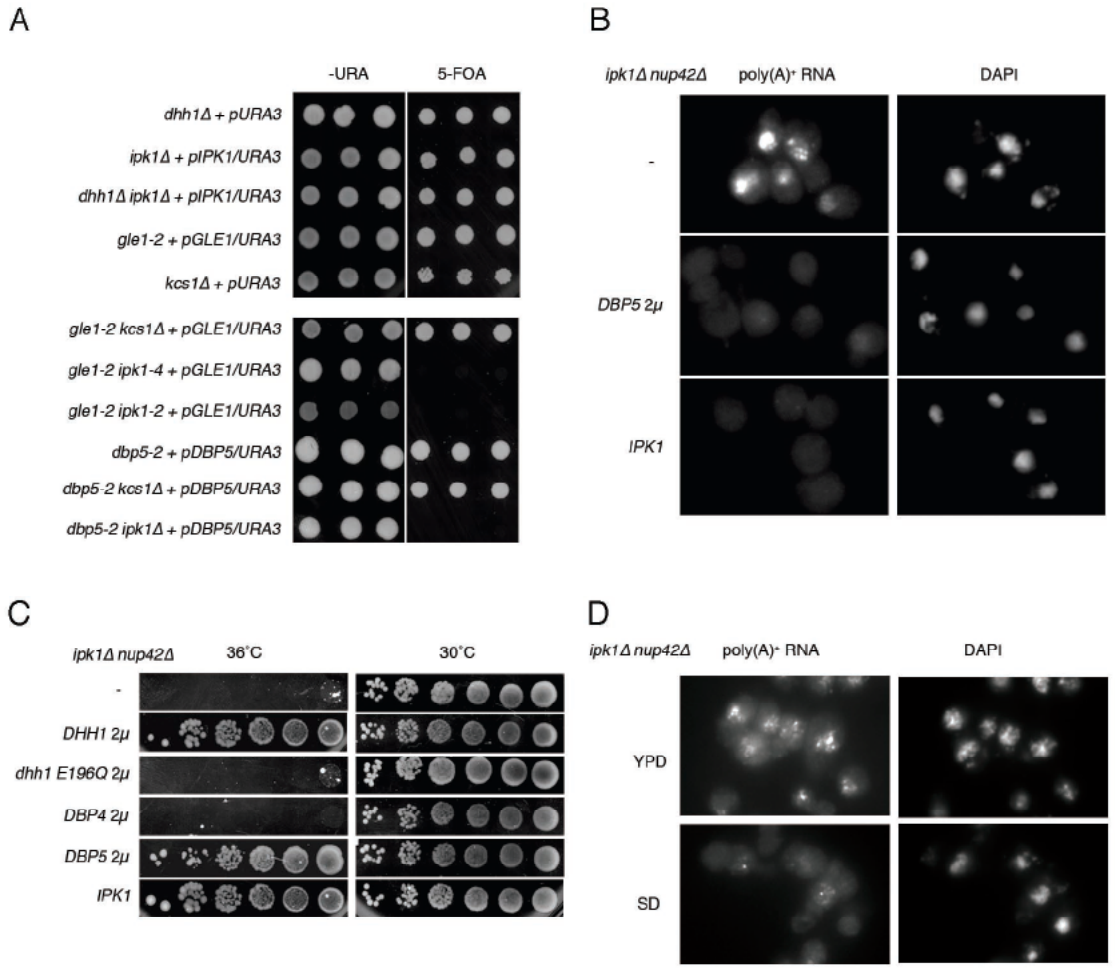

Figure 9: *DBP5* overexpression rescues the mRNA export and growth defects of the *ipk1Δ nup42Δ* mutant. (A) Yeast strains containing the indicated plasmids were spotted in 1:1 serial dilutions and grown on synthetic minimal media lacking uracil (–URA) or synthetic complete media containing 1.0 mg ml 5-FOA at 23 °C. (B) *In situ* hybridization with a digoxigenin-coupled oligo(dT) probe was conducted after shifting to growth at 36 °C for 1 hour. Poly(A)$^+$ RNA localization was visualized by indirect immunofluorescence microscopy with a FITC-coupled anti-digoxigenin antibody (left) and nuclei were visualized with DAPI staining (right). (C) The *ipk1Δ nup42Δ* strain was transformed with either an empty plasmid (-), a plasmid harboring *IPK1*, or a *2μ* plasmid harboring *DHH1, dhh1E196Q, DBP4* or *DBP5–Myc*. Cells were spotted in fivefold serial dilutions and incubated at 30 or 36 °C. (D) *In situ* hybridization was conducted as in (A) after shifting to growth at 37 °C for 1 hour. Cells were either grown in complete rich media (YPD), or synthetic media (SD).

Overexpression of *DBP5* rescues both growth and mRNA export defects of *ipk1Δ nup42Δ* cells

As Nup42 provides the only reported NPC interaction site for yeast Gle1 (Murphy and Wente 1996; Rollenhagen et al. 2004; Strahm et al. 1999) we predicted this strain was compromised in two specific ways and could be used to isolate mRNA export factors that required both IP_6 production and efficient Gle1 localization at the NPC cytoplasmic fibrils. A genetic screen was conducted in *Saccharomyces cerevisiae* for high-copy extragenic suppressors of the *ipk1Δ nup42Δ* double mutant temperature-sensitive lethality. A yeast 2*μ* genomic library was transformed into the *ipk1Δ nup42Δ* cells and colonies that grew at the non-permissive temperature were selected. Two of the genes identified, *PDE2* and *SSD1*, were tested independently for their ability to interact with other mRNA export factors genetically (Figure 10). Interestingly, overexpression of *PDE2* also rescues the lethality of *gle1-2 ipk1-5* mutant strains (Figure 10 A-B), but not *dbp5-2 ipk1Δ* cells (data not shown). *PDE2* is one of two cyclic AMP (cAMP) phosphodiesterase enzymes that regulate cAMP levels in yeast (Mitsuzawa 1993). Additionally, *PDE2* has been found to have genetic interactions with *SSD1* (Matsuura and Anraku 1994), also isolated in this screen, and *GLC7 (Uesono et al. 1997)*, a protein phosphatase with roles in many processes including mRNA export. *SSD1* overexpression rescues the defect of *mex67-5, ipk1Δ nup159-1,* and *ipk1Δ nup116Δ* mutants (Figure 10 C-D). However, it did not rescue the defects of *nup116Δ, nup159-1, dbp5-2* or *gle1-2* mutant strains (data not shown). *SSD1* is a polymorphic locus with numerous genetic interactions (Kaeberlein et

al. 2004). Importantly, two alleles, designated *SSD1-V* and *ssd1-d*, have been identified for *SSD1* and are found in laboratory and natural isolates. The *ssd1-d* allele is present in the parental strains used in this screen, and it is likely to produce a nonfunctional Ssd1 protein.

One novel high-copy plasmid was further characterized and found to harbour *DHH1*, the gene that was necessary and sufficient for rescue of lethality of *ipk1Δ nup42Δ* mutants (Figure 9 C). Dhh1 is a cytoplasmic DEAD-box helicase that stimulates mRNA decapping (Rocak and Linder 2004). To test whether the Dhh1 ATPase activity was required, a catalytically deficient *dhh1-E196Q* mutant was generated. This mutant did not rescue the *ipk1Δ nup42Δ* temperature-sensitive growth (Figure 9 C). It has been reported that *dhh1* mutants enhance the lethality of *dbp5* mutants, suggesting functional overlap between Dhh1 and Dbp5 (Tseng-Rogenski et al. 2003). In direct tests, it was found that *DBP5* overexpression suppressed the *ipk1Δ nup42Δ* growth defect (Figure 9 C). Interestingly, *DBP5* overexpression also rescued the mRNA export defects (Figure 9 B), whereas *DHH1* did not (data not shown). Overexpression of *DBP4* (an unrelated DEAD-box helicase) (Rocak and Linder 2004), *GLE1* or *GFD1* (a Gle1 and Dbp5 interacting protein) (Hodge et al. 1999; Strahm et al. 1999) did not rescue the *ipk1Δ nup42Δ* lethality (Figure 9 B) and data not shown). The *dhh1Δ ipk1Δ* double mutant was viable (Figure 9 A), whereas the *dbp5-2*

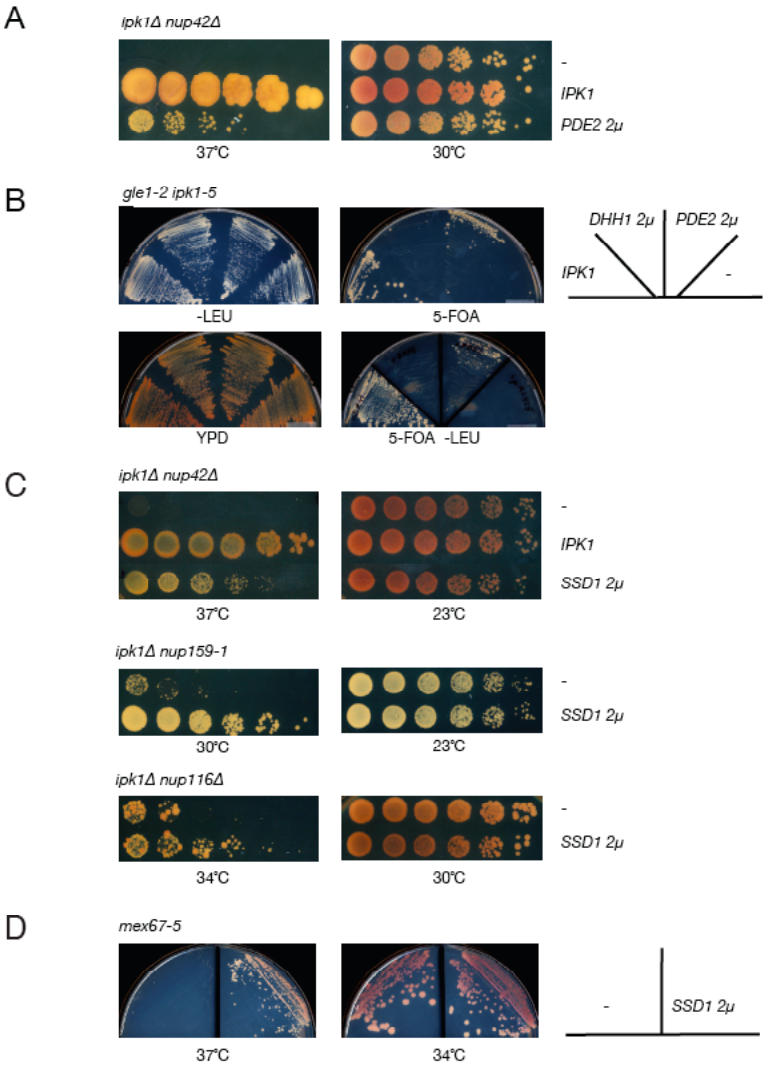

Figure 10: *PDE2* and *DHH1* partially rescue the growth defects of the *ipk1Δ nup42Δ* mutant. (A) The *ipk1Δ nup42Δ* strain was transformed with either an empty plasmid (-), a plasmid harboring *IPK1*, or a *2μ* plasmid harboring *PDE2*. Cells were spotted in fivefold serial dilutions and incubated at 30 or 37 °C. (B) A *gle1-2 ipk1-5* strain harboring a *GLE1/URA3* plasmid was transformed with empty plasmid (-), a plasmid harboring *IPK1* or *2μ* plasmid harboring *DHH1/LEU2* or *PDE2/LEU2*. Resulting strains were streaked at 23˚C in rich media (YPD), synthetic minimal media lacking leucine (-LEU), synthetic media containing 5-FOA, or synthetic media lacking leucine and containing 5-FOA. (C) The *ipk1Δ nup159-1* and *ipk1Δ nup116Δ* strains were transformed with either an empty plasmid (-) or a *2μ* plasmid harboring *SSD1*. Additionally, the *ipk1Δ nup42 Δ* mutant was transformed with a plasmid harboring *IPK1*. Cells were spotted in fivefold serial dilutions and incubated at 23, 30, 34, and 37˚C (best rescuing temperature shown). (D) A *mex67-5* mutant strain was transformed with either and empty plasmid (-) or a *2μ* plasmid harboring *SSD1* and streaked on –LEU at 34˚ and 37˚C.

ipk1Δ mutant is inviable at all growth temperatures (Miller 2004). This suggested that bypassing the mRNA export defect in the *ipk1Δ nup42Δ* mutant specifically required Dbp5. We concluded that the partial rescue of the *ipk1Δ nup42Δ* mutant by overexpressing *DHH1* was indirect and due to either the partial functional overlap between Dbp5 and Dhh1 (Tseng-Rogenski et al. 2003), or due to altered flux in the global mRNA biosynthetic pathway and potential connections between mRNA export and turnover (Moore 2005). Based on these results and published genetic data tightly linking *GLE1*, *IPK1* and *DBP5* function (Hodge et al. 1999; Miller 2004; Strahm et al. 1999; York et al. 1999), we speculated that IP_6 and Gle1 were directly required for Dbp5 activity.

IP_6 and Gle1 stimulate Dbp5 ATPase activity *in vitro*

To test this hypothesis, an *in vitro* system for assaying the RNA-dependent ATPase activity of Dbp5 was established using bacterially expressed purified factors (Figure 11 A). ATPase reactions were determined to be dose and time dependent. It has been reported that purified recombinant Dbp5 has ATPase activity; however, *in vitro* unwinding of RNA was demonstrated only with Dbp5 in cell extracts, suggesting unidentified cellular cofactors are required for optimal function (Schmitt et al. 1999; Tseng et al. 1998). Recombinant Dbp5 (500 nM) did have ATPase activity (Figure 11 B). Addition of only IP_6 had no effect on the RNA-dependent Dbp5 ATPase activity level; however, Gle1 addition alone resulted in twofold activation of Dbp5 (Figure 11 B). Gle1 alone, or Gle1 plus IP_6,

had no apparent ATPase activity (data not shown). Surprisingly, addition of both Gle1 (250 nM) and IP_6 (100 nM) stimulated the Dbp5 ATPase activity nearly fivefold (Figure 11 B). The same effects were observed with either a heteropolymeric ssRNA (data not shown) or a poly(A)$^+$ ssRNA. The half-maximal effective concentration (EC-50) for IP_6 activation was 27.3 nM, with a 95% confidence interval from 20.3–36.7 nM at 500 nM Dbp5 and 250 nM Gle1 (Figure 11 C). At higher IP_6 concentrations the activation data did not fit a simple sigmoidal curve and this may reflect an additional level of complexity. In comparison, IP_5 was significantly less effective at all concentrations tested (see Figure 12 and below). This correlated with prior observations that the elevated IP_5 level in *ipk1Δ* cells did not rescue *dbp5* or *gle1* mutants, and indicated that specific stimulation of the ATPase activity of Dbp5 by IP_6 is the physiologically relevant mechanism. Michaelis-Menten saturation kinetics were determined for Dbp5-driven ATP hydrolysis in the presence and absence of Gle1 and/or IP_6 (Figure 11 D). The apparent k_{cat} was as follows: for Dbp5 alone it was 0.10 +/- 0.01 sec^{-1}; for Dbp5 + Gle1 it was 0.15 +/- 0.02 sec^{-1}; and for Dbp5 + Gle1 + IP_6 it was 0.29 +/- 0.02 sec^{-1}. Thus, optimal activity required the presence of both Gle1 and IP_6. Under the same conditions plus 1 mM ATP, the RNA concentration for half-maximal activity was also markedly impacted (Figure 11 E). With Gle1 (250 nM) and IP_6 (100 nM), approximately 470 nM RNA was required for half-maximal activity. This was at least twentyfold lower in direct comparison with addition of

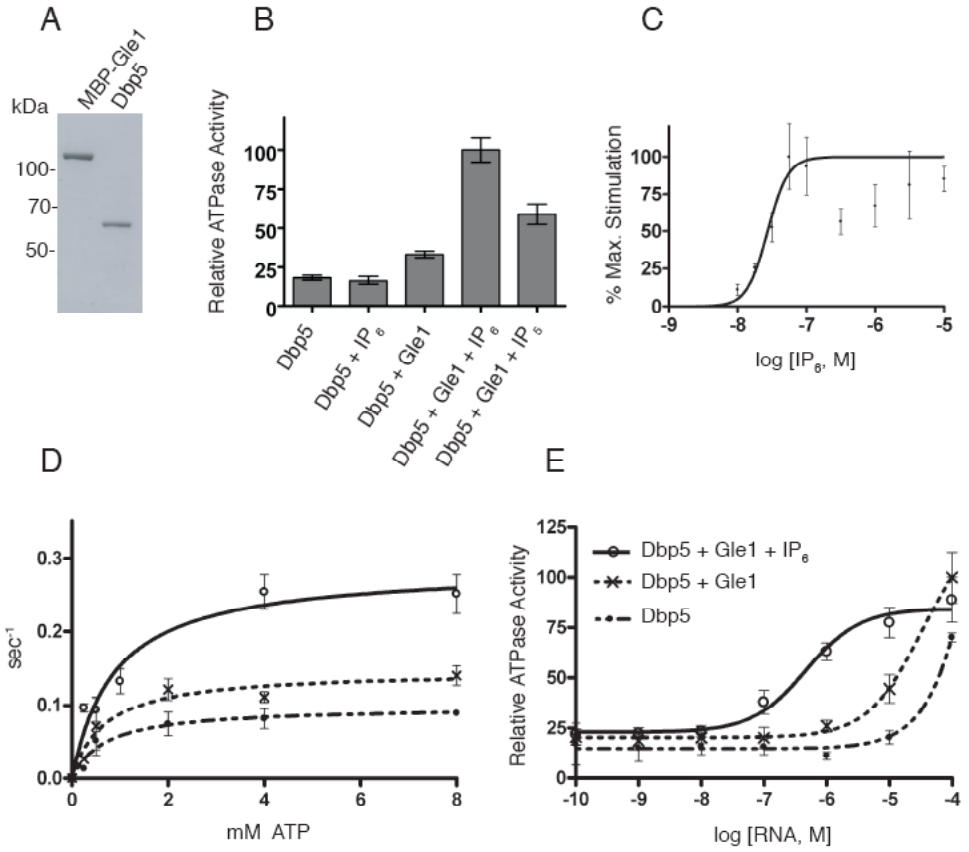

Figure 11: (A) Gle1 and IP_6 stimulate the ATPase activity of Dbp5. Bacterially expressed recombinant MBP–Gle1 and Dbp5 were separated on a 7.5% SDS-polyacrylamide gel and stained with Coomassie. (B) Dbp5 ATPase assays (500 nM Dbp5, 1 mM ATP and 1 μM RNA with 250 nM Gle1 and/or 100 nM IP_6 or IP_5) were conducted in triplicate with hydrolysis levels normalized relative to the highest mean level (percentage) in the assay set. (C) Dose-response curves showing the effect of IP_6 concentration on the RNA (1 μM) dependent Dbp5 (500 nM) ATPase activity in the presence of Gle1 (250 nM). Triplicate assay data for each concentration point were normalized relative to the highest hydrolysis level in the assay set (percentage). (D) Dependence of Dbp5 ATP hydrolysis rate on ATP concentration through Michaelis-Menten saturation kinetic experiments utilizing 500 nM Dbp5, 1 μM RNA with 250 nM Gle1 and/or 100 nM IP_6. (E) Dependence of Dbp5 ATPase activity on RNA concentration with concentration of factors as in (D) with 1 mM ATP. The mean and s.e.m. were calculated from three independent experiments in (B–E).

Gle1 alone, which required an RNA concentration in the 10–100 μM range. Gle1 and IP_6 work together to increase the catalytic efficiency of Dbp5 and decrease the RNA concentration threshold needed for Dbp5 activity.

IP_6 binds Gle1-Dbp5

To analyse the mechanism of IP_6 activation, IP_6 binding was tested in an equilibrium-binding assay with purified recombinant Gle1 and Dbp5. Although Gle1 alone bound IP_6, maximal binding required both Gle1 and Dbp5 (Figure 12 A). Binding was specific, as increasing the concentration of unlabelled IP_6 effectively competed for radiolabelled ^{3}H-IP_6. In contrast, increasing the level of unlabelled IP_5 or inositol hexakissulphate (IS_6) did not decrease binding of IP_6 (Figure 12 B). To test the effect of Dbp5 substrates, IP_6 binding was analysed in the presence of RNA and/or the non-hydrolysable analogue ATPγ-S. As shown in (Figure 12 C), no significant difference was noted suggesting IP_6 binding to Gle1–Dbp5 does not require Dbp5 catalytic activity. To test whether IP_6 was altered during the assays, the Dbp5 ATPase reaction was carried out for 2 hours in the presence of 1 μM ^{3}H-IP_6 and Gle1. The inositide composition was analysed by HPLC and the IP_6 level was not altered under these conditions (Figure 12 D). Thus, we conclude that IP_6 is a stable binding partner that affects Gle1 and Dbp5.

Discussion: New model for mRNA export

Taken together, we have defined a role for Gle1 and IP_6 in activating Dbp5 during mRNA export. We conclude that Gle1 and IP_6 are previously uncharacterized Dbp5 cellular cofactors for stimulating its ATPase activity. We speculate that Gle1 and IP_6 may also be required for Dbp5 RNA unwinding and/or the displacement of proteins from RNA–protein complexes. Our previous studies showed that Gle1 is recruited to the NPC independent of Dbp5 and Mex67 (Strawn et al. 2001) and it has also been shown that the Nup159–Dbp5 interaction is key to Dbp5 activity in mRNA export (Hodge et al. 1999; Schmitt et al. 1999; Weirich et al. 2004). We speculate that mRNA export is dependent on the juxtaposition of Gle1 and Dbp5 at the cytoplasmic NPC fibrils during mRNP extrusion (Figure 13). The respective binding sites on Nup42 and Nup159 would effectively serve as an interaction scaffold and facilitate this corecruitment. Indeed, under heat shock conditions in *nup42Δ* cells, NPC localization of both Gle1 and Dbp5 is diminished coincident with a block in mRNA export (Rollenhagen et al. 2004).

These results provide evidence for spatial control of the mRNA export mechanism that may aid the establishment of directionality in mRNA export. It is predicted that DEAD-box proteins use the hydrolysis of ATP to either unwind RNA duplexes or dissociate RNA–protein complexes (Rocak and Linder 2004).

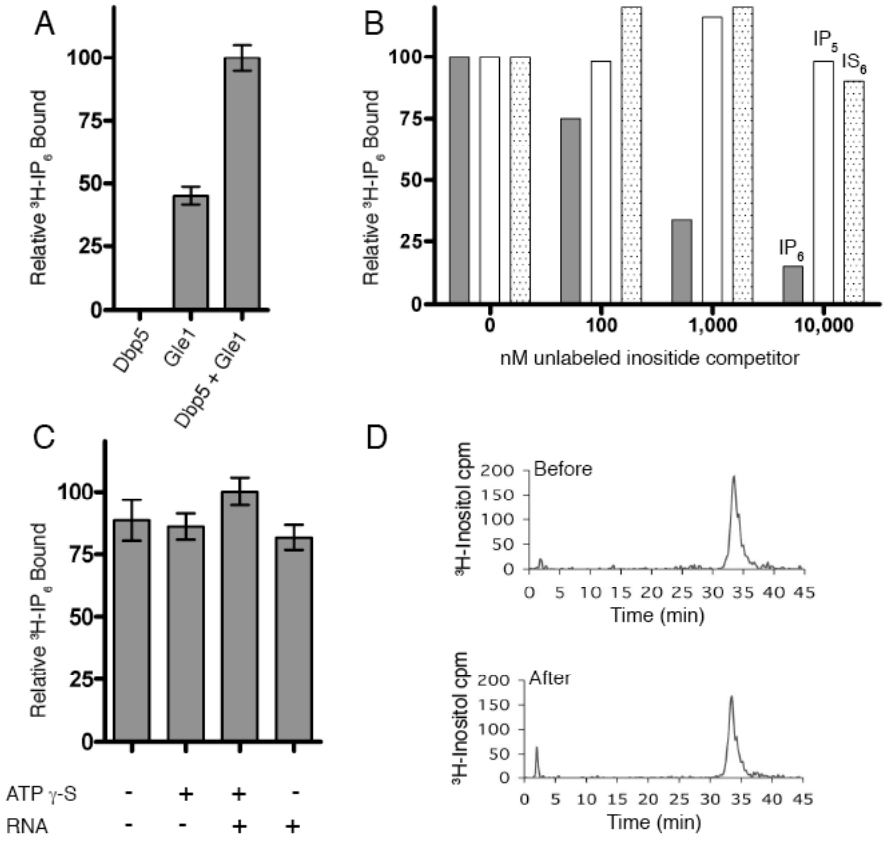

Figure 12: (A) Gle1 binds IP_6. IP_6 binding assays were performed with 500 nM Dbp5, 250 nM Gle1 and 10 nM ^3H-IP_6 as indicated. (B) Competition binding assays were performed as in (A), with the addition of unlabelled inositides as indicated. (C) Effect of cofactors on IP_6 binding was assayed by conducting incubations as in (A) with the addition of 1m M ATP-γS and/or 1 μM RNA. The percentage bound IP_6 in (A-C) was calculated by subtraction of background binding (BSA only), divided by the maximum bound in the given data set, and the mean and s.e.m. were calculated from three independent experiments. (D) High Pressure Liquid Chromatography analysis of ^3H-IP_6 stability before and after a Dbp5 ATPase assay. The mean and s.e.m. were calculated from three independent experiments in (A–C).

To date, two DEAD-box protein family members have been shown to remove or displace proteins from RNA (Fairman et al. 2004; Jankowsky et al. 2001). It has also been proposed that Dbp5 acts to remodel the mRNP protein composition during export (Schmitt et al. 1999; Snay-Hodge et al. 1998; Tseng et al. 1998). Recent work showing Dbp5 is a necessary component for proper regulation of RNA-bound versus free Mex67 provides supporting evidence for this model (Lund and Guthrie 2005). Gle1 and IP_6 activation of the Dbp5 ATPase may provide an input of energy to drive the release of critical mRNA binding proteins.

Yra1, Mex67 and Nab2 are candidates for release (Lund and Guthrie 2005; Windgassen et al. 2004), whereas Npl3 is a candidate for being retained as a component of cytoplasmic mRNPs (Moore 2005; Windgassen et al. 2004). Such a bias between two different mRNP composition or conformational states may drive transport in a directional manner analogous to the classic Brownian ratchet. This work also reveals striking parallel control mechanisms for dictating transport directionality in both the Kap and mRNA export pathways — at the final export step the Kap pathways require a cytoplasmic GAP for stimulating Ran GTPase activity (Pemberton and Paschal 2005). Here, we show that Gle1 and IP_6 can work as ATPase activating factors (AAFs) for stimulating Dbp5 at the NPC cytoplasmic face.

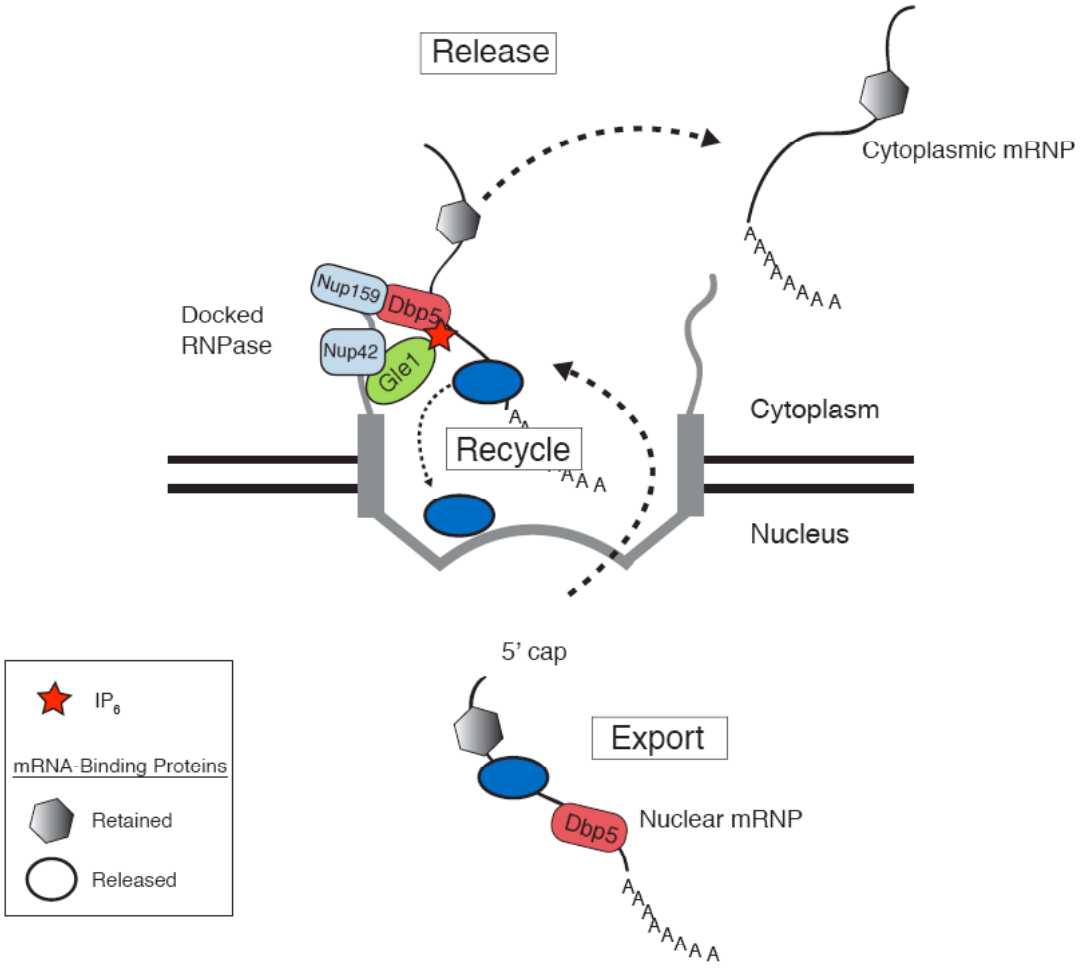

Figure 13: Dbp5 associates with nascent transcripts in the nucleus and is recruited to the cytoplasmic NPC fibrils by Nup159 interaction. This juxtaposes Dbp5 and Gle1 — docked to the NPC through Nup42 — allowing maximal IP_6 binding and activation of the Dbp5 ATPase activity. This activity may facilitate the release of mRNA binding proteins, or export factors, from the exporting mRNP. Triggering release and recycling of select mRNA binding proteins would impart directionality by shifting the mRNP preferentially to the cytoplasmic mRNP state during a terminal export step.

Interestingly, Dbp5, Gle1 and IP_6 are not exclusively localized to the NPC. Dbp5 shuttles between the nucleus and cytoplasm and interacts with components of the transcriptional elongation complex factor TFIIH (Estruch and Cole 2003) and Yra1, a nuclear mRNP protein involved in mRNA processing prior to export (Schmitt et al. 1999). There is evidence that human Gle1 also shuttles between the nucleus and the cytoplasm and interacts with both the vertebrate Nup42 orthologue (hCG1) and a different Nup, human Nup155 (Kendirgi et al. 2003; Kendirgi et al. 2005; Rayala et al. 2004). Yeast Gle1 and Dbp5 both independently bind Gfd1, a non-essential cytoplasmic protein that associates with the shuttling mRNP protein Nab2 (Hodge et al. 1999; Strahm et al. 1999; Suntharalingam et al. 2004), and IP_6 and soluble inositides are capable of diffusing throughout the cell (Miller 2004). Such localization at multiple cellular sites may result in Gle1–IP_6 control of Dbp5 activation at other steps in the mRNP biosynthesis, processing, export, and/or turnover pathway.

Total cellular IP_6 levels are presumably high (in the micromolar range) (Shears 2001); however, the concentration of the free IP_6 pool is unknown. We found that altering IP_6 levels in the cell can impact Gle1 function and mRNA export. The complete lack of IP_6 production, in addition to lowered IP_6 levels in a catalytically compromised *ipk1-5* mutant, are not sufficient for function of a *gle1-2* mutant (Ives et al. 2000; York et al. 1999). Conversely, elevated IP_6 levels rescued a *gle1-4* mutant (York et al. 1999). Although *ipk1* cells are viable, growth is compromised at elevated temperatures (York et al. 1999). Thus, we speculate

that changes in IP_6 levels may impact on mRNA export *in vivo* and allow highly efficient export under different growth conditions. Alternatively, the ability of IP_6 to activate Dbp5 in a Gle1-dependent manner may reflect a general property of highly phosphorylated inositides to serve as enzyme cofactors. This is consistent with a recent report showing that IP_6 is required for the activity and stability of adenosine deaminases that act on RNA (Macbeth et al. 2005). Dbp5 is a member of a large family of DEAD-box proteins (including approximately 30 in budding yeast) that have proposed functions in a range of RNA processing events, including ribosome biogenesis, mRNA splicing, mRNA export, mRNA translation and mRNA turnover (Rocak and Linder 2004). The mechanisms for regulating many of the DEAD-box proteins have not determined. Our findings suggest that these other cellular processes may follow this emerging paradigm and be controlled by soluble inositol polyphosphates.

Table 2: Yeast strains and plasmids used in Chapter II

Strain Name	Genotype	Source
SWY2114	*MATa leu2 ura3 his3 ade2-1 trp1-1 ipk1::KAN^R nup42::HIS3*	(Miller et al., 2004)
SWY2649	*MATa leu2 ura3 his3 trp1-1 can1-100 ipk1::KAN^R nup159-1*	(Miller et al., 2004)
SWY2246	*MATa leu2 ade2-1 ura3 his3 trp1-1 can1-100 ipk1::KAN^R nup116::HIS3*	(Miller et al., 2004)
SWY2117	*MATα ade2-1 ade3::hisG ura3-1 his3-11,15 trp1-1 leu2-3,112 lys2 ipk1::KAN^R*	This Study
SWY3547	*MATα ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100 dhh1::KAN^R*	This Study
SWY3608	*MATa ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3,112 ipk1::KAN^R dhh1::KAN^R*	This Study
LSY541	***MATa ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100 kcs1::HIS3***	(York et al 2005)
SWY1181	*MATα ade2-1 ura3-1 his3-11,15 leu2-3,112 lys2 gle1-2*	This Study
CSY550	*MATa leu2Δ1 trp1Δ63 ura3-52 dbp5-2*	(Snay-Hodge et al., 1998)
SWY1793	*MATa ade2-1 ade3 ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100 gle1-2 ipk1-4* *	(Miller et al., 2004)
SWY1835	*MATα ade2-1 ade3 ura3-1 his3-11,15 leu2-3,112 lys2 gle1-2 ipk1-2* *	This Study
SWY1837	*MATα ade2-1 ade3 ura3-1 his3-11,15 leu2-3,112 lys2 gle1-2 ipk1-5* *	This Study
SWY3467	*MATa ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3,112 gle1-2 kcs1::HIS3*	This Study
SWY2642	*MATα ura3 his3 trp1 leu2 can1-100 dbp5-2 ipk1::KAN^R* *	This Study
SWY3474	*MATa ura3 his3 trp1 leu2 dbp5-2 kcs1::HIS3*	This Study
SWY2287	*Mata ura3 trp1 leu2 mex67::HIS3 + MEX67/TRP1*	This Study

Plasmid Name	Description	Source
pRS316	*CEN/URA3*	(Christianson et al., 1992)
pRS315	*CEN/LEU2*	(Christianson et al., 1992)
pSW1273	*IPK1/ADE3/CEN/LEU2*	(Miller et al., 2004)
pSW1272	*IPK1/ADE3/CEN/URA3*	This Study
pDBP4	*DBP4/2μ/URA3*	(Liang et al., 1997)
pSW612	*GLE1/CEN/URA3*	(York et al., 1999)
pCA5005	*DBP5/CEN/URA3*	(Teng et al., 1998)
pSW449	Bacterial expression of MBP-Gle1	(Murphy and Wente 1996)
pCS830	*DBP5-myc/2μ/LEU2*	(Snay-Hodge et al., 1998)
pSW1319	Bacterial expression of GST-Dbp5	This Study
pSW3174	*SSD1/2μ/LEU2*	This Study
pSW3177	*DHH1/2μ/LEU2*	This Study
pSW3181	*PDE2/2μ/LEU2*	This Study
pSW3180	*dhh1-E196Q/2μ/LEU2*	This Study
pSpo12$_{76\text{-}130}$-GFP	*SPO12* NLS fused to GFP under TPI promoter *2μ/LEU2*	(Chaves and Blobel 2001)
pGAD-GFP	SV40 NLS fused to three GFP under *PHO4* promoter *CEN/URA3*	(Shulga et al., 1996)

* Requires pSW612 or pCA5005 for viability

CHAPTER III

IDENTIFICATION OF MOLECULAR DETERMINANTS IN Gle1 FOR IP_6-MEDIATED Dbp5 ACTIVATION

Introduction

Directional transport of mRNA from the nucleus to the cytoplasm is a highly orchestrated process that bridges nuclear mRNA processing events to protein synthesis in the cytoplasm. Soluble mRNA export factors that mediate NPC interaction and translocation associate with the mRNA throughout the maturation process, serving a dual role of transport receptors and mRNA processing regulators (Hieronymus and Silver 2004; McKee and Silver 2007; Rodriguez 2004; Saguez et al. 2005). Properly capped, spliced and polyadenylated mRNAs combined with their associated RNA binding proteins constitute mature mRNPs that are targeted for export. Members of the conserved Mex67/Mtr2 heterodimer family (also known as TAP/p15 or NXF1/NXT1) are thought to be the major mRNA export receptors, directly bridging mRNA to FG-domains within the aqueous NPC channel (Gruter et al. 1998; Kohler and Hurt 2007; Segref et al. 1997).

EM studies of the Balbiani ring mRNP suggest that NPC translocation involves mRNP remodeling events (Daneholt 2001a) presumably resulting in displacement of nuclear resident proteins from the mRNP (Pinol-Roma and Dreyfuss 1992). These steps are thought to be required for subsequent rounds of

export. Even though the general steps required for mRNA export are thought to be followed by most mRNAs, the protein signature present in mature mRNPs is not homogeneous. For instance, intron-containing pre-mRNAs are loaded with exon-exon junctions during splicing, a mark that is absent in intronless mRNAs (Le Hir et al. 2001). Additionally, specific mRNPs subsets have been found to have differential association of Mex67 and Yra1 (Hieronymus and Silver 2003). The physiological relevance of mRNP protein signatures is illustrated by transcript specific mRNA export defects in *yra1-1* and *mex67-5* temperature-sensitive mutants (Hieronymus and Silver 2003) and the lack of a *SSA4* (hsp70) mRNA export defect during the general mRNA export block imposed by the heat shock response (Saavedra et al. 1997). Similar protein signatures in mRNP populations may be used to coordinate considerable modifications to the gene expression profile at the level of mRNA export. Importantly, transcript specific regulation has been observed in the splicing machinery by inhibiting splicing of a subset of messages during different environmental conditions in yeast (Pleiss et al. 2007a, b).

Dbp5, a member of the DEAD-box helicase family of RNA-dependent ATPases, is thought to mediate mRNP remodeling through the displacement of protein from mRNAs (Snay-Hodge et al. 1998; Tseng et al. 1998). Dbp5 is cotranscriptionally recruited to the mRNP (Estruch and Cole 2003; Zhao et al. 2002) and its ATPase activity is thought to be activated at the NPC by the conserved mRNA export factor Gle1 (Alcazar-Roman et al. 2006; Weirich et al.

2006). Importantly, further ATPase stimulation is provided by the small molecule IP_6, the fully phosphorylated member of the soluble IPs signaling pathway. Thus, Gle1 and IP_6 function as Dbp5 ATPase activating factors, promoting Dbp5 ADP accumulation. Dbp5 binding to ADP has been shown to induce important intramolecular changes in the structure of Dbp5 (Tran 2007). In fact, these changes allow Dbp5 to trigger specific RNA:protein remodeling events. Thus, the nuclear export of mRNAs from the nucleus to the cytoplasm is mediated by the temporal and spatial coordination of biochemical interactions between mRNAs, mRNA export factors, and nucleoporins. Here we define the biochemical interaction between Gle1 and IP_6, and their relation to Dbp5. Furthermore, we identify residues in Gle1 required for IP_6 binding and potentiation of Dbp5 stimulation *in vitro* and *in vivo*.

Materials and methods

Yeast media and manipulations

Strains were grown in YPD (1% yeast extract, 2% peptone, 2% glucose) at 23 ˚C unless otherwise noted. Synthetic media lacking appropriate amino acids was supplemented with 2% Glucose (SD). Media containing drugs for plasmid loss or drug resistance were supplemented as follows: 5-FOA (US Biological) was used at 1.0 mg/ml. Yeast cells were transformed using a lithium acetate method (Ito et al. 1983).

Yeast strains

A list of strains used in this study in is Table 4. To generate strains expressing different alleles of *GLE1*, we utilized a *gle1Δ* strain (Murphy and Wente 1996) with a plasmid harboring *GLE1/URA3*. This strain was transformed with plasmids containing wildtype or mutant alleles of *GLE1* and *LEU2*. Resulting colonies were selected in –LEU media and streaked in 5-FOA plates to select for colonies that had lost the *GLE1/URA3* plasmid. Resulting strains were grown in YPD for all studies thereafter.

Plasmids

The plasmids used in this study are detailed in Table 4. *GLE1/LEU2/CEN* (pSW399) and pMAL-TEV-Gle1 (pSW3242) vectors were used in oligonucleotide-based site-directed *in vitro* mutagenesis to create yeast (pSW3343, pSW3344, and pSW3345) and bacterial (pSW3291, pSW3292, pSW3293, and pSW3295) plasmids to express mutant alleles of *gle1*.

Protein purification and IP_6

Recombinant, bacterially expressed, and untagged Dbp5 and Gle1 protein were purified as described previously (Tran 2007). After purification, proteins were dialyzed in Buffer B (20 mM Hepes-HCL pH 7.5, 150 mM NaCl, 20% w/v glycerol). IP_6 was prepared by resuspending phytic (Sigma) acid in 50 mM

Hepes-HCL pH 7.5 and adjusting pH with 10 N NaOH to reach ~pH 7.5. pH was tested by placing a drop of solution on pH paper indicator (Litmus) and comparing to standards. IP_6 solution was stored at 4 ˚C and prepared fresh every 3 months.

ATPase assays

Dbp5 ATPase assays were conducted as previously described (Huang and Hackney 1994) with the stated modifications. The ATPase reaction was conducted in a total volume of 60 μl containing: 10 mM Hepes-HCL pH 7.5, 45 mM NaCl, 3 mM MgCl2, 1 mM DTT, 3 mM PEP, 0.21 mM NADH, 0.333 U/μl SUPERasin (Ambion), and 0.777 units of pyruvate kinase-lactate dehydrogenase (Sigma). Varying amounts of protein, RNA (25 mer poly(A), and IP_6 was used and reactions were started by addition of ATP/Mg^{2+}. Measurements were taken every 20 seconds by detecting the OD_{340} using Synergy HT Multi-mode mircroplate reader (Biotek). Rate of OD_{340} signal decline was then utilize to measure steady state ATPase activity.

IP_6 binding assays

A modified PEG-precipitation assay was used to measure IP_6 binding (Shears 1997). In brief, 60 μl binding reactions were conducted with 10 nM ^{3}H-IP_6 (21.4 Ci mmol-1; PerkinElmer LAS) in Buffer A (16 mM HEPES at pH 7.5, 120 mM NaCl, 3 mM MgCl2, 16% w/v glycerol, 1 mg ml-1 BSA) supplemented with 1

U/μl SUPERasin (Ambion, Austin, TX). Gle1, Dbp5, RNA (25 mer poly(A)), and/or nucleotides were added as indicated. Samples were mixed and incubated at room temperature for 10 min. Proteins were precipitated by adding 40 μl of 30% PEG 3350 (Sigma), followed by severe mixing and incubation at 4 ˚C for 10 min. Precipitated proteins with bound ^{3}H-IP$_6$ were separated by centrifugation for 25 min at full speed at 4 °C in a Beckman benchtop centrifuge. The supernatant was aspirated and the pellets were solubilized with 300 μl 1% SDS overnight. Radioactivity was measured by scintillation counting. Equilibirum binding kinetics and dissociation constants were determined using GraphPad Prism v.4 software.

Microscopic analysis of mRNA export defects, Gle1 localization, and immunoblots

The localization of poly(A)$^+$ RNA was analyzed by growing strains in rich at 23°C prior to shifting to 37°C for one hour. Cells were collected, fixed in 3.7% formaldehyde and 20% methanol, and washed before processing for indirect fluorescence *in situ* hybridizations (FISH). *In situs* were performed using an digoxigenin-oligo(dT)30 probe as previously described (York et al. 1999). Indirect immunofluorescence were preformed as previously described (Wente et al. 1992). Cells were incubated at 4 ˚C overnight with Gle1 antibodies (1:100) prepared in guinea pig (Cocalico) and affinity-purified . Primary antibody was detected using FITC-conjugated anti-guinea pig IgG (1:1000) for 60 min at room temperature. Nuclei were visualized by DAPI staining. Cells were observed using a fluorescent microscope (model BX50; Olympus, Lake Success, NY) using an

Uplan 100x/1.3 objective. Images were taken using a digital camera (Photometrics Cool Snap HQ: Roper Scientific) and process using MetaVue software (Universal Imaging, West Chester, PA) and Adobe Photoshop 7.0. Analysis of Gle1 protein levels in selected strains was performed using immunobloting. Cells grown at 23 ˚C were shifted to higher temperatures for 1 hr as indicated, collected, washed and processed for crude cell lysis as previously described (Yaffe and Schatz 1984). Proteins were separated by electrophoresis in SDS polyacrylamide gels for immunoblot analysis with anti-Gle1 (1:1000) derived from guinea pig (Cocalico) and anti-Pgk1 (1:4000) (mAb22c5 M. Probes).

Results

Dbp5 binding to Gle1 enhances IP_6 binding *in vitro*

We and others have previously demonstrated that Gle1 is the physiological target of IP_6 during mRNA export and that Dbp5 stimulates the physical association of these two factors (Alcazar-Roman et al. 2006; Weirich et al. 2006). In order to define the precise impact of Dbp5 on the Gle1:IP_6 interaction we experimentally determined the dissociation constant (Kd) between full length Gle1 and IP_6. Equilibrium binding assays showed that Gle1 and IP_6 interact strongly with a Kd of 94 nM with a 95% confidence interval from 67 to 121 nM (Figure 14 A). The apparent association (K_{on}) and dissociation (K_{off}) rate were too fast for our assays to determine (data not shown). Next we measured the

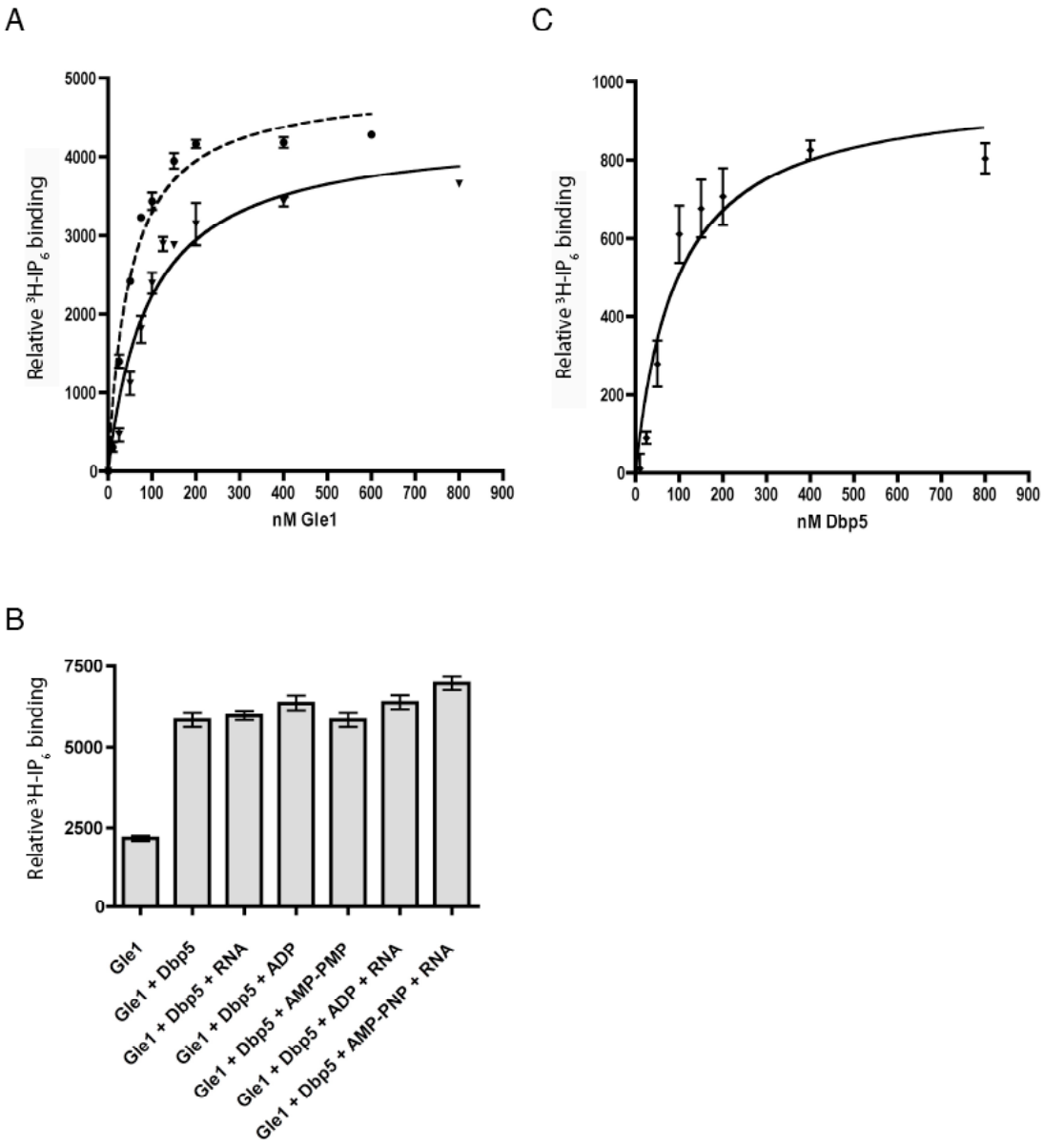

Figure 14: Binding affinities of IP_6, Gle1, and Dbp5. (A) Equilibrium binding assays utilizing 10 nM ^3H-IP_6 and Gle1 (solid line) or Gle1 + 1 mM Dbp5 (dashed line) were used to determine the Kd of Gle1 for IP_6. (B) Equilibrium binding assays as in (A) were used to test the effect of known regulators of Dbp5 activity on Gle1-IP6 binding. (C) Equilibrium binding assays as in (A) were used to measure the stimulation kinetics of Dbp5 for Gle1-IP_6 binding using 25 nM Gle1 and 10 nM IP_6. The mean and s.e.m. were calculated from three independent experiments in (A-C).

impact of Dbp5 on the Gle1-IP_6 interaction. Gle1 bound IP_6 with a Kd of 49.72 nM with a 95% confidence interval from 39.0 to 60.44 nM in the presence of 1 mM Dbp5 (Figure 14 A and Table 3). This two-fold increase in binding affinity is independent and not impacted by RNA and/or ATP (Figure 14 B). We then tested the ability of Dbp5 to stimulate Gle1:IP_6 binding in the presence of saturating levels of the non-hydrolysable ATP analog AMP-PNP or ADP (Figure 14 B), factors known to induce structural changes on Dbp5 (Tran 2007). Interestingly, both Dbp5:AMP-PNP and Dbp5:ADP, in the presence or absence of RNA, were able to stimulate Gle1:IP_6 binding. We conclude that the structural changes in Dbp5 resulting from ADP and/or ATP binding do not interfere with its ability to interact with Gle1. Furthermore, both reported conformations of Dbp5 are able to stimulate Gle1:IP_6 binding.

Dbp5 and Gle1 interact *in vitro*

To gain insights into the association of Gle1 and Dbp5 we investigated the kinetics of Gle1-IP_6 binding stimulation by Dbp5. With a Gle1 concentration of 25 nM and an IP_6 concentration of 10 nM, the half-maximal concentration of Dbp5 needed for Gle1-IP_6 binding stimulation was of 69 nM (Figure 14 C) with a 95% confidence interval from 51 to 137 nM. This result suggests that Dbp5 binds Gle1 with high affinity, and that the apparent Kd of 70 nM defines the binding interaction of Dbp5 for Gle1. This was a surprising result since we have been unable to detect a stable interaction between these two proteins *in vitro*. We

conclude that transient interactions between Gle1-IP_6 and Gle1-Dbp5 are a result of very fast K_{on} and K_{off} values, a property that fits with the model of transient interactions required for rapid mRNA export.

Identification of residues in Gle1 necessary for IP_6 binding

The solution of the crystal structures of the C-terminal domain of hADAR2 (Macbeth et al. 2005) and the F-box protein subunit of the ubiquitin ligase complex SCF (Tir1) (Tan et al. 2007) revealed a molecule of IP_6 embedded in their tertiary structure. To accomplish binding of the very negative IP_6 molecule, both proteins contain a highly positively charged pocket decorated with several lysine (K) and arginine (R) residues. Of note, the residues are spread through several hundred residues of the primary sequence. In the case of hADAR2, the residues responsible for IP_6 binding are highly conserved from yeast to humans. Since depletion of IP_6 coincides with accumulation of mRNA in the nucleus in human cells overexpressing SopB (Feng et al. 2001) and *Schizosaccharomyces pombe ipk1Δ* cells exhibit a mRNA export defect (Sarmah unpublished results), we hypothesize that the residues coordinating IP_6 binding are also conserved in many Gle1 homologues. Sequence alignments identified four conserved positively charged residues present within stretches of medium to high conservation in the C-terminal domain of Gle1 (Figure 15a). We selected those residues for targeted mutagenesis. We replaced codons for lysine or arginine residues with glutamine (Q)-encoding codons to conserve the polarity and

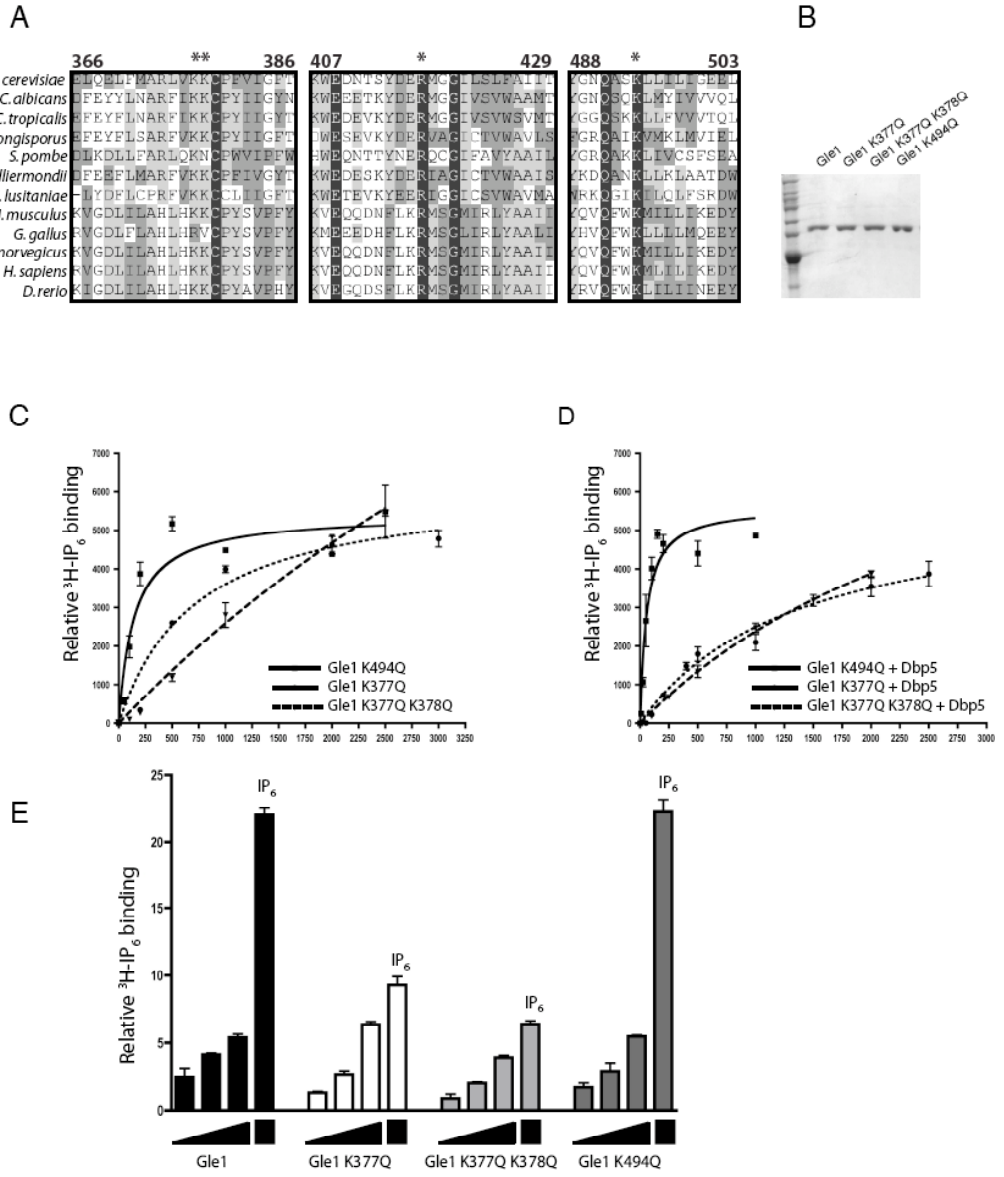

Figure 15: Gle1 residues K377 and K378 are required for the IP_6-mediated Dbp5 ATPase stimulation. (A) Sequence alignment of conserved regions of the CTD of Gle1 from selected fungal and metazoan species. Sections with grater homology are depicted in increasingly darker gray. Stars denote amino acids selected as putative IP_6 binding residues. (B) Bacterially expressed recombinant Gle1, Gle1 K377Q, Gle1 K377Q K378Q, and Gle1 K494Q were separated in a 7.5% SDS-polyacrylamide gel and stained with Coomassie (C) Equilibrium binding assays utilizing 10n M ^3H-IP_6 and increasing amounts of Gle1 were used to calculate the Kd of Gle1 proteins for IP_6 (D) Equilibrium binding assays as in (C) in the presence of 1 mM Dbp5. (E) Dbp5 ATPase assay utilizing 100 nM, 400 nM, or 800 nM Gle1, 1 mM ATP, 10 μM RNA, and 200 nM Dbp5. 100 nM IP_6 was added to each sample containing 800 nM Gle1. The mean and s.e.m. were calculated from three independent experiments in (C-D).

relative size of the side chains. Gle1 expressed harboring a Q at position 417 (R417Q) resulted in insoluble protein (data not shown). However, Gle1-K377Q, Gle1-K377Q K378Q, and Gle1-K494Q were completely soluble and were purified to homogeneity (Figure 15 B). To evaluate the role of these amino acids in the binding of IP_6 by Gle1, we performed ^3H-IP_6 equilibrium binding assays and (Figure 15 C). The binding of Gle1 for IP_6 was unaffected in Gle1-K494Q. However, Gle1-K377Q and especially Gle1-K377Q K378Q had close to 10- fold reduction in Gle1-IP_6 binding affinity, 724.1 nM and 8251 nM respectively. Addition of 1 mM Dbp5 stimulated IP_6 binding of Gle1-K494Q to similar levels to that observed for Gle1-wt. However, Gle1-K377Q and Gle1-K377Q K378Q still had a drastic deficiency in IP_6 binding (Figure 15 D and Table 3).

Conserved Gle1 residues K377 and K378 facilitate IP_6-mediated Dbp5 stimulation *in vitro*

We and others have previously shown that Gle1 is able to stimulate the ATPase activity of Dbp5 in an IP_6-independent fashion and that IP_6 greatly potentiates this stimulation (Alcazar-Roman et al. 2006; Weirich et al. 2006). In order to test the role of Gle1 residues K494, K377 and K378 in the IP_6-dependent stimulation of Dbp5 ATPase activity, we performed ATPase assays with increasing concentrations of Gle1 (Figure 15 E). Interestingly, Gle1, Gle1 K377Q, Gle1 K377Q K378Q, and Gle1 K494Q exhibited similar Dbp5 ATPase activation in the absence of IP_6. We further stimulated Dbp5 ATPase activity by adding 100 nM IP_6 to the samples containing the highest Gle1 concentration (800 nM). In

Table 3: Dissociation constants of Gle1 and IP_6

Protein	K_d	95% confidence
Gle1	94.37 nM	67.25 to 121.5 nM
Gle1 K377Q	724.1 nM	244.7 to 1203 nM
Gle1 K377Q K378Q	8251 nM	0 to 16680 nM
Gle1 K494Q	141.2 nM	65.3 to 217.1 nM
Gle1 + 1mM Dbp5	49.72 nM	39 to 60.44 nM
Gle1 K377Q + 1mM Dbp5	1375 nM	811.2 to 1940 nM
Gle1 K377Q K378Q + 1mM Dbp5	3374 nM	1751 to 4994 nM
Gle1 K494Q + 1mM Dbp5	55.51 nM	26.67 to 84.35 nM

samples containing Gle1 and Gle1 K494Q, the addition of IP_6 induced ATPase activity dramatically. However, little or no increase in ATPase activity was observed in samples containing Gle1-K377Q and Gle1-K377Q K378Q upon IP_6 addition. These results indicate that the K377Q and K378Q are necessary for the IP_6 mediated stimulation of Dbp5 ATPase activity, and that it is possible to decouple IP_6 binding from Dbp5 binding and stimulation. Taken together, these *in vitro* assays strongly argue that the conserved Gle1 K377 and K378 residues are required for coordinating IP_6-binding and potentiating the Gle1 activation of Dbp5 ATPase activity by IP_6.

Cells harboring Gle1 K377Q and Gle1 K377Q K378Q have mRNA export defects

It has been demonstrated that cells harboring the *ipk1Δ* mutation are viable at all temperatures but exhibit a weak mRNA export defect at 37 ˚C (York et al. 1999). This phenotype is presumably due to lack of cellular IP_6 to target Gle1 and stimulate the ATPase activity of Dbp5. Our biochemical analysis has now characterized Gle1 proteins with a strong defect in IP_6 binding while maintaining proper Dbp5 stimulation. Based on the above studies we predict that yeast cells expressing Gle1-K377Q and Gle1-K377Q K378Q will have a mRNA export defect when grown at 37 ˚C similar to *ipk1Δ* cells. Additionally, cells expressing Gle1-wt and Gle1-K494Q should have a wildtype phenotype. We generated strains using a plasmid shuffle strategy in which the chromosomal copy of *GLE1* had been deleted and containing plasmid harboring either wildtype

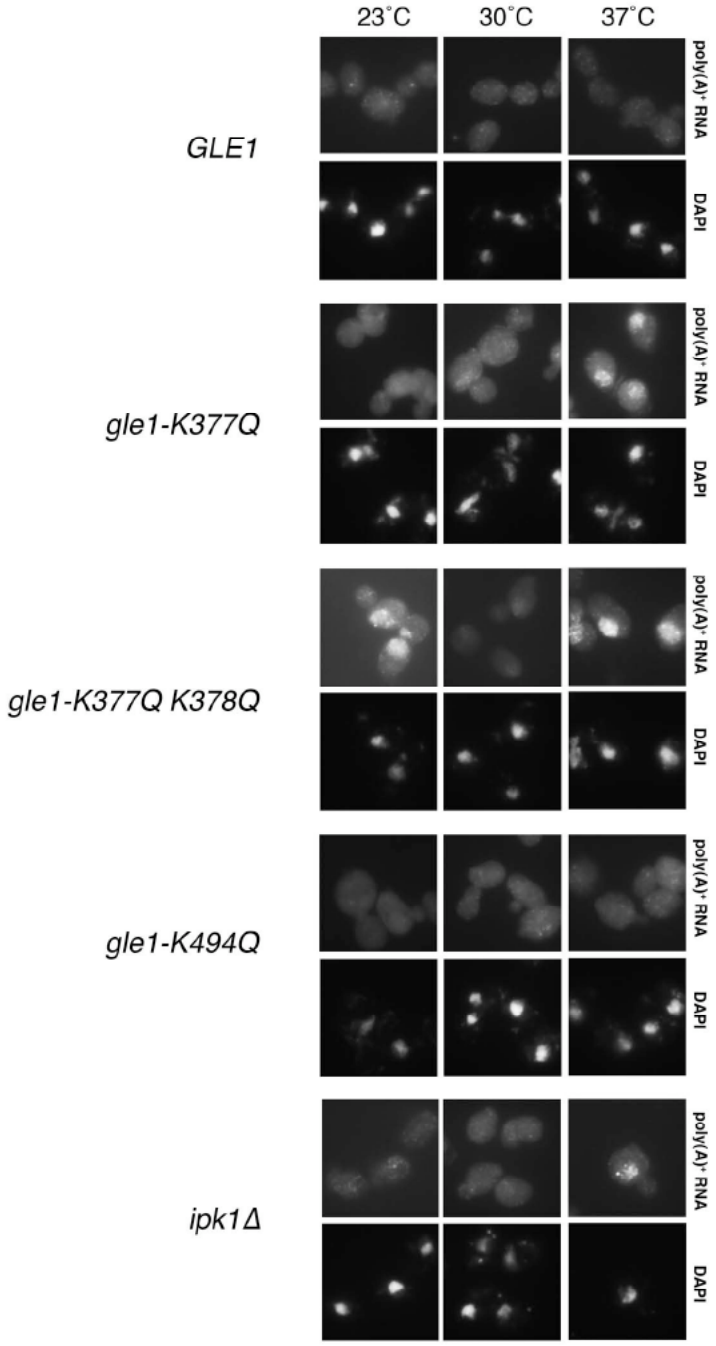

Figure 16: In vitro IP_6-binding defects correlate to mRNA export defects in vivo. An *ipk1Δ* strain and *gle1Δ* mutant strains carrying a plasmid harboring either *GLE1, gle1-K377Q, gle1-K377Q K378Q*, or *gle1-K494Q*, were used for *in situ* hybridization with a digoxigenin-coupled oligo(dT) probe after growing at 23˚C and shifting to 23˚C and 37°C for one hour. Poly(A)$^+$ RNA localization was visualized by indirect immunofluorescence microscopy with a FITC-coupled anti-digoxigenin antibody and nuclei were visualized with DAPI staining as indicated.

or mutant alleles of *GLE1*. In situ hybridization with an oligo (dT) probe detected nuclear mRNA accumulation in both Gle1-K377Q- and Gle1-K377Q K378Q-expressing strains at 37 ˚C but not in the Gle1-wt or Gle1-K494Q-expressing strains (Figure 16). Importantly, similar to *ipk1Δ* strains, all strains showed normal mRNA export at 30 ˚C. Strikingly, *gle1-K377Q K378Q* also showed mRNA accumulation at 23 ˚C, a phenotype not detected in the *ipk1Δ* strain or in the other Gle1 point mutant strains. This defect suggests the presence of additional defects in the *gle1-K377Q K378Q* strain not detected in our biochemical assays. In order to rule out defects in expression, stability and localization of Gle1 point mutants, we utilized anti-Gle1 polyclonal antibodies. We demonstrated that all tested alleles of Gle1 were stable within one hour of the temperature shift (Figure 17 A), which was the standard condition used for all assays in this study. Additionally, immunofluorescence microscopy using anti-Gle1 antibodies showed proper nuclear rim localization for all strains tested (Figure 18). Next we assessed the viability of strains expressing the different Gle1 alleles at different temperatures. The mRNA export detected in the strain expressing Gle1-K377Q K378Q nicely correlated with growth defects evident in doubling times (data not shown) and serial dilution assays (Figure 17 B), as this strain had both high and low temperature sensitivity. Taken together, these observations confirm our biochemical analysis and corroborate the role of Gle1 residues K377 and K378 in IP_6-binding and potentiating of Gle1 stimulation of Dbp5 ATPase activity *in vivo* and *in vitro*.

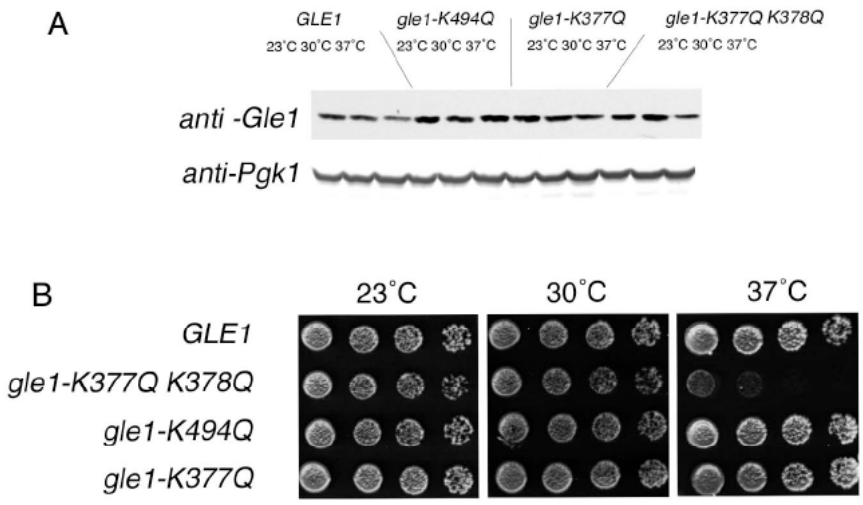

Figure 17: Protein stability and growth phenotype of strains expressing gle1 mutant alleles. (A) Indicated strains were grown at 23°C and shifted to 30°C and 37°C for one hour prior to western blot analysis utilizing Gel1 polyclonal antibodies. anti-Pgk1 was used as a loading control. (B) Indicated strains were spotted in fivefold serial dilutions and incubated in rich media at 23°C, 30°C and 37°C.

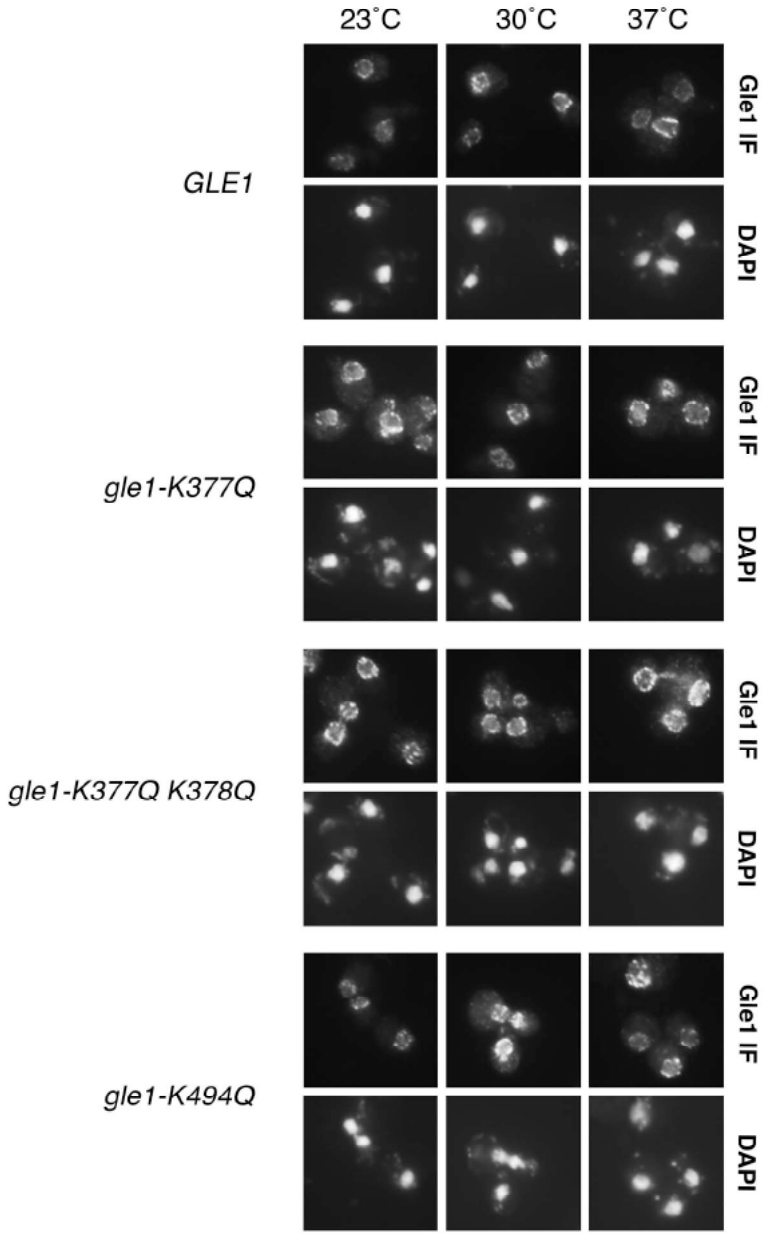

Figure 18: Proper localization and stability of Gle1 proteins. Gle1 localization was observed by indirect immunofluorescence microscopy utilizing Gle1 polyclonal antibodies and nuclei were visualized with DAPI. *gle1Δ* mutant strains carrying plasmids harboring *GLE1, gle1-K377Q, gle1-K377Q K378Q*, or *gle1-K494Q* were grown at 23˚C and shifted to 30˚C and 37˚C for one hour prior to conducting immunofluorescence.

Discussion: IP_6 as a regulator of mRNA export

Taken together, we have defined the biochemical relationship between IP_6 binding to Gle1 and stimulation of Dbp5. We conclude that the tight but transient association of Gle1 to IP_6 is due to fast K_{on} and K_{off} rates that are mediated through conserved positively charged amino acids in the C-terminal domain of Gle1. Furthermore, we demonstrate that these amino acids are responsible for transducing the IP_6-driven stimulation of Dbp5 ATPase activity *in vitro* and *in vivo*. Our previous studies have identified a pivotal role for the Gle1/IP_6-driven ATPase stimulation of Dbp5 in displacement of Nab2 from RNA (Tran 2007). We speculate that IP_6 potentiation of Dbp5 activity will be crucial for displacement of proteins in specific mRNPs.

Conceptually, transient protein interactions are expected to occur during the rapid mRNA export process. Consistent with this model, we determine that even though Gle1-IP_6 and Gle1-Dbp5 dissociation constants are in the low nanomolar range, they cannot be captured as a stable complex *in vitro* (data not shown). We speculate that the NPC, through Nup159 and Nup42, provides a scaffold for the interaction of these proteins *in vivo*, and that additional cellular factors may regulate and participate in this interaction. Gfd1 is a prime candidate for this role, as it has genetic and direct physical interactions to Gle1, Dbp5, and Nab2 (Hodge et al. 1999; Strahm et al. 1999; Suntharalingam et al. 2004). Interestingly, mass spectrometry analysis has identified Gfd1 and Nup159 as protein whose phosphorylation profile changes in response to mating pheromone

(Gruhler et al. 2005)), a signal that activates one of the five mitogen-activated protein kinase (MAP) kinase pathways in yeast (Buehrer and Errede 1997). Regulation of these and other factors may be essential for the export of mRNPs with specific remodeling requirements during signaling or special growth conditions.

Selective regulation of mRNA export has been reported for heat shock mRNAs (Farny et al. 2008; Saavedra et al. 1997); intronless and intron-containing transcripts; (Luo et al. 2001); and histone mRNAs (Erkmann et al. 2005). In some cases the mRNA nucleotide sequence allows direct association with export factors (Erkmann et al. 2005); whereas in other cases it impacts the global protein composition of the mRNP. As a result, the precise protein signature of a mature mRNP varies from message to message. We speculate that Dbp5 could be effective at displacing proteins present in a given mRNP, whereas being inefficient at remodeling other mRNPs. We have previously demonstrated that Dbp5 cycles between an ATP- and an ADP-bound forms (Tran 2007). We now show that IP_6 and Gle1 are able to bind both Dbp5 conformations. We propose that IP_6 is able to associate with Dbp5-Gle1 complexes during and after ATP hydrolysis and regulate the remodeling of specific mRNPs as they exit the nucleus. Direct control over the efficiency of remodeling for these mRNPs could be mediated by changes of local IP_6 levels that impact Gle1's ability to stimulate Dbp5.

Efficient mRNA export during cell signaling would aid in a rapid cellular

response. The phosphate-responsive signaling pathway (*PHO)* pathway is an attractive candidate to test for differential regulation of mRNA export by IP_6. During phosphate starvation IP_7 promotes the nuclear localization of the Pho4 transcription factor (Lee et al. 2007) whereas IP_4/IP_5 regulate chromatin remodeling at Pho4 responsive promoters (Steger et al. 2003). Pho4 promotes the production of *PHO5, PHO11, PHO12* and *PHO84* mRNAs during the *PHO* signaling pathway (Carroll et al. 2001). I hypothesize that the export of these messages would be slower in a cell that does not produce IP_6.

Pharmacological induction of the *PHO* pathway can be achieved by the utilization of a *PHO85*^{F82G} allele and 4 -Amino-1-tert-butyl-3-(1'-naphthyl) pyrazolo [3,4-d] pyrimidine (1-Na-PP1) (Carroll et al. 2001). This pharmacological strategy bypasses the requirement of producing IP_7, induces rapid nuclear localization of Pho4, and is sufficient for the production of *PHO* responsive mRNAs. mRNA export of *PHO* specific messages would be compared in *ipk1Δ PHO85*^{F82G} and *IPK1 PHO85*^{F82G} cells in the presence of 1-Na-PP1. Since chromosome remodeling of Pho4-responsive genes is not dramatically perturbed by the *ipk1Δ* mutation (Steger et al. 2003), *PHO* messages should be efficiently expressed in this strain. As a control, the export of *PGK1,* an abundant message in the cell, would need to be monitored. Additionally, the *gle1-K377Q* mutation would be added to the *PHO85*^{F82G} strain to test the requirement of IP_6 regulation through Gle1 in *PHO* mRNA export. Finally, the *mex67-5* mutation would be added as a positive control of general mRNA export defects. Detection of *PHO* messages

would be conducted through FISH utilizing message-specific probes with 4 to 5 fluorophores attached (Dong et al. 2007). Individual probes would be designed to recognize areas of high sequence homology of *PHO5, PHO11*, and *PHO12* mRNAs to increase FISH signal. As a precaution, the *PHO3* gene, which is highly homologous to *PHO5* and is not under the control of the *PHO* signaling pathway, would be chromosomally deleted from all strains to be used.

IP_6 is an abundant molecule in the cell. However, the concentration of free IP_6 at specific cellular locales is unknown. Genetic manipulations of *GLE1* have now allowed us to uncouple IP_6 signaling from mRNA export. Additionally, recent studies in our laboratory, focused on translation initiation and termination, suggest that Gle1 and IP_6 do not always work together (Bolger T. et al., Submitted). Thus, IP_6 may only associate with Gle1 to coordinate specific cellular functions.

This study adds to the growing list of PIPs and IPs found to be regulators of gene expression. In addition the nuclear production of IP_4/IP_5 and IP_7 that regulate *PHO* responsive genes, $PI(4,5)P_2$ has been recently shown to regulate the expression of oxidative stress response genes by regulating the polyadenylation of their mRNAs (Mellman et al. 2008). Additionally, Plc1 has been connected to the regulation of high osmolarity glycerol response (*HOG*) pathway messages (Guha et al. 2007). Signal transduction pathways that utilize PIPs and IPs as secondary massagers are being uncovered as message specific gene expression modulators at the level of transcription, export, and translation.

These signaling mechanisms are poised to facilitate the dynamic expression profiles manifested in different cell types during metazoan development.

Table 4: Yeast strains and plasmids used in Chapter III

Strain Name	Genotype	Source
SWY1831	*Mat @ gle1::HIS3 ade2 ADE3 ura3 his3 leu2 trp1 LYS2 can1 +* pSW410	(Murphy and Wente 1996)
pSW3826	*Mat @ gle1::HIS3 ade2 ADE3 ura3 his3 leu2 trp1 LYS2 can1 +* pSW399	This Study
pSW3823	*Mat @ gle1::HIS3 ade2 ADE3 ura3 his3 leu2 trp1 LYS2 can1 +* pSW3343	This Study
pSW3825	*Mat @ gle1::HIS3 ade2 ADE3 ura3 his3 leu2 trp1 LYS2 can1 +* pSW3345	This Study
pSW3824	*Mat @ gle1::HIS3 ade2 ADE3 ura3 his3 leu2 trp1 LYS2 can1 +* pSW3344	This Study
SWY2117	*MATα ade2-1 ade3::hisG ura3-1 his3-11,15 trp1-1 leu2-3,112 lys2 ipk1::KAN*^{R}	(Alcazar-Roman et al., 2006)

Plasmid Name	Description	Source
pSW410	*GLE1/CEN/URA3*	(Murphy and Wente 1996)
pSW399	*GLE1/CEN/LEU2*	(Murphy and Wente 1996)
pSW3343	*gle1-K377Q/CEN/LEU2*	This Study
pSW3345	*gle1-K377Q K378Q/CEN/LEU2*	This Study
pSW3344	*gle1-K494Q/CEN/LEU2*	This Study
pSW3242	Bacterial expression of MBP-TEV-Gle1	(Tran et al., 2007)
pSW3319	Bacterial expression of GST-Dbp5	This Study
pSW3291	Bacterial expression of MBP-TEV-Gle1 K377Q	This Study
pSW3293	Bacterial expression of MBP-TEV-Gle1 K377Q K378Q	This Study
pSW3292	Bacterial expression of MBP-TEV-Gle1 K494Q	This Study
pSW3295	Bacterial expression of MBP-TEV-Gle1 R417Q	This Study

CHAPTER IV

FUTURE DIRECTIONS

Introduction

mRNA export is vital for the fluent communication between the nucleus and the cytoplasm, a property required for rapid changes in gene expression profile (Rodriguez 2004; Saguez et al. 2005) (Gross et al. 2007; McKee and Silver 2007). The budding yeast *Saccharomyces cerevisiae* has been in the forefront of these studies due to its facile genetic manipulation and tractability. These techniques have allowed the field to identify numerous mRNA export factors and regulators. However, our understanding of mRNA export has undoubtedly reached a point at which biochemical dissection is required. In this aspect too, budding yeast proteins and complexes have been selected for purification and crystallization (Madrid and Weis 2006). The strength of combined biochemical and genetic approaches is manifested by the ability to test *in vivo* predictions derived from biochemical studies.

The utilization of yeast as a model system for the study of mRNA export is at its prime. However, it is important to underline that metazoan cells have specialized, and in some cases, developed alternative strategies for the export of mRNAs. Studies like the genome-wide RNAi screen performed by Pam Silver

and colleagues to detect factors required for mRNA export in drosophila culture cells are crucial to expand our knowledge of this process in metazoans.

We have now established a biochemical mechanism for Gle1, Dbp5 and IP_6 in the later stages of mRNA export in yeast. The most urgent future direction is to test if hGle1B and hGle1A bind IP_6 and activate hDbp5. Considering their primary sequence similarity, hGle1B and yGle1 should have similar biochemical properties. In fact, sequence alignment of both proteins suggests that hGle1 would be able to bind IP_6 (Figure 15 A). Once the biochemical properties of these human proteins have been determined, we could exploit the advantages of cell size provided by cell culture microscopy to decipher the roles of these proteins *in vivo*.

Dynamics of Gle1 and Dbp5 regulation

Both Gle1 and Dbp5 have a steady state NPC localization *in vivo* in yeast and human cells (Murphy and Wente 1996; Snay-Hodge et al. 1998; Tseng et al. 1998; Watkins et al. 1998). However, it is now well established that Dbp5 and hGle1 shuttle between the nucleus and the cytoplasm. In the case of *A. nidulans*, Gle1 remains localized to the NPC during mitosis while FG-nups disperse throughout the cell (Osmani et al. 2006). It will be important to uncover what are the functional requirements and impact of Gle1 and Dbp5 localization and shuttling.

hGle1 shuttles between the nucleus and the cytoplasm by means of its shuttling domain (SD) (Kendirgi et al. 2003). The SD domain is present in both hGle1A and hGle1B, suggesting that both isomers are subject to nucleocytoplasmic exchange. However, a striking difference is observed when the localization of ectopically expressed hGle1A and hGle1B are compared. Unlike hGle1B, hGle1A does not contain an hCG1 binding site and does not have a strong steady state NPC localization (Kendirgi et al. 2005). Thus, hGle1B has a similar localization pattern to yGle1, and may have homologous roles in mRNA export. What is then the role of hGle1A? The high sequence similarity of yeast and human Dbp5 and Gle1 suggest that hGle1B and hGle1A are able to regulate hDbp5 activity. A possible role for hGle1A would be to regulate Dbp5 at different cellular locales. In fact, hGle1A is enriched in the cytoplasm. In light of the recent discovery of a role for yDbp5 in translation (Gross et al. 2007) an attractive possibility is that hGle1B regulates Dbp5 in mRNA export whereas hGle1A regulates Dbp5 in translation. Alternatively, hGle1B and hGle1A may play overlapping roles in mRNA export in different cell types. A careful examination of cell-type expression preferences for one isoform versus the other would be an important contribution to this dilemma. Even more provocative is the possibility that hGle1B and hGle1A have different subcellular localization patterns in different cell types. A simple experiment would be to conduct immunofluorescence and transient transfections of GFP-tagged hGle1 isoforms in a number of cell types, especially neurons.

It is unknown whether yGle1 shuttles. The SD contained in hGle1 is not conserved in the yeast homologue (Kendirgi et al. 2003). However, there is evidence to suggest nucleocytoplasmic shuttling is mediated by other molecular determinants. yGle1 has been shown to have an NPC steady-state localization *in vivo* by GFP-tagging strategies (Strawn et al. 2001) and *in vitro* by indirect immunofluorescence (Murphy and Wente 1996). However, higher resolution studies utilizing immuno-EM places yGle1 at both the cytoplasmic and nucleoplasmic sides of the NPC, albeit, with a strong prevalence towards the cytoplasmic face of the NPC (Miller 2004; Rout et al. 2000). Localization of yGle1 at both faces of the NPC may represent transitory interactions during nucleocytoplasmic shuttling. Moreover, a small nine amino acid stretch of yGle1 (aa 350-358) is sufficient to mediate nuclear export of an exogenous cargo (Murphy and Wente 1996).

Gle1 shuttling in yeast and humans could be important to regulate Dbp5 or other DEAD-box helicases during mRNA processing, export and translation. Shuttling may also allow early Gle1 association with the mRNP during pre-mRNA processing. Gle1 shuttling raises another important question. How is Dbp5 regulated at terminal steps of mRNA export if Gle1 shuttles? NPC components like Nup42/hCG1 and Nup159/CAN may facilitate local activation of Dbp5 by providing a scaffold platform that facilitates their interaction, or by removing inhibitory proteins allowing proper ATPase activation. Determination of the

precise dynamics of Gle1 localization during mRNA export is of crucial importance, and should follow the studies presented in this thesis.

Gle1 localization dynamics might depend on gene expression. The model of mRNA export places Gle1 at the cytoplasmic face of the NPC awaiting the mature mRNP containing Dbp5 and Mex67 among other proteins. As the mRNP traverses the NPC, local activation of Dbp5 would be facilitated by its recruitment to Nup159, geographically situating Dbp5 next to Gle1 (see below). In this model, Gle1 localization is independent of mRNA transcription, export, and translation. To test this, genetic or pharmacological inhibition of transcription and translation would be used to observe Gle1-localization dynamics. Gle1 mislocalization during Pol II transcriptional inhibition should occur if Gle1 shuttles between the NPC and other sites of action. Furthermore, mislocalization might reveal additional sites of Gle1 activity. Similar studies could be performed utilizing pharmacological translational inhibitors. Additionally, energy depletion successfully utilized in the study of Mex67-GFP localization dynamics (Terry and Wente 2007) could be utilized to elucidate the energy requirements of Gle1 localization. This question is of great importance as hGle1 is able to shuttle and its yeast homologue could play additional roles in the gene expression apparatus.

A similar approach could be utilized to study Dbp5 localization dynamics. Are there populations of Dbp5 insensitive to energy depletion? Are there any changes in Dbp5 localization upon blocks to transcription or translation? In yeast, Dbp5-GFP localizes to the cytoplasm but mainly to the NPC (Snay-Hodge et al.

1998) whereas immunofluorescence utilizing Dbp5 antibodies detects Dbp5 at the NPC and in the cytoplasm at similar levels (Snay-Hodge et al. 1998; Tseng et al. 1998). This discrepancy could be attributed to background signal from Dbp5 antibodies or defects in the tagged Dbp5-GFP protein. Immunofluorescence with Dbp5 antibodies on Dbp5-GFP expressing cells could solve this discrepancy. If cytoplasmic fluorescence remains, then it is likely due to background signal. On the contrary, if signal is higher at the NPC, then it is likely that GFP tagging of Dbp5 is deleterious to proper Dbp5 localization. Solving this dilemma would allow us to properly study Dbp5 localization dynamics. In fact, yDbp5 localization changes during different environmental conditions (Izawa et al. 2005a; Izawa et al. 2005b; Takemura et al. 2004), underlining the importance of this level of regulation. How are these changes achieved? What are the implications of these changes? Under ethanol stress, yDbp5 accumulates in the nucleus (Takemura et al. 2004). This localization is rapid and reversible. A cell under stressful conditions might require a cease of translational activity, thus restricting Dbp5 localization to the nucleus. Conversely, cells recovering from stress might require high levels of cytoplasmic Dbp5 to cope with translational demands. Thus, studying Dbp5 localization dynamics could also provide a tool to better understand roles of Dbp5 apart from the NPC. Additionally, utilization of a TAP-tagged strategy under these conditions could enrich for Dbp5 binding partners in the nucleus or cytoplasm.

Due to the predominantly steady NPC localization, Dbp5 and Gle1 colocalize in yeast and human cells. *In vitro* studies have now indicated that both proteins directly interact, albeit not forming a stable complex. To determine if Gle1 and Dbp5 interact at all sites of subcellular colocalization, I would utilize fluorescence resonance energy transfer (FRET). Proteins able to undergo FRET must be within 10 nm of each other (Hailey et al. 2002). A FRET signal in the cytoplasm would strongly argue for a role of Dbp5 and Gle1 in cytoplasmic processes such as translation.

Dbp5 has reported roles in transcription, export and translation (Estruch and Cole 2003; Gross et al. 2007; Snay-Hodge et al. 1998; Tseng et al. 1998). How does Dbp5 regulate all these processes? Recent biochemical evidence suggests that Dbp5 might work as a switch, shifting from ADP to ATP bound forms ((Tran 2007) and Introduction) (Figure 19 B). Furthermore, the relative affinity of Dbp5 for ADP is much higher than the one for ATP. Given that intracellular ATP levels range from 2-10 mM and that ADP levels are ~10 fold lower (Delumeau et al. 2002; Lee et al. 2004b; Lu et al. 2002), a lower Kd for ADP would assure the stability of Dbp5-ADP during mRNA export. An attractive model centered on this cycle predicts that Dbp5-ATP plays roles in the nucleus while Gle1/IP_6-driven ATP hydrolysis at the cytoplasmic side of the NPC would result in Dbp5-ADP playing roles in mRNA export and other cytoplasmic events. This model is strikingly similar to RanGTP hydrolysis cycles that regulate protein transport (Stewart 2007). If Dbp5 and Ran are regulated in a similar fashion, the

activator Gle1 represents the Dbp5 ATPase activating factor (AAF), stimulating Dbp5-ADP formation. How does Dbp5 cycle from an ADP- to an ATP-bound form? The simplest explanation would be that ATP could bind Dbp5 by displacing ADP. In support of this model ATPase assays *in vitro* suggest that Dbp5 can undergo many cycles of ATP hydrolysis in the absence of an exchange factor. This requires release of ADP followed by ATP binding. Importantly, early studies describing the GTPase activity of Ran indicates that, at least *in vitro*, Ran does not require an activator protein or an exchange factor for GTPase activity (Bischoff et al. 1994). So, similarly to Dbp5, Ran can cycle between NTP- and NDP-bound forms *in vitro* to support a low level of NTPase activity. However, in the cell RanGAP and RanGEF regulate Ran activity in a spatial manner. Is there a Dbp5 AEF to facilitate the exchange of ADP for ATP? If Dbp5 requires an AEF, this activity would likely be localized in the nucleus. Furthermore, Dbp5 AEF would bind Dbp5 only in a Dbp5-ADP form and not in a Dbp5-ATP form. Given these predictions, future efforts should concentrate in a biochemical strategy to identify Dbp5 binding proteins that associate with Dbp5 differentially in ADP versus ATP-bound forms. Recombinant GST-Dbp5-ADP, -AMP-PNP, or -APO could be used in pull down assays utilizing yeast extracts. Isolated proteins would be grouped in AMP-PNP dependent, ADP-dependent, or nucleotide impendent Dbp5 binding proteins. A potential Dbp5-AEF would be present in the ADP-dependent group.

Visualization of Dbp5 structural changes *in vivo* is an extremely challenging task. However, modern technology is available to potentially allow tests of hypotheses in real time. Our model predicts that Dbp5 behaves like a molecular switch, changing from an ATP- to an ADP-bound conformation (Tran 2007). These cycles have been shown to result in important structural changes *in vitro*. It is possible that Dbp5 remains in an ATP-bound form in the nucleus attached to mRNPs, and switches to an ADP-bound form at the NPC. Utilization of intramolecular FRET could indicate different structural conformations at distinct cellular locales . For instance, FRET might be facilitated by nuclear Dbp5-ATP and inhibited due to structural changes of Dbp5 when bound to ADP in the cytoplasm. GFP-hDbp5 is localized in the cytoplasm and at the NPC (Schmitt et al. 1999) and could provide an interesting model for these studies.

Co-transcriptional recruitment of Dbp5 has been suggested by genetic and physical interactions of Dbp5 with the transcriptional machinery (Estruch and Cole 2003). Furthermore, Dbp5 only binds RNA in the presence of AMP-PNP (Weirich et al. 2006). A possibility is that Dbp5 is kept on an ATP-bound form as it associates with the pre-mRNP. However, an important question remains: How many Dbp5 molecules are present in a mature mRNP? If Dbp5 decorates mRNP molecules throughout their length, long messages should have a greater number of associated Dbp5 molecules as compared to short messages. To test this hypothesis mRNP reporters of different lengths could be designed with MS2 binding sequences. After isolation of individual mRNPs, Dbp5 antibodies could

be used to detect if Dbp5 isolation increases proportionally to mRNA length. UV cross-linking of yeast mutants with mRNA export defects and with disabled nuclear exosomes could be used to enrich for such messages in the nucleus. If in contrast, mRNPs contain a fixed number of Dbp5 molecules, the same amount of Dbp5 should be isolated with any message. These studies will be crucial in understanding the mechanism of mRNA export. If larger mRNPs require more Dbp5 for export, it might indicate that Dbp5 is a limiting factor in extruding the mRNP through the NPC, and that it acts at different points during this process. Additionally, if the same number of Dbp5 molecules is required for the export of short and long mRNPs, Dbp5 may operate at a define step or steps during mRNP NPC protrusion. Similar studies could be done for Mex67, Nab2, Pab1, and even Gle1. The composition and stoichiometry of an export competent mRNP is likely to play an important role in the export of diverse mRNP cargoes.

The recent computational model of the NPC structure highlights the platform for local Dbp5 activation by Gle1 at the cytoplasmic side of the NPC (Alber et al. 2007a; Alber et al. 2007b) (Figure 1 A-C). Each of the eight pillars forming the NPC contains two columns that support adjacent Dbp5 and Gle1 binding sites. An NPC engaged at maximal mRNA export efficiency could potentially support activation of eight Dbp5 molecules at the same time. In fact, computational analysis predicts that the stoichiometry of Gle1 at the NPC is identical to the one for Nup42 and Nup159 (Alber et al. 2007a). Whether each Dbp5 and Gle1 at the NPC is engaged in mRNA export or if eight mRNPs could

be exported at the same time is unknown and should be studied in the future. To understand this mechanism it will be critical to determine the residence time of an activated Dbp5 (Dbp5-ADP) at the NPC.

Possible mechanisms of Dbp5 stimulation by Gle1

In order to elucidate the precise role of Dbp5 in mRNA export, it is necessary to understand its biochemical properties. Biochemical characterization of Dbp5 has revealed binding to ATP/ADP, RNA, Nup159, Gle1, Gfd1, and Nab2 (Cole and Scarcelli 2006). Furthermore, Dbp5 enzymatic activity is sensitive to Mg^{2+} levels, a conserved property in other DEAD-box helicases (Linder 2006; Schmitt et al. 1999; Tseng et al. 1998). How is Gle1 able to drastically stimulate Dbp5 ATPase activity?

One possibility is that Gle1 may be able to stabilize a closed conformation of Dbp5, much like eIF4G regulates eIF4A (Oberer et al. 2005). Dbp5 in a closed conformation would then be able to hydrolyze ATP and bind RNA more efficiently (Figure 19 C). One prediction of this model is that Gle1 could interact with both Dbp5 N- and C-termini. Despite our efforts, detecting a stable interaction between Gle1 and Dbp5 has been elusive. In this model, repeated cycles of ATP-hydrolysis would require constant assembly and disassembly of Dbp5-Gle1 complexes, which might result in fast K_{off} and K_{on} rates. Nevertheless, we detected an interaction between Gle1 and Dbp5 in the low nanomolar range by means of Gle1-IP_6 binding stimulation. I am currently using this method to

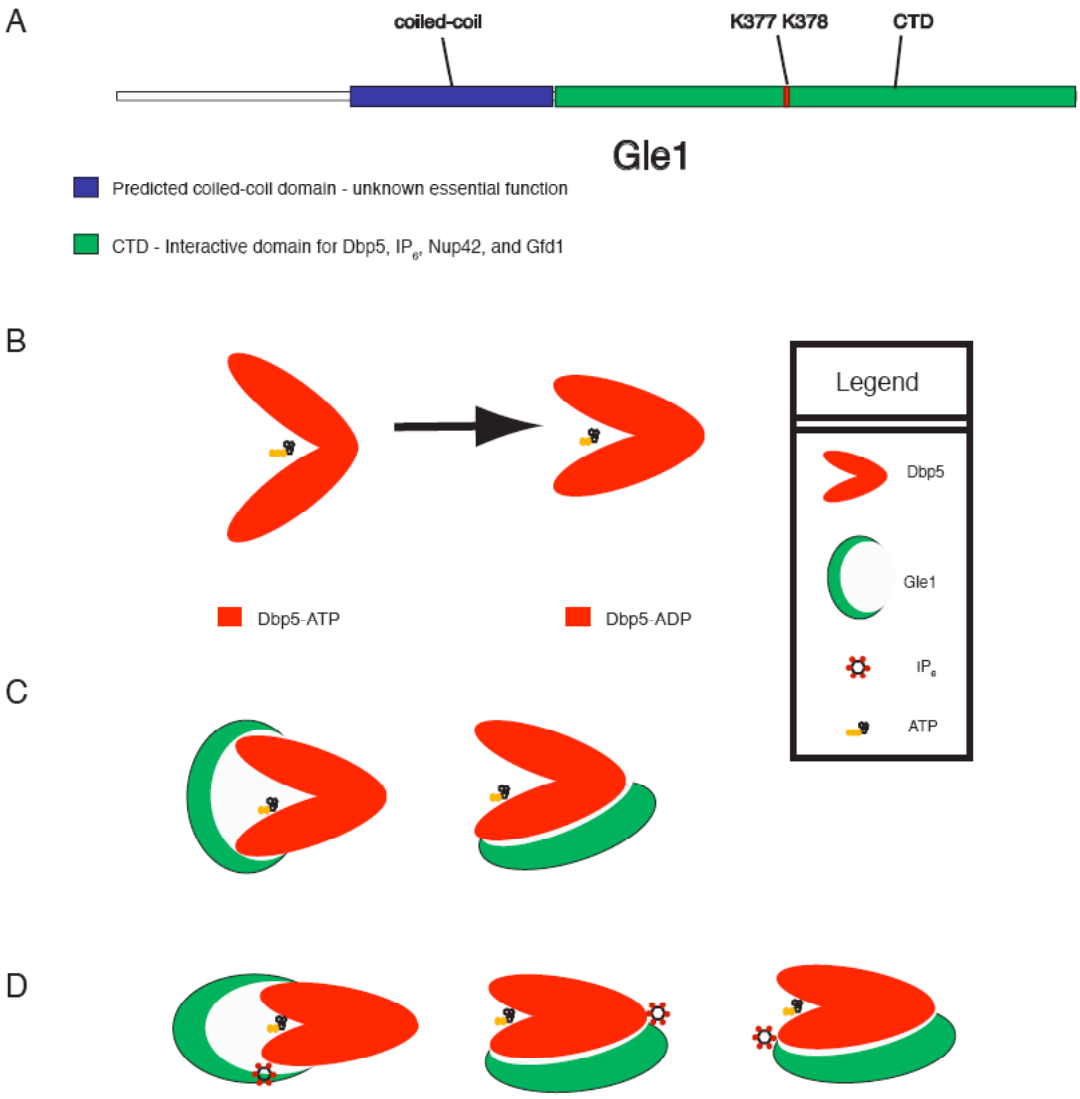

Figure 19: (A) Schematic of Gle1 CTD and putative coiled-coil domains with indicated binding partners. (B) Model of Dbp5 regulation. Dbp5 (red) may cycle between ATP- and ADP-bound forms, resulting in structure changes that regulate its activity. (C) Models of stimulation of Dbp5 ATPase activity by Gle1. Gle1 may interact with both domains of Dbp5 working as a molecular clamp, facilitating a close structural conformation (left). Alternatively, Gle1 may bind one domain of Dbp5 and producing allosteric changes that stimulate Dbp5 ATPase activity (right). (D) Models of stimulation of Dbp5 ATPase activity by IP_6. IP_6 may bind Gle1 and induce an even more stimulatory fold for Dbp5 ATPase activity (left). Alternatively, IP_6 may operate as a molecular glue, increasing the Gle1-Dbp5 interaction, resulting in grater stimulation of Dbp5 ATPase activity (center). Finally, IP_6 may interact with the Dbp5 catalytic domain, helping to stabilize an intermediate step or even to coordinate the Mg^{2+} ion required for ATPase activity.

determine whether the N-, C- terminus or both regions of Dbp5 are sufficient for stimulation of IP_6-binding by Gle1. RNA and nucleotide binding is thought to occur through interactions with residues in the cleft formed between the two globular domains of DEAD-box helicases (Linder 2006). It is likely that that recombinant bacterially purified Dbp5- C-terminal domain (CTD) and N-terminal domains (NTD) will not bind RNA or nucleotides independently or when combined. However, if Gle1 stimulates a closed conformation by simultaneously binding both the NTD and CTD of Dbp5 (or increase their association), partial recapitulation of RNA and nucleotide binding might be achieved.

An additional possibility is that Gle1 binds to only one domain of Dbp5 (Figure 19 C). This binding could result in allosteric changes in Dbp5 that account for increased sensitivity to RNA in ATPase assays. Since in this model Gle1 does not have to interact with two globular domains in Dbp5, a small region of Gle1 could be sufficient for Dbp5 ATPase stimulation. Additionally, one of the globular domains of Dbp5 could be sufficient for full stimulation of Gle1-IP_6 binding.

In vitro, the CTD of Gle1 is sufficient for Dbp5 activation (Weirich et al. 2006). *In vivo*, however, both the NTD and the CTD of Gle1 are essential (Watkins et al. 1998). What is the role of Gle1-NTD? Gle1-NTD can be divided in the extreme NTD and a predicted coiled-coil domain (Figure 19 A). The extreme NTD is not essential whereas the coiled-coil domain is (Watkins et al. 1998). To determine the function of the predicted coiled-coil domain, a battery of temperature sensitive mutants could be generated with amino acid substitutions

in the coiled-coil domain. These could be tested for mRNA export upon temperature shift. If the resulting Gle1 proteins are stable and localize to the NPC, accumulation of poly(A)$^+$ RNA would suggest a role for that region of Gle1 in mRNA export. Additional defects could be tested for mRNA processing or translation.

The dynamics of Dbp5 activation cycles at the NPC suggests that Dbp5 may simultaneously bind Nup159 and Gle1. Nup159 is able to stably interact with Dbp5 in the presence of AMP-PNP, ADP and in the absence of nucleotides (Weirich et al. 2004), which suggests that ATP hydrolysis can occur while Dbp5 is bound to Nup159. At some point, Dbp5 must be released from Nup159 to achieve nucleocytoplasmic shuttling. How is temporal association of Nup159 mediated? Candidate proteins necessary for this process are most likely contained within the mRNP (perhaps at the 3' end of the message). Furthermore, Dbp5 binding partners like Gle1, Nab2 and Gfd1 might facilitate Nup159 removal by competitive binding. It is likely that mutations in *DBP5* resulting in constitutive Dbp5-NPC localization will not associate with proteins that facilitate detachment from Nup159.

Taken together, establishing an assay to calculate and determine Dbp5-Gle1 interactions in the presence of Nup159, Nup42, and Gfd1 will allow direct testing of the importance of these proteins in Dbp5 ATP-ADP cycles and elucidate a potential mechanism for regulation Dbp5 localization dynamics.

Possible mechanisms of Dbp5 stimulation by IP_6

IP_6 has been found to have many roles in cellular physiology (Alcazar-Roman and Wente 2008; York 2006). IP_6 might play regulatory roles in some processes whereas it might act as a cofactor in others. As a member of the IP signaling pathway, IP_6 levels potentially change. In light of Ipk1 localization at the nuclear periphery, it is likely that IP_6 levels can be spatially regulated. In mRNA export, IP_6 drastically augments Dbp5 ATPase activity in a Gle1-dependent manner. During Dbp5 ATPase stimulation, IP_6 interacts with the Gle1-CTD and is not hydrolyzed or modified in any way (Alcazar-Roman et al. 2006). How does IP_6 stimulate Dbp5 activity? Elucidating the mode of IP_6 regulation is tightly dependent on uncovering how Gle1 stimulates Dbp5 in the absence of IP_6. Whether IP_6 works as a regulatory molecule or as a cofactor, I propose three modes of action.

IP_6 might impact Gle1 through allosteric regulation. In this manner, IP_6 binding could increase the affinity of Gle1 for Dbp5, resulting in better activation *in vitro* and *in vivo*. Alternatively, if Gle1 stabilizes a closed conformation in Dbp5 by behaving like a molecular clamp, IP_6 binding might facilitate Gle1's action in bringing the two globular domains of Dbp5 closer together. In other words, IP_6 binding could adjust the tightness of the clamp, and facilitate RNA/ATP binding and hydrolysis (Figure 19 D).

Another possibility is that IP_6 facilitates an interaction between Dbp5 and Gle1 by complementing a positively charged pocket formed when the two

proteins interact. By this mechanism, IP_6 would behave as molecular glue (Figure 19 C). This would account for the higher binding affinity for IP_6 when Gle1 and Dbp5 are present together. Recent studies by O'Shea and co-workers have determined that IP_7 induces structural changes in the Pho80/Pho85/Pho81 complex (Lee et al. 2008). In short, upon IP_7 binding, an additional segment of Pho81 is able to interact with the complex and inhibit Pho81 kinase activity. Similar to IP_7, IP_6 may induce a new Gle1 motif for interaction with Dbp5 further stimulating ATPase activity.

Yet another possibility is that IP_6 might participate in the ATPase reaction by contributing to the stabilization of an intermediate state of ATP/Dbp5 during the ATPase reaction. For instance, IP_6 may help coordinate the Mg^{2+} needed for ATP hydrolysis. In fact, biochemical evidence suggests that IP_6 associates with divalent cations in the cell, specifically Mg^{2+} (Veiga et al. 2006). Additionally, *ipk1Δ* mutants depend on proper Mg^{2+} homeostasis (Unpublished data). Although there is no evidence to support this model, it is appropriate to consider all possibilities.

Recent biochemical and genetic studies in the Wente laboratory have demonstrated that Dbp5 displaces Nab2 from mRNA (Tran 2007). Furthermore, *in vitro*, Dbp5-ADP is sufficient for this activity. These observations suggest that Gle1 and IP_6 are only required for the hydrolysis-driven change of Dbp5-ATP to Dbp5-ADP, and that ATP hydrolysis is not required for protein displacement. These observations are surprising and support the hypothesis of a role for Dbp5

as a ATP/ADP switch. Yet, many questions remain regarding the biochemical properties of Dbp5-ADP that allow such protein displacement. Undoubtedly, a combination of biochemical assays and structural biology data on these components engaged in ATP hydrolysis and protein-RNA remodeling will be required to reveal the secrets of mRNA export.

It can then be speculated that Dbp5-ADP driven protein displacement at the cytoplasmic side of the NPC is crucial for mRNA export. For instance, Dbp5-ADP could displace Mex67, Nab2 and other proteins to be recycled back in the nucleus for future rounds of export. Additionally, Dbp5 may allow the release of the mRNP from the NPC by releasing the interaction between NPC-bound proteins and the mRNA. Yet another possibility is that removal of specific mRNP proteins might expose binding sites for cytoplasmic factors to the mRNP important for mRNA localization and translation. It is likely that the entire collection of proteins targeted by Dbp5 include some factors that fit all the roles described above; nevertheless, a crucial molecular mechanism must exist to allow correct targeting of proteins to be removed by Dbp5. Direct binding to Dbp5 might allow local remodeling in defined mRNP domains. Alternatively, Dbp5-ADP could be targeted to specific proteins by adaptors. These proteins would bridge the interaction by binding to both Dbp5-ADP and proteins to be displaced from the mRNA. Gfd1 fits both criteria by binding Dbp5 and Nab2 *in vitro* (Suntharalingam et al. 2004). Once the target is defined, Dbp5-ADP must drive dissociation of mRNA bound proteins. What is the mechanism of protein

displacement from RNA? Biochemical and biophysical data on the processive ATP-dependent dsRNA unwinding by DEAD-box helicases has defined that individual ATP-hydrolysis events are responsible for defined steps in the unwinding and translocation processes, and that these proteins work by locally melting of the dsRNA ahead of the opening fork (Cheng et al. 2007; Dumont et al. 2006). Given that protein displacement by Dbp5 does not require ATP hydrolysis, it is possible that Dbp5-ADP bind the mRNP and undergoes conformational changes upon binding. These changes could force the local bending of the RNA and promote protein release. Alternatively, Dbp5-ADP could work by competing with mRNP proteins for RNA binding. Once the protein has been displaced, these could be re-imported into the nucleus. Additionally, the removal of these proteins would have a global effect on mRNP structure, which could be important for translation and localization.

Dynamics of IP_6 production, localization, and regulation

Ipk1 is localized at the nuclear periphery in yeast cells (York et al. 1999). Thus, IP_6 production is potentially situated at the site of Gle1-Dbp5 function. However, IP_6 production specifically localized at the plasma membrane is able to regulate its target at the NPC and facilitate mRNA export (Miller 2004). What is then the evolutionary advantage for Ipk1 localization at the nuclear periphery? When thinking of IP_6, it is imperative to consider the other nuclear functions reported for this molecule: mRNA editing, DNA repair, and chromatin remodeling

(Hanakahi et al. 2000; Macbeth et al. 2005; Shen et al. 2003; Steger et al. 2003). In this light, localization of Ipk1 at the nuclear periphery might serve an important role in these or other unknown nuclear functions by placing production of IP_6 at the gate of the NPC. A common pattern between the targeted production of IP_6 and its functions in the cell is also illustrated by studies of zebrafish Ipk1 (zfIpk1). In zebrafish, IP_6 has a determinant role in the left-right asymmetric distribution of internal organs by controlling ciliary beating at the Kupffer's vesicle (Sarmah et al. 2005; Sarmah et al. 2007). Strikingly, zfIpk1 is enriched at the centrosomes and basal bodies of ciliated cells (Sarmah et al. 2007). Taken together, eukaryotic cells might establish and regulate the localization of IPs-producing enzymes to increase the local concentration of specific IPs and regulate cellular processes. Future studies aimed at understanding the full role and regulation of IPs will need to determine the molecular determinants for Ipk1 targeting to the nuclear periphery/basal body, and test if this localization is regulated.

Subcellular concentrations of IP_6 and other IPs might be regulated. In fact, the localization of Ipk1 near NPCs suggests that IP_6 levels might be higher at these sites. On the other hand, enzymes responsible for IP_6 turnover could be localized at sites where IP_6 is not required. A potential mechanism for regulating subcellular concentrations of IPs could also be achieved by active transport of IPs through membrane transporters. Eukaryotic cells have in place inositol transporters for extracellular intake of inositol (described above). Furthermore, in yeast, localization of these transporters is not restricted to the plasma membrane

(Huh et al. 2003). However, classic and recent studies have established that, at least for IP_3 and IP_7, a rapid burst of IP production is sufficient for signaling. A recent report suggests that Plc1 activation through G-protein-coupled receptors could potentially change cellular levels of most IPs (Otto et al. 2007). But how are these bursts achieved? There is no data describing the enzymatic activation or inhibition of IPs-kinases and phosphatases. We know, however, that Vip1 produces IP_7 upon phosphate starvation in yeast. Is Vip1 activated? Is the IP_6 pool made available to it? Yeast-two-hybrid screens utilizing IPs enzymes as baits, coupled to genetic studies in yeast should be utilized to identify these components. The localization and regulation of IPs kinases, and subcellular localization of their products are completely unknown and represents, in my opinion, the most fertile grounds for research in the IP field.

IPs and regulation of gene expression

Rapid and decisive changes in gene expression lie at the center of the cell's innate ability to react to environmental changes or to complete cellular differentiation programs. In the nucleus, regulation of gene expression requires proper activation of chromatin-remodeling complexes, modulation of transcription factor activity, faithful mRNA processing, and efficient mRNA export. In metazoans, distinct and independent machineries exist for the regulation of nuclear PIPs from those in the cytoplasm (Cocco et al. 1987; Divecha et al. 1991; Martelli et al. 1991). In fact, dynamics of nuclear PIP_2 serves in the regulation of

insulin growth factor 1 signaling (Cocco et al. 1998; Divecha et al. 1991), chromatin structure (Yu et al. 1998; Zhao et al. 1998), and indirectly in the pre-mRNA processing machinery (Boronenkov et al. 1998; Osborne et al. 2001), thus impacting gene expression (Bunce et al. 2006; Gonzales and Anderson 2006; Irvine 2003). Interestingly, recent studies have revealed roles for specific soluble IPs in each in gene expression. Taken together, these studies suggest that IPs might coordinate cellular responses and orchestrate nuclear functions that impact the global gene expression mechanism.

Transcriptional regulation

The newly emerging paradigm of IPs as nuclear-signaling molecules is clearly evidenced by a series of recent discoveries in the *PHO* pathway which carefully coordinates cellular responses to phosphate starvation. In budding yeast, the cyclin-dependent kinase (CDK) Pho85 regulates a vast number of cellular functions (Carroll and O'Shea 2002). One of its associated cyclins, Pho80, directs Pho85 in its roles during *PHO* signaling. The CDK1 inhibitor Pho81 binds the CDK complex but only inhibits the CDK in response to P_i starvation (Figure 20). In the presence of high P_i levels, Pho80–Pho85 is active and phosphorylates the transcription factor Pho4. Phosphorylated Pho4 is exported to the cytoplasm and the Pi-responsive gene targets remain turned off. In response to P_i starvation, Pho81 inhibits Pho80–Pho85 phosphorylation of Pho4; thus, Pho4 remains nuclear and binds activation sequences in the PHO5

promoter (Fascher et al. 1990). Pho4 further recruits the chromatin-remodeling complexes INO80 and SWI/SNF (Steger et al. 2003), which help displace four positioned nucleosomes from the PHO5 promoter (Almer et al. 1986) facilitating PHO5 transcription.

A recent report has biochemically identified IP_7 as a signal for negative regulation of the Pho80–Pho85 CDK in a Pho81-dependent manner (Lee et al. 2007). During P_i starvation, yeast cells increase IP_7 levels sevenfold. This production of IP_7 is required for *in vivo* Pho4-green fluorescent protein nuclear localization. This is presumably accomplished by IP_7 binding to the Pho80–Pho85–Pho81 complex. By this action, IP_7 is sufficient to inactivate recombinant Pho80–Pho85 in a Pho81-dependent manner. Thus, the transcription factor Pho4 is not phosphorylated by the CDK, not exported from the nucleus, and as such can activate PHO5 transcription (Komeili and O'Shea 1999). Importantly, IP_7 from the Vip1-driven phosphorylation of IP_6 and not the Kcs1-derived IP_7 isoform is required for inactivation of the Pho80–Pho85 CDK. Vip1 may produce two forms of IP_7 (Figure 8) (Mulugu et al. 2007). Future studies will determine whether 6PP-IP_5, its stereoisomeric cousin 4PP-IP_5, or both participate in *PHO* signaling. To date, a number of studies have strengthened the role of IPs in transcriptional control (Auesukaree et al. 2005; El Alami et al. 2003; Odom et al. 2000; Romero et al. 2006), creating fertile ground for the biochemical dissection of these mechanisms.

Alteration of chromatin structure

It is interesting to note that there are further independent connections between the production of IPs and function of the PHO-signaling pathway. In a genetic screen, mutant cells deficient in IP_4 and IP_5 production were found to have defects in nucleosome remodeling and PHO5 transcription (Steger et al. 2003). Specifically, *ipk2Δ* mutants are deficient for recruitment of the INO80 and SWI/SNF chromatin-remodeling complexes to the *PHO5* promoter. In an independent study, IP_4 and IP_5 were found to stimulate *in vitro* nucleosome remodeling by the SWI/SNF complex (Shen et al. 2003). IP_6, on the other hand, inhibits the remodeling and ATPase activity of ISW2, INO80, and nucleosome-remodeling factor complexes. The requirement for Plc1 and Ipk2 in the transcription of *INO1* (Shen et al. 2003), a gene controlled by the SNF2, ISW2, and INO80 chromatin-remodeling complexes (Ebbert et al. 1999; Goldmark et al. 2000; Peterson et al. 1991), suggests that the *in vitro* effects of IPs on chromatin remodeling are physiologically relevant.

PHO is not the only cellular signaling pathway that utilizes IPs. Adaptation to osmotic changes in budding yeast utilizes the *HOG* MAP kinase pathway (Brewster et al. 1993; O'Rourke et al. 2002). Cells in hyperosmotic conditions activate a MAP kinase pathway that results in the phosphorylation and subsequent nuclear import of the Hog1 MAP kinase. Nuclear Hog1 regulates a number of transcriptional activators and repressors and results in a broad transcriptional response encompassing more than 500 genes (Hohmann 2002;

Posas et al. 2000; Rep et al. 2000). Concomitantly, osmotic changes trigger production of IPs (Ongusaha et al. 1998; Perera et al. 2004; Pesesse et al. 2004). Indeed, *plc1Δ* mutant cells are osmosensitive (Flick and Thorner 1993) suggesting that production of IPs is essential for the proper response to osmotic stress. It is interesting to note that IP_4 and IP_5 have been recently implicated in the recruitment of the Snf1p-dependent Spt-Ada-Gcn5-acetyltransferase (SAGA) chromatin-remodeling complex to osmotically inducible promoters (Guha et al. 2007). As opposed to wild-type cells, *plc1Δ* and *ipk2Δ* mutants do not recruit SAGA or the TATA-binding protein to *HOG* inducible promoters during hyperosmotic shock. Thus, *plc1Δ* and *ipk2Δ* cells are not viable under high osmolarity conditions. Taken together, both the *PHO*- and *HOG*-signaling pathways utilize IPs to modulate transcription of their target genes.

The impact of IPs on multiple different steps in the gene expression pathway allows for a potentially integrated cellular response system (Figure 20). An attractive model could be centered on *PHO* signaling and IPs, based on the studies referenced above. Under conditions of P_i starvation and by yet unknown mechanisms, Plc1 and Vip1 localized in the nucleus are activated. Nuclear Ipk2, Ipk1, and Kcs1 rapidly convert IP_3 into IP_4 and other IPs, including IP_7. IP_7-mediated inactivation of the Pho80–Pho85 kinase stimulates Pho4 nuclear retention. Nuclear Pho4 then binds accessible activation sequences in the *PHO5* promoter and recruit chromatin-remodeling complexes in an IP_4/IP_5-dependent manner. Transcription and maturation of *PHO*-responsive mRNPs could

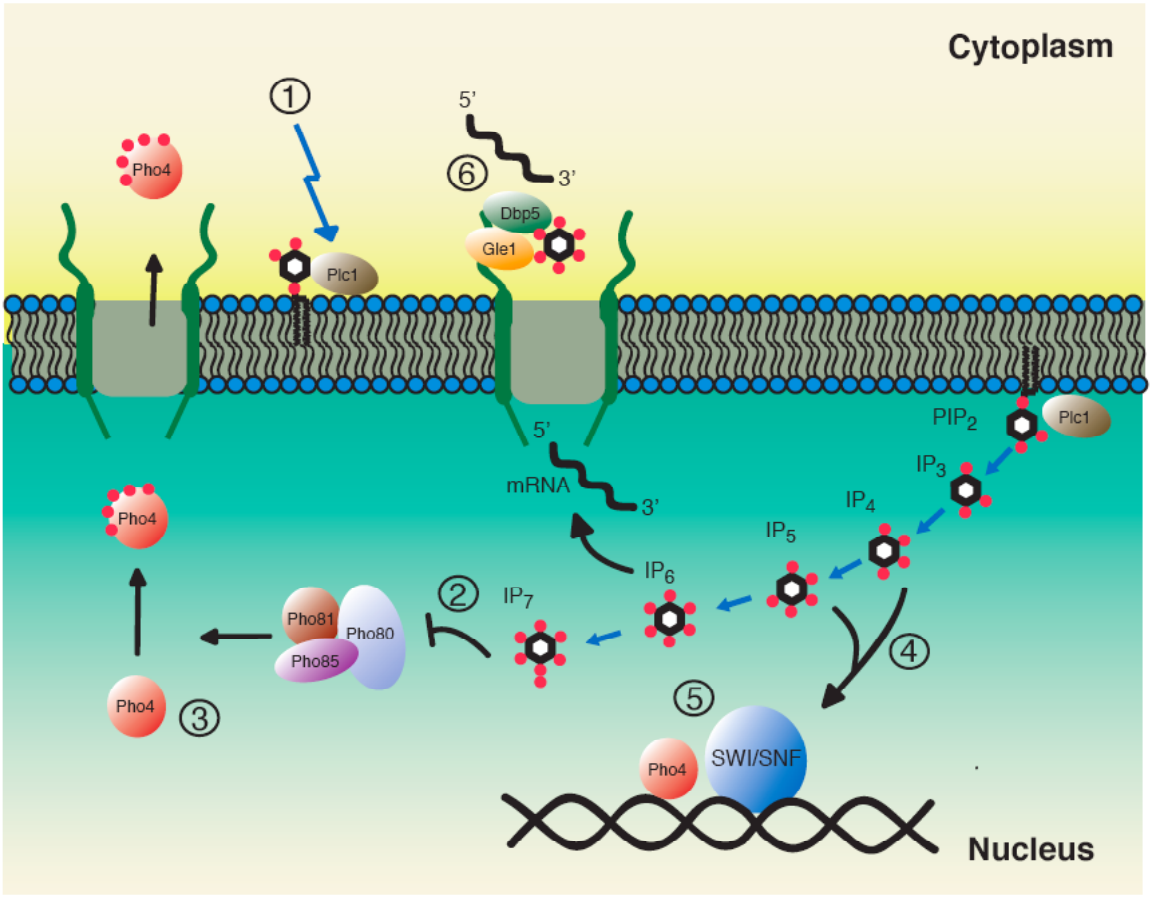

Figure 20: Model for global control of gene expression by IPs in the *PHO* pathway. A sequential signaling pathway is shown. 1- P_i starvation results in activation of Plc1 and Vip1. The mechanism for this activation is unknown. 2- Vip1-derived IP_7, produced from phosphorylation of pre-existing and/or new IP_6 pools, inhibits the Pho85 CDK in a Pho81 dependent fashion. 3- Inactive Pho81 fails to phosphorylate the transcription factor Pho4 resulting in nuclear retention and Pho4 recruitment to *PHO*-responsive promoters. 4- The production of IP_4 and IP_5 also aids in the recruitment and activation of the SWI/SNF chromatin-remodeling complex at *PHO*-responsive promoters. 5- Chromatin remodeling results in transcriptional activation and mRNA synthesis. 6- Dynamic IP_6-levels control mRNA export by regulating Dbp5 activity in a Gle1-dependent manner at the NPC cytoplasmic face. This regulation could preferentially expedite export of *PHO*-responsive mRNAs and result in a rapid global cellular response. Red balls indicate phosphates in IPs, IPPs, and Pho4.

potentially be coupled with IP_6/Gle1 regulation of Dbp5 at the NPC, expediting directional release of key mRNPs in the cytoplasm. Thus, *PHO*-responsive genes have the potential to be regulated at multiple levels by different IP species. Many questions remain unsolved: Are all IP species produced during *PHO* signaling? Is IP_7 produced from a pre-existing IP_6 pool or from newly synthesized IP pools triggered by Plc1 signaling? How is the signal terminated? Such multitargeted cellular adjustments and positive feedback loops are often employed to ensure decisive and penetrant cellular responses. It is tantalizing to consider that the control of IPs allow coupling of multiple steps in the gene expression pathway for effective *PHO* signaling.

Editing of messenger and tRNA

The coordinated processing of transcribed RNA is directly linked to proper gene expression. ADARs or adenosine deaminases acting on tRNA (ADATs) are a family of enzymes that mediate the site-specific catalytic deamination of adenosine to produce inosine in RNA (Bass 2002). Deamination of targeted adenosines on mRNA happens cotranscriptionally within the nucleus. During mRNA splicing and translation, the inosine is recognized as guanosine resulting in differential splicing and codon usage (Bass 2002). It is interesting to note that the crystal structure of the hADAR2 C-terminal domain revealed a molecule of IP_6 buried within a basic pocket of the protein structure (Macbeth et al. 2005). This domain is necessary and sufficient for editing, and the enzyme activity requires

the presence of IP_6. Similarly, Tad1, a member of the ADAT family in yeast, has a reduced capacity to edit tRNA in the absence of IP_6. For both hADAR2 and yeast Tad1, the requirement for IP_6 was surprising and suggests possible mechanisms for regulation of editing activity.

Is IP_6 directly regulating editing as a signaling molecule or serving as a cofactor for protein folding? Based on the structural analysis of the hADAR2 C-terminal domain (Macbeth et al. 2005), the IP_6 appears to play a structural cofactor role and is distinct from the catalytic site. Importantly, the established RNA targets of hADAR2 in human cells are found in the nervous system (Paul and Bass 1998) such as the 2C subtype of the serotonin receptor (Burns et al. 1997) and the glutamate receptor (Lomeli et al. 1994). In fact, the level of editing on glutamate receptor mRNAs is developmentally regulated (Bernard and Khrestchatisky 1994). In parallel, Ipk1, the kinase that produces IP_6, has dynamic expression patterns during embryogenesis and is present in adult neural tissue (Sarmah et al. 2005; Verbsky et al. 2005a) providing a platform for spatial and temporal regulation of ADAR2 activity during development. Furthermore, IP_6-coordinated mRNA editing could regulate gene expression by editing splice sites that would result in splice variants with greater stability and/or export potential. Alternatively, an altered splice site could produce an export-incompetent mRNA or a dysfunctional protein, as has been demonstrated by the self-regulation of hADAR2 activity (Rueter et al. 1999). Hyperedited RNAs are also shown to be retained in the nucleus (Zhang and Carmichael 2001), providing an additional

regulatory function for hADAR2/IP_6 in gene expression. Future studies of the full-length hADAR2 should provide a more complete story of the physiological role for IP_6 function in RNA editing.

Other nuclear roles for IPs

In addition to gene expression, several other nuclear processes have been linked to the repertoire of IP-regulated mechanisms. Genetic, biochemical, and cell biological studies have shown that IPs regulate DNA repair, telomere homeostasis, and kinase-free phosphorylation of proteins within the nucleus.

DNA repair

Eukaryotic cells use two distinct mechanisms for repairing double-stranded DNA breaks, homologous recombination (HR) and nonhomologous end-joining (NHEJ) (van Attikum and Gasser 2005; Zhou and Elledge 2000). The first evidence for the role of IPs in DNA repair came from a genetic screen in which a *kcs1* mutant was found to be defective for DNA hyperrecombination characteristic in a protein kinase C1 (*pkc1*) mutant (Huang and Symington 1995). As the IP enzymatic properties of Kcs1 were uncovered, it was found that the abnormal HR of pkc1 mutants was linked to IP_7 and IP_8 production (Luo et al. 2002). Independent support for the role of IPs in DNA repair was demonstrated in biochemical studies of the mammalian NHEJ mechanism. Using *in vitro* assays,

IP_6 was identified as a potent stimulator of NHEJ (Hanakahi et al. 2000). The DNA-dependent protein kinase (DNA-PK) complex is recruited to double-stranded DNA breaks where it plays a crucial role in NHEJ. IP_6 binds the Ku70/80 subunit of the DNA-PK complex (Hanakahi and West 2002; Ma and Lieber 2002) and presumably results in allosteric changes that impact kinase activity through increased stability or DNA binding of the holoenzyme (Hanakahi and West 2002). However, the mechanism for how IPs impact DNA repair remains elusive, and additional work is required to demonstrate the physiological impact. Although Ku70 protein dynamics are possibly altered in cells treated with calmodulin antagonists that reduce levels of all cellular IPs (Byrum et al. 2004), this effect could be indirect because of multiple other perturbations associated with disrupted Ca^{2+} homeostasis. It is interesting to note that ATP-dependent chromatin-remodeling complexes have roles in DNA repair (Downs et al. 2004; Morrison et al. 2004; van Attikum et al. 2004), and three components of the INO80 complex (Ino80, Arp5, and Arp8) are recruited to sites of double-strand breaks. Given the proposed roles for IP_4/IP_5 in INO80 chromatin remodeling, it is also intriguing to consider that IPs could regulate DNA repair through multiple mechanisms.

Telomere length

Two studies have provided *in vivo* evidence in budding yeast for the role of IPPs in maintenance of telomere length (Saiardi et al. 2005; York et al. 2005). As

described in Fig. 2, Kcs1 utilizes IP_5 or IP_6 and phosphorylates a pre-existing phosphate in the 5-position to generate PP-IP_4 and PP-IP_5 (Saiardi et al. 1999), respectively. In kcs1 mutant cells, the telomeres are longer. Genetic studies suggest that PP-IP_4 and not PP-IP_5 impact telomere length (Saiardi et al. 2005; York et al. 2005). This indicates that an undefined target of PP-IP_4 is able to distinguish the phosphorylation state of the axial 2-phosphate on the inositol ring. However, the telomere defects cannot be entirely attributed to lack of PP-IP_4 production. Expression of one of the three human inositol diphosphoryl synthases (hIP6K1) completely restores PP-IP_4 production but fails to fully rescue telomere length defects (York et al. 2005). Nonenzymatic roles of Kcs1 and/or controlled timing and localization of PP-IP_4 production might also be necessary for proper regulation of telomere length. Finally, Tel1, a protein kinase required for proper telomere maintenance, is indispensable for Kcs1 telomere regulation and thus seems to have roles downstream of Kcs1 (Saiardi et al. 2005; York et al. 2005). It is possible that PP-IP_4 binds and regulates Tel1. Moreover, because both DNA-PK and Tel1 are members of the phosphatidyl inositol 3-kinase-related kinase family and have roles in DNA damage response (Durocher and Jackson 2001; Pennaneach and Kolodner 2004; Zhou and Elledge 2000), Tel1 might be regulated by IPs in a manner similar to DNA-PK in the DNA repair mechanism.

Direct phosphorylation of nuclear proteins

One of the most surprising functions attributed to IPs is the nonenzymatic phosphorylation of proteins by Kcs1-derived IP_7 (5PP-IP_5) (Saiardi et al. 2004). Using *in vitro* assay conditions, Kcs1-derived IP_7 directly phosphorylates Nsr1, Srp40, and Ygr130c in the absence of a protein kinase. Nsr1 and Srp40 are both found in the nucleolus and are involved in ribosome biogenesis (Meier 1996; Stage-Zimmermann et al. 2000), suggesting that IP_7 may have roles in ribosome maturation, processing, and/or transport. However, this has not been demonstrated. Although kcs1 mutant cells have reduced levels of Nsr1 phosphorylation (Saiardi et al. 2004), nearly 40% of Nsr1 phosphorylation remains. Whether this non-Kcs1-linked Nsr1 phosphorylation represents protein kinase-driven or Vip1-derived IP_7 (4/6PP-IP_5) phosphorylation remains unknown. Overall, many questions remain regarding the mechanism, specificity, and requirements of the chemical reaction of phosphate transfer by IP_7.

Concluding remarks

The abundance of IP species and their emerging roles in diverse aspects of nuclear cellular physiology suggests that cells utilize *myo*-inositol as an immensely versatile backbone for the production of second messengers. As described, these insights have come from a series of unrelated genetic and biochemical studies of fundamental nuclear processes (mRNA export, chromatin

remodeling, transcriptional activation, DNA repair, and RNA editing) that serendipitously discovered roles for IPs in their respective mechanisms. Taken together, this represents a fascinating new paradigm that places IPs as linchpins for coordinating multiple steps of the gene expression pathway from upstream signaling to mRNA export.

The budding yeast model system has been especially fruitful in terms of revealing these nuclear functions because of a potential specialization and limited diversification of its IP signaling roles. In contrast to metazoan cells, *S. cerevisiae* has no reported IP_3 receptor that modulates Ca^{2+} release, nor is there DAG regulation of protein kinase C (York 2006). However, it is well documented that IP_3 serves as the precursor for the formation of the highly phosphorylated IPs (York et al. 1999). *S. cerevisiae* might possess only the nuclear highly phosphorylated IP functions as the rudimentary pathway required in all eukaryotes. Multicellular metazoan organisms and more recently evolved eukaryotes would have not only the nuclear functions for the highly phosphorylated IPs (e.g., IP_5, IP_6, IPPs) but also cytoplasmic roles for them. For example, recent studies have documented roles for Vip1 production of IP_7 in cytokinesis of *S. pombe* (Mulugu et al. 2007). In addition, Ipk1 production of IP_6 is required for ciliary beating in left–right asymmetry establishment in vertebrates (Sarmah et al. 2005; Sarmah et al. 2007), and enzymatic production of IPs is essential for in mouse early embryonic development (Frederick et al. 2005; Verbsky et al. 2005a). Thus, in both the nucleus and the cytoplasm, the spatial

and temporal activation of inositol-signaling pathways modulates protein machines and enables eukaryotic cells to sense changes in their environment. Unraveling the complexity of IP fluxes and potential coupling between the nuclear and cytoplasmic IP functions will be frontiers for future investigations.

To conclude, the studies presented here have focused on mRNA export regulation by IP_6, Gle1, and Dbp5. In short, Gle1 directs mRNA export by spatially activating the ATPase activity of the DEAD-box protein Dbp5 at the NPC. Importantly, these studies have also demonstrated that IP_6, a member of the IP-signaling pathway, can potentiate the Gle1-driven Dbp5 ATPase stimulation *in vitro* and impact Gle1 activity *in vivo*. Localized activation of ATPase activity at the cyoplasmic face of the NPC could account for the directionality of the mRNA export process. As a result, cytoplasmic NTPase activation seems to be a conserved mechanism governing nuclear export of proteins and mRNA. Additionally, the studies presented here enrich our understanding of how DEAD-box helicases could be regulated in a spatiotemporal manner. Finally, our studies contribute to the grater appreciation of IP control of gene expression. As a result of these studies, the field now seeks to understand the molecular mechanism by which Gle1 activates Dbp5, the mechanism by which Dbp5 is able to remodel a mRNP, and the regulation of transcript specific mRNA export. Significantly, these findings will bee important for the elucidation of abnormal functions of these players in viral pathogenesis and genetic disorders. In fact, a recent study has unveiled that 1% of the

population of Finland are heterogeneous for a specific mutation in hGLE1 (Nousiainen et al. 2008). Homozygous embryos with hGLE1 lesions develop but die during gestation due to lethal congenital contracture syndrome 1 (LCCS1) and lethal arthrogryposis with anterior horn cell disease (LAAHD). Postmortem analyses reveals severe neurological defects. Importantly, similar conditions known as LCCS2 and LCCS3 are due to genetic lesions of IP signaling components (Narkis et al. 2007a; Narkis et al. 2007b). The study presented in this thesis describes a biochemical mechanism that strongly argues that hGle1 and IP_6 might play a common role in the regulation of a neuronal DEAD-box helicase, providing a potential platform for the studies of defective biochemical mechanism in these human diseases.

Appendix

A. RESPONSE TO HIGH OSMOLARITY IMPACTS mRNA EXPORT AND GROWTH OF mRNA EXPORT MUTANTS

A number of reports have established that yeast cells under stress respond by altering its mRNA export capacity and blocking the export of general mRNAs (Saavedra et al. 1997; Carroll et al. 2001; O'Rourke et al. 2002). In yeast, the *HOG* pathway controls a cellular response to high osmolarity by activating a MAP-kinase pathway terminating in the phosphorylation and subsequent nuclear import of the Hog1 MAP-kinase. Nuclear Hog1 regulates a number of transcription factors and promotes transcription of *HOG* responsive genes.

Since osmotic changes result in accumulation of IP_6, and *PLC1* is required for coping with high osmotic stress, we tested if high osmolarity would have an impact on mRNA export of wild type and cells carrying mutations in genes encoding mRNA export factors. Cells grown in high concentration of sorbitol, NaCl, $MgCl_2$ or $CaCl_2$ activate the *HOG* pathway. We have tested the impact in growth and mRNA export efficiency of mRNA export mutants in high osmolarity.

We found that high osmolarity rescues the growth defect of strains lacking both IP_6 production and containing mutations in *NUP159, NUP42, NUP116,* or *GLE2.* Further tests revealed that high osmolarity rescues both the growth and mRNA export defects of *nup159-1, gle1-4,* and *mex67-5.* Of note, the growth

defect suppression is remarkable using 300mM $CaCl_2$ media at 37˚C for *mex67-5* and *nup159-1.* Additionally, high osmolarity partially rescues the growth defect of *nup116Δ, dpb5-2* and *glc7-5.* Thus, suppression mRNA export defects overlap with suppression of growth defects at high temperatures. Additionally, these effects do not require IP_6 production and affect different steps of the mRNA export process such as targeting by Mex67, and release by Dbp5.

What is the physiological mechanism for this suppression of mRNA export defects? It is unknown whether or not Hog1 is required for this effect. We could utilize a *hog1Δ mex67-5* mutant and test for the mRNA export defects at 37˚C in high osmolarity. It is possible that the activation of the *HOG* pathway alters the path of mRNA export through the NPC by modification of the mRNPs. An alternative hypothesis is that the NPC itself is a target of *HOG* regulation. It would be beneficial to study the defects of import and export in *xpo1-1* mutants under high osmotic stress. If high osmolarity rescues the defects of *xpo1-1* mutants, a general study of NPC permeability and composition should follow. Regulation of transport may allow rapid gene expression changes to cope with stress in harsh environmental conditions to which yeast cells are exposed in their natural habitat.

B.

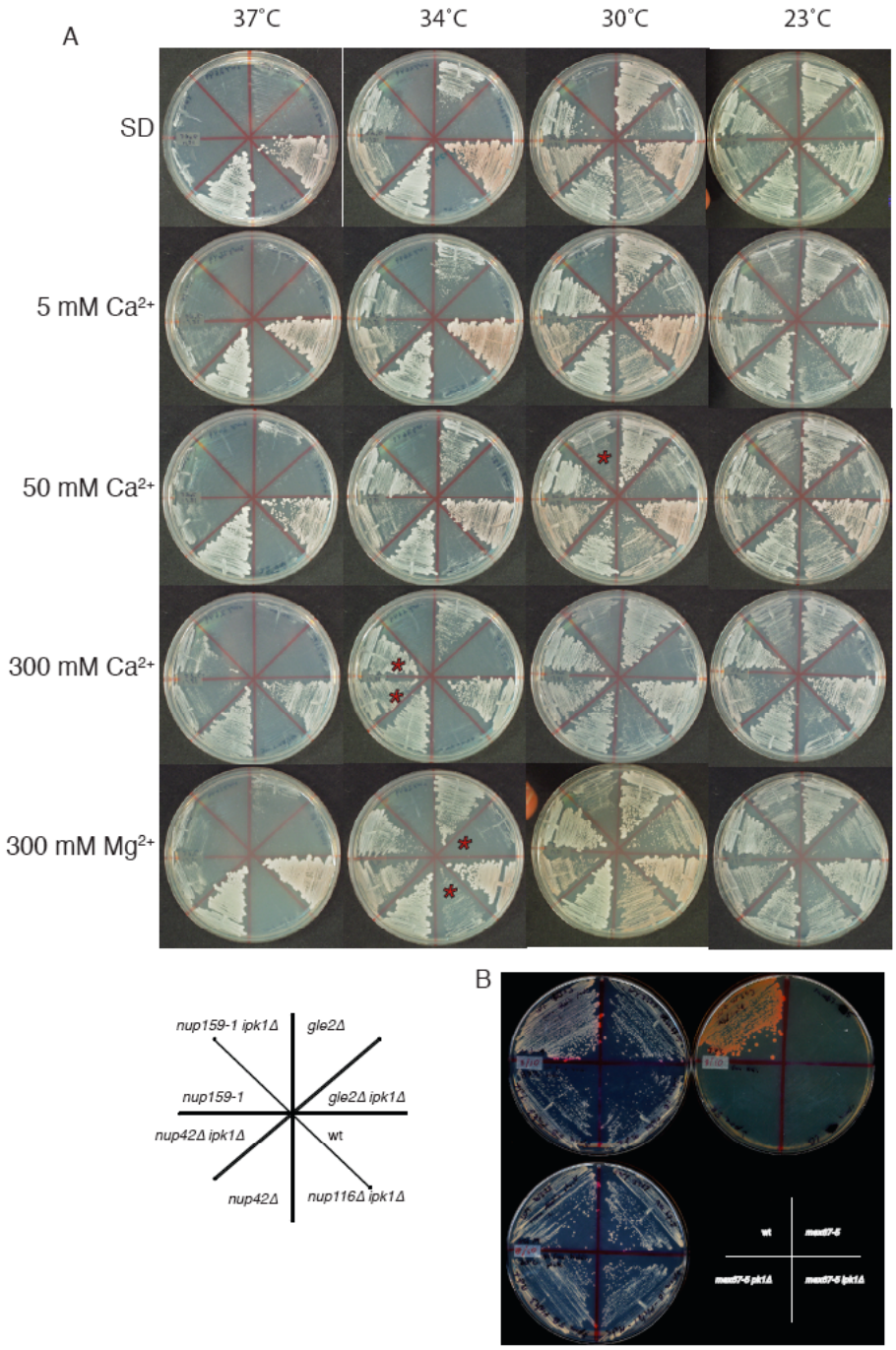

Figure A1: High osmolarity rescues the growth defect of several mutant strains. (A) Indicated strains were streaked and grown in plates containing high osmolarity as indcated. (B) Indcated strains were grown in 1M Sorbitol (left), YPD, (right), and 1M KCl (below) at 37˚C. Red stars indicate growth defect suppressed in high osmolarity (A).

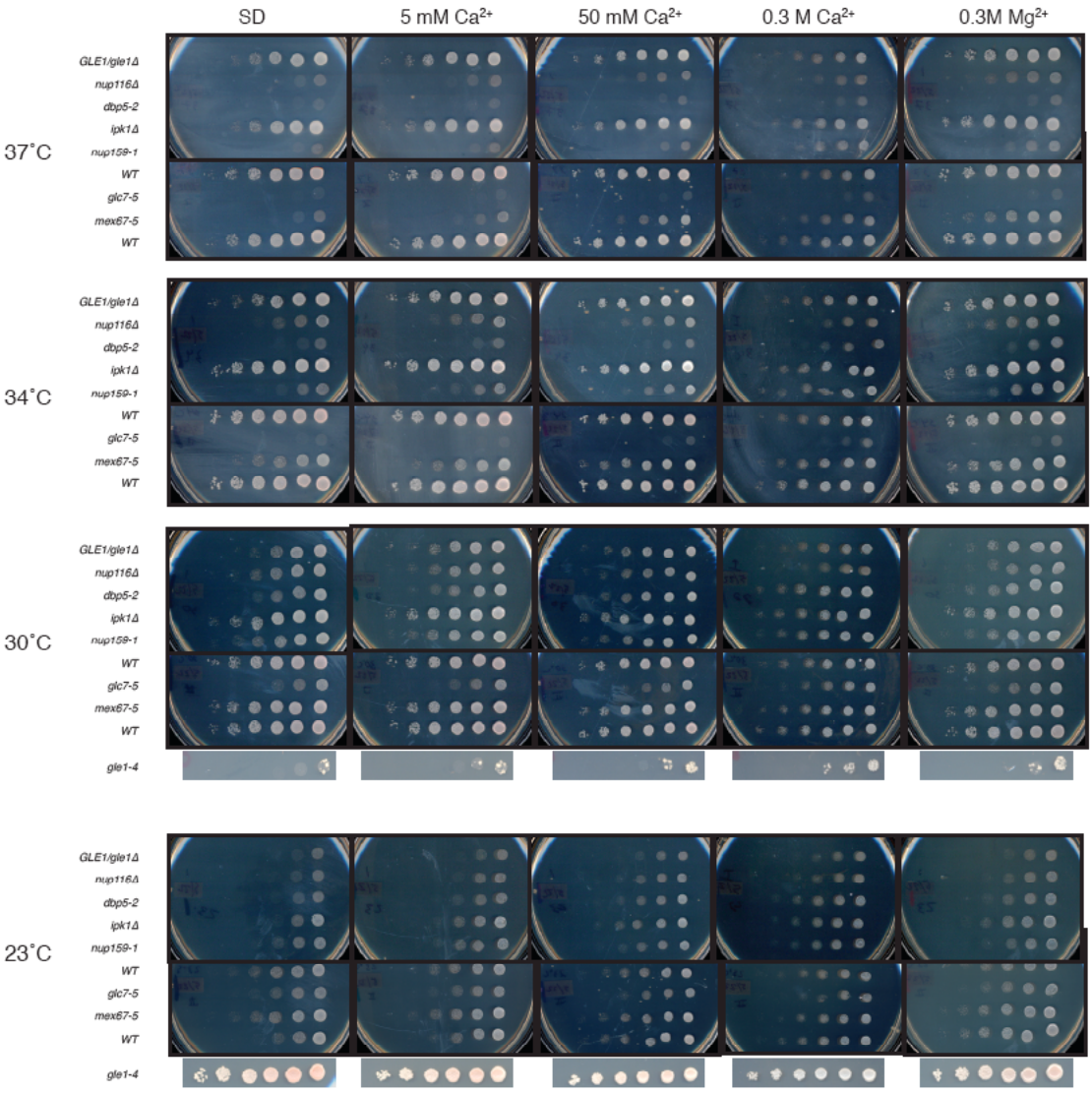

Figure A2: High osmolarity rescues the growth defect of several mutant strains. Serial dilutions were plated on plates containing high osmolarity as indcated.

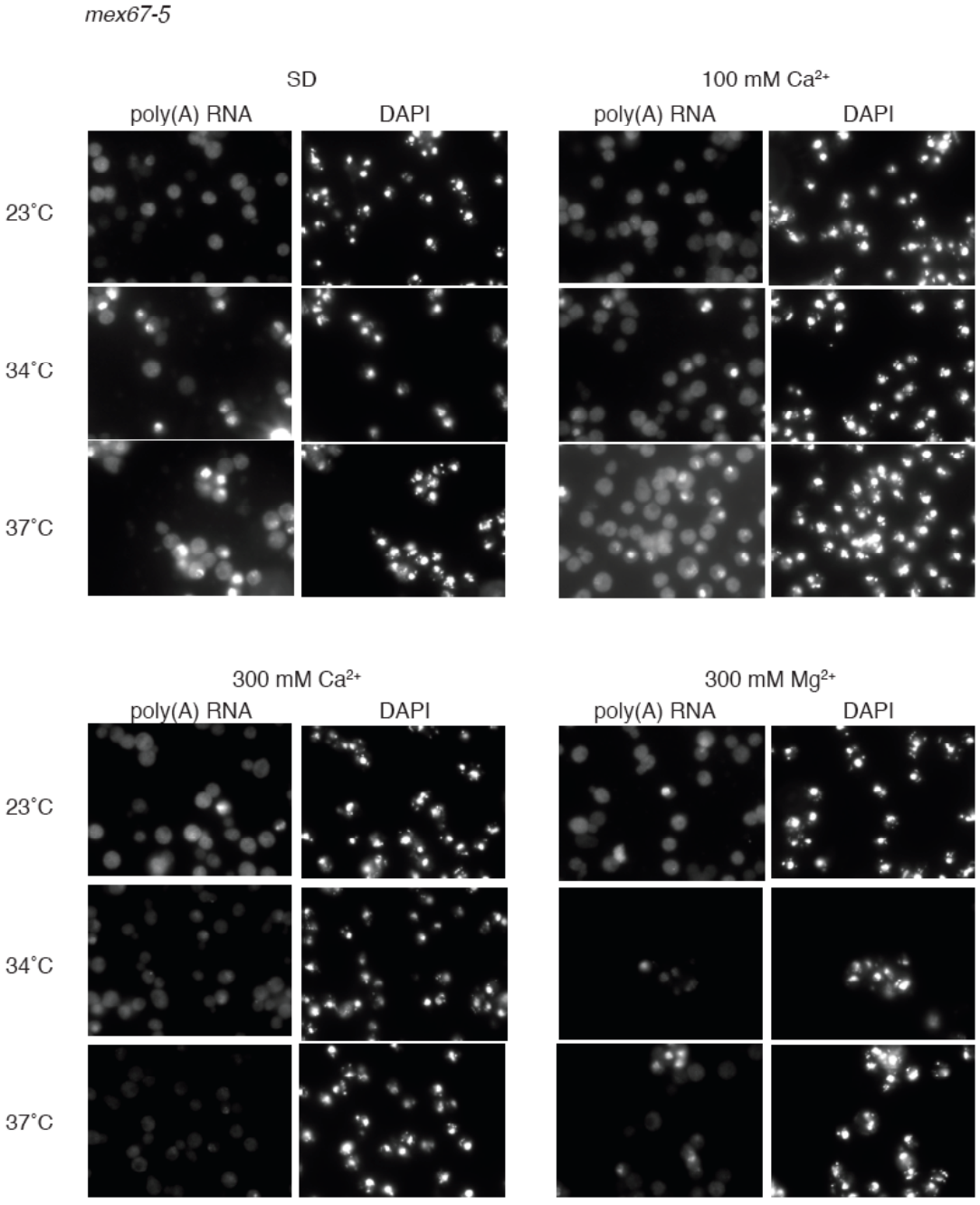

Figure A3: High osmolarity rescues the mRNA export defect of *mex67-5* mutants. Strains were grown at 23˚C and shifted for 3hrs in high osmolarity media (or synthetic media) prior to shifting temperatures for 1hr. Strains were processed by *in situ* hybridization with an oligo d(T) probe and DAPI staining to visualize nuclei.

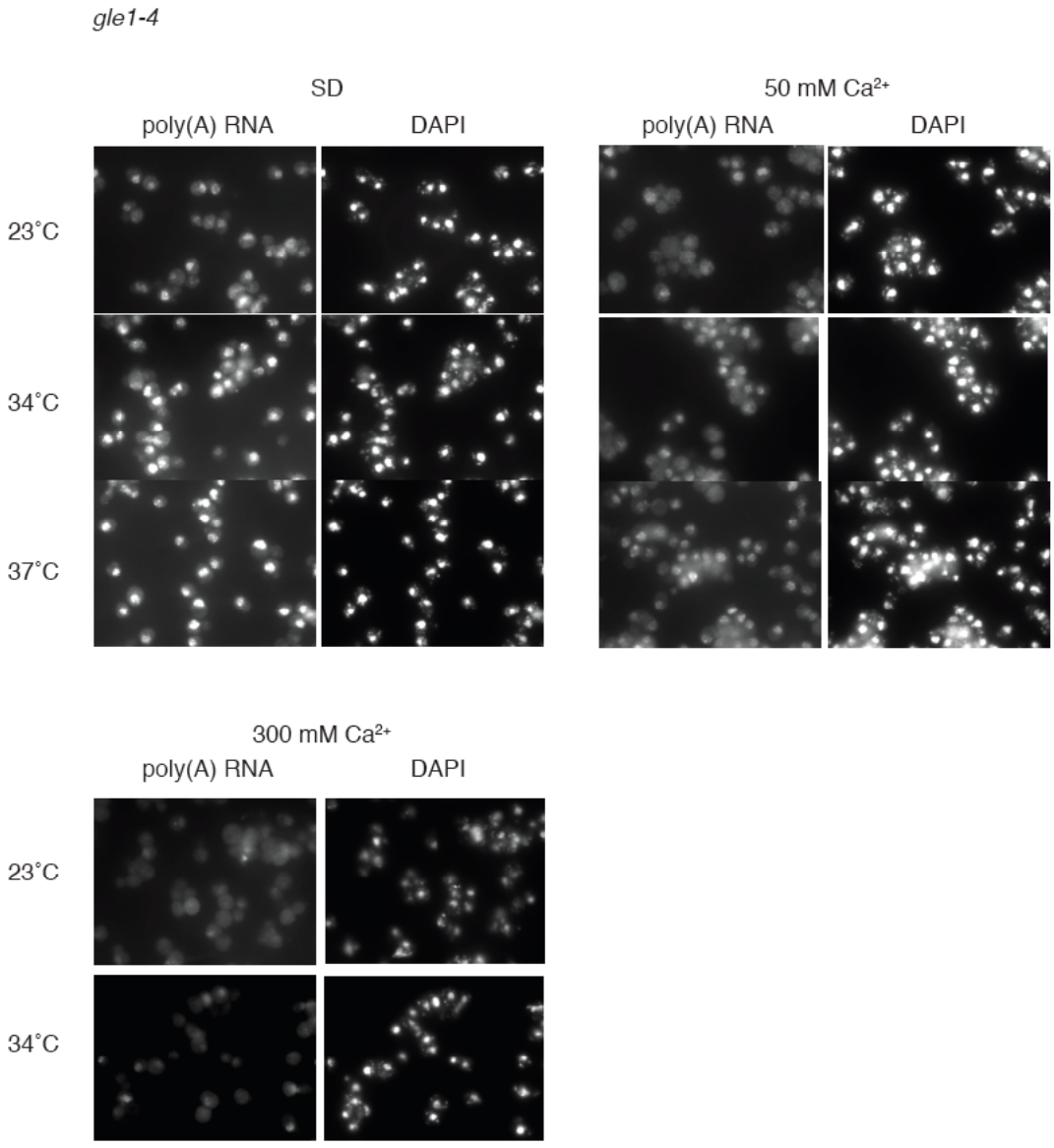

Figure A4: High osmolarity rescues the mRNA export defect of *gle1-4* mutants. Strains were grown at 23˚C and shifted for 3hrs in high osmolarity media (or synthetic media) prior to shifting temperatures for 1hr. Strains were processed by *in situ* hybridization with an oligo d(T) probe and DAPI staining to visualize nuclei.

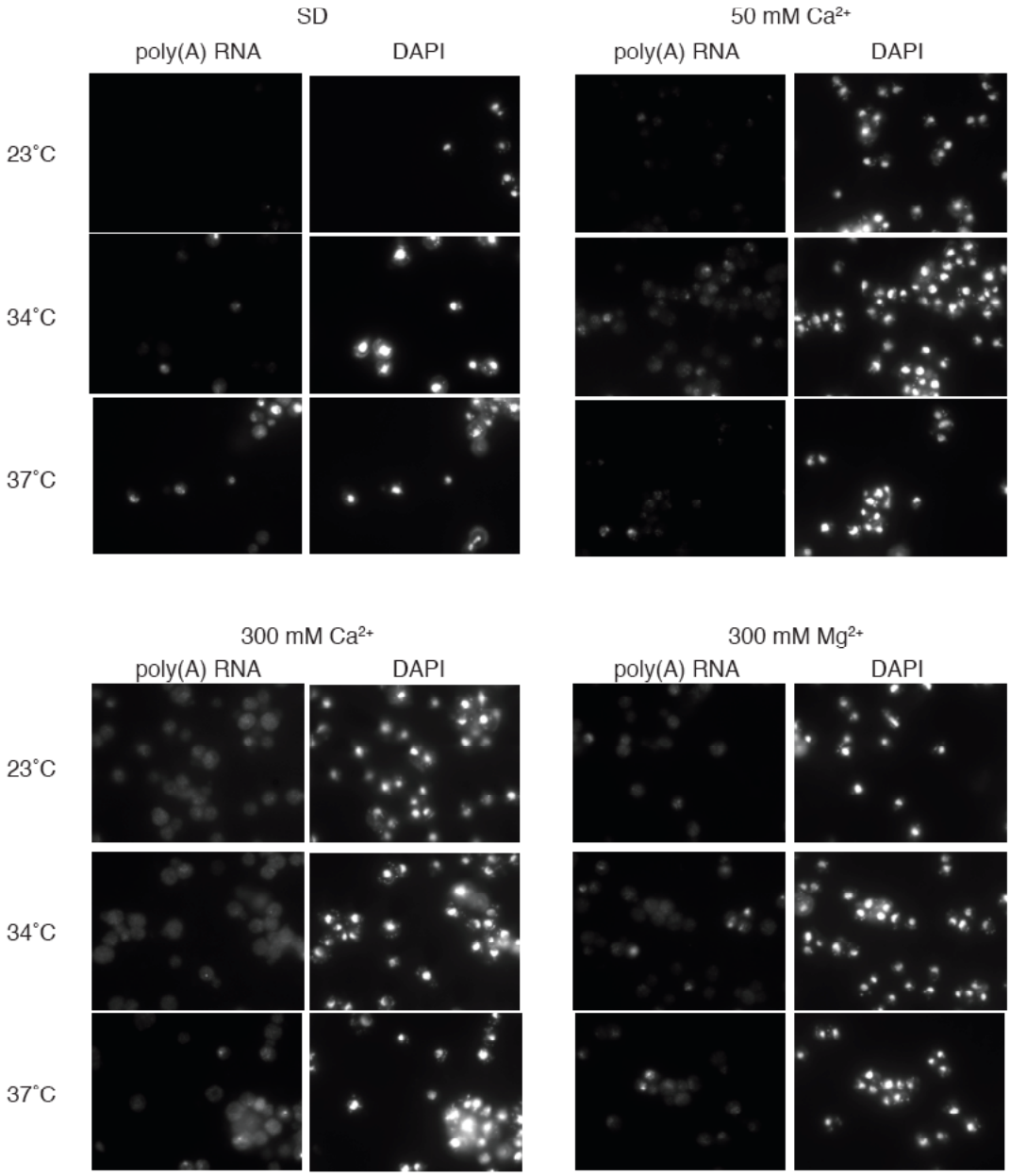

Figure A5: High osmolarity rescues the mRNA export defect of *gle1-4* mutants. Strains were grown at 23˚C and shifted for 3hrs in high osmolarity media (or synthetic media) prior to shifting temperatures for 1hr. Strains were processed by *in situ* hybridization with an oligo d(T) probe and DAPI staining to visualize nuclei.

Analysis, cloning, and expression of Gle1 proteins from fungal species.

Gle1 is a conserved protein in Eukarya with a defined essential role in mRNA export. Studies in *Saccharomyces cerevisiae* have revealed a C-terminal domain that binds IP_6 and stimulates Dbp5 ATPase activity. A sequence alignment with other fungal and metazoan species reveals areas of high homology within the C-terminal domain. The N-terminal domain, however, lacks areas of high homology and is challenging to analyze with bioinformatics. Figure B3 presents an alignment of Gle1 from different species. Interestingly, a marked difference is evident between higher eukaryotes and fungal species at many motifs in the C-terminus.

Analysis of the C-terminus domain from all species identifies many conserved motifs. I described them here using the *Saccharomyces cerevisiae* numbering.

E-A-P motif sc326-365

The E-A-P motif is the area of most homology in Gle1. It is called E-A-P due to the conserved E, A, and P residues in that region. In Gle1 sequences from Group 1 and Group 3 (see below) two proline residues form a boundary of this domain.

DILA-KKCP motif sc369-386

The DILA-KKCP motif is the second area of high homology in Gle1. It contains amino acids important for IP_6 binding.

E-R-AAII motif sc329-409 and 413-429

This motif contains to sub-motifs with good homology, specially following the glutamic acid at position 392. The arginine at position 417 seems to be required for the solubility of Gle1 (see above).

W-N-L motif sc451-507

This motif is much less conserved than the previous ones. However, it contains W451, N459, and L473 that are well conserved.

B motif sc514-523

The B-motif (also be called RLK/R motif for residues found in sc518-519) is called B due to its presence in hGle1B and not in hGle1A. This motif is interesting since it is probably responsible for binding Nup42, the hCG1 homologue in yeast. Additionally, the positively charged residues of the RLK/R sequence are only present in Gle1 from groups 2 and 3 (see below).

We can divide Gle1 sequences in three groups: Group1 (*B. cinerea, H. capsulatum, A. terreus, M. grisea, C. immitis, N. crassa,* and *A. nidulans),* Group 2 (*C. albicans, S. cerevisiae, C. tropicalis, C. guilliermondii, C. lusitaniae, L. elongisporus,* and *S. pombe)*, and Group 3 (*M. musculus, G. gallus, R. norvegicus, H. sapiens,* and *D. rerio)*. Group 1 contains a DILA motif but does not contain a complete KKCP motif. Instead, they contain a VV, IV, or VS instead of KK. Interestingly, these amino acids are required for IP_6 in scGle1. Group 2 contains a sequence LNxAKA within the E-A-P motif. Additionally, they contain a complete KKCC motif but not a DILA sequence. Group 3, corresponding to the Gle1 sequences from higher eukaryotes, contains a DILA-KKCP motif but lack the LNxAKA sequence in the E-A-P motif.

Interestingly, several positively charged amino acids are present only in Group 2 and Group 3 Gle1 sequences. These amino acids include K377, K378, K494, R517, and R287. R519 is also present in most Group 2 and 3 Gle1 sequences, but *A. nidulans* and *A. terreus* Gle1 also contain R519.

In order to gain a deep understanding of Gle1/IP_6-driven stimulation of Dbp5 ATPase activity, we require the purification, concentration, and crystallization of Gle1 and Gle1 domains. We have cloned Gle1 fragments from fungal species that might facilitate the rapid purification and concentration of these domains. From *S. cerevisiae* we have cloned C-terminal fragments: sc241-538, sc244-538, sc272-538, and sc435-538. From *S. pombe* we have cloned C-terminal fragments: sp188-479 and sp193-479. From *N. crassa* we have cloned C-terminal fragments: nc283-541 and nc320-541. From *A. nidulans* we have cloned C-terminal fragments: an235-481 and an260-481. Constructs were tested for expression in bacteria. All *S. cerevisiae* and *S. pombe* fragments expressed robustly (Figure B1). Gle1 from *N. crassa* expressed only in some cases. However, none of the *A. nidulans* clones expressed significant amount of protein. (Figure B2). Future goals are to purify and crystallize full length Gle1 protein from yeast or other fungal species.

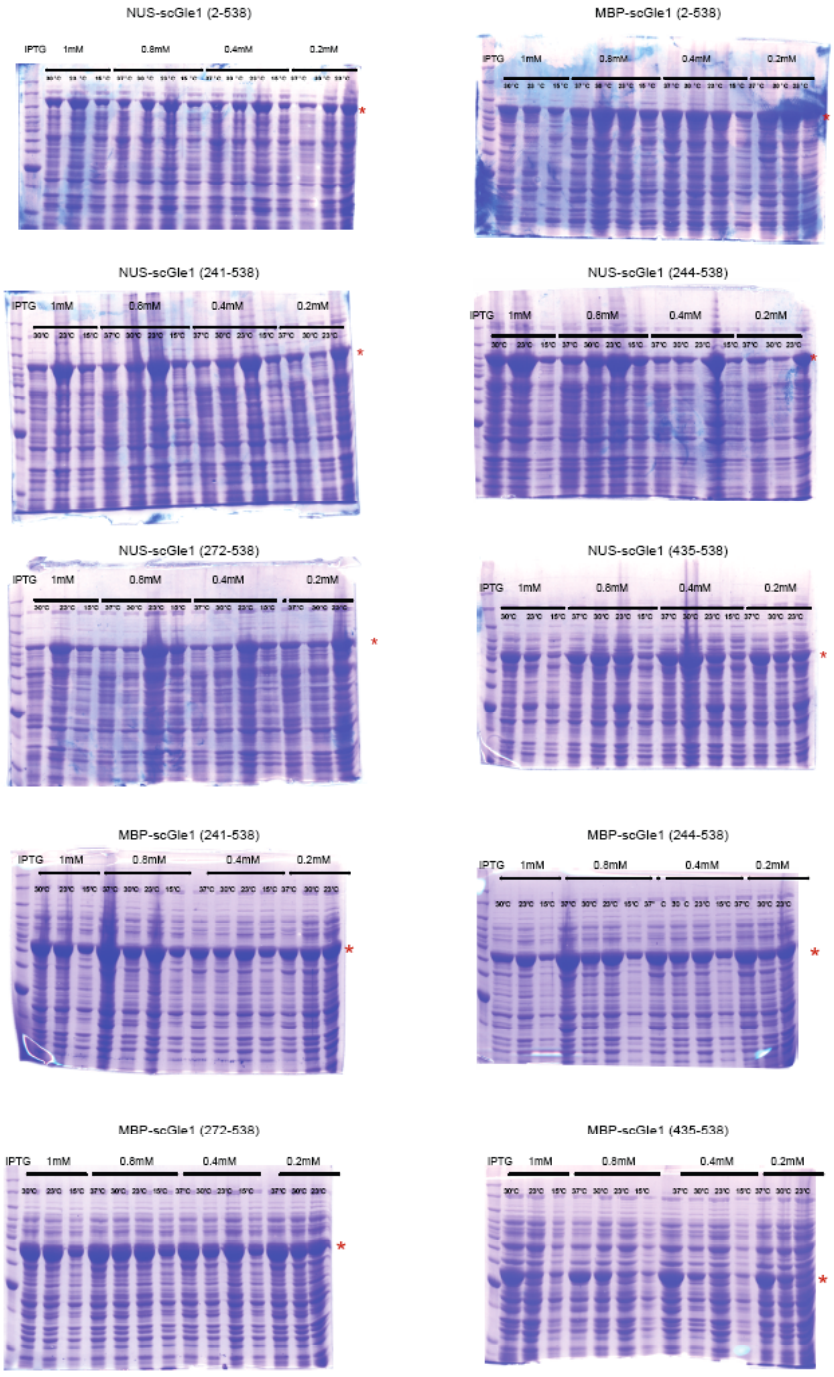

Figure B1: Bacterial expression of scGle1 fragments. Bacteria were induced with IPTG at the indicated concentration and temperature. Cells at 37˚C and 30˚C were grown for 3 hours, whereas cells at 23˚C and 15˚C were grown overnight.

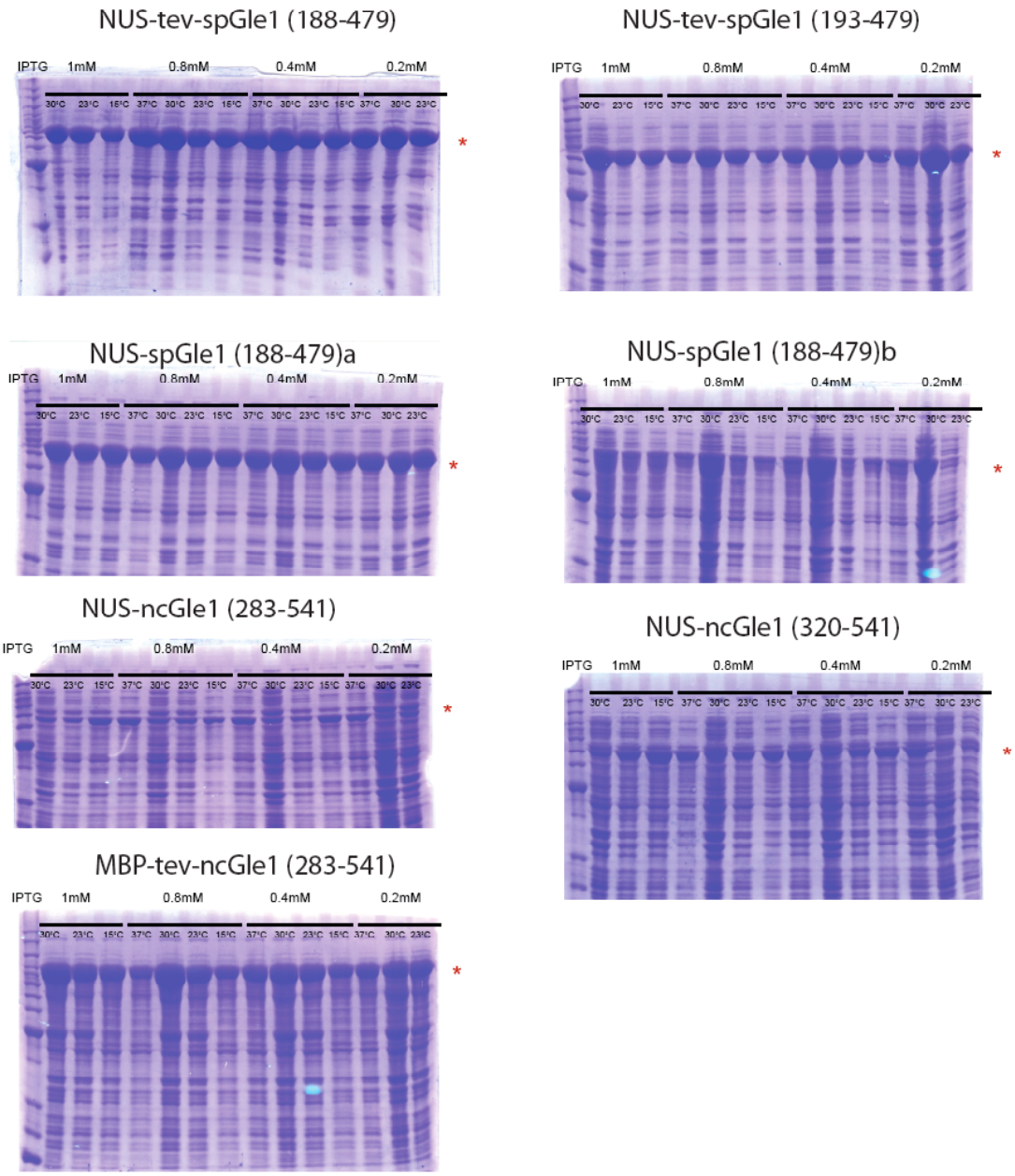

Figure B2: Bacterial expression of spGle1and ncGle1 fragments. Bacteria were induced with IPTG at the indicated concentration and temperature. Cells at 37˚C and 30˚C were grown for 3 hours, whereas cells at 23˚C and 15˚C were grown overnight.

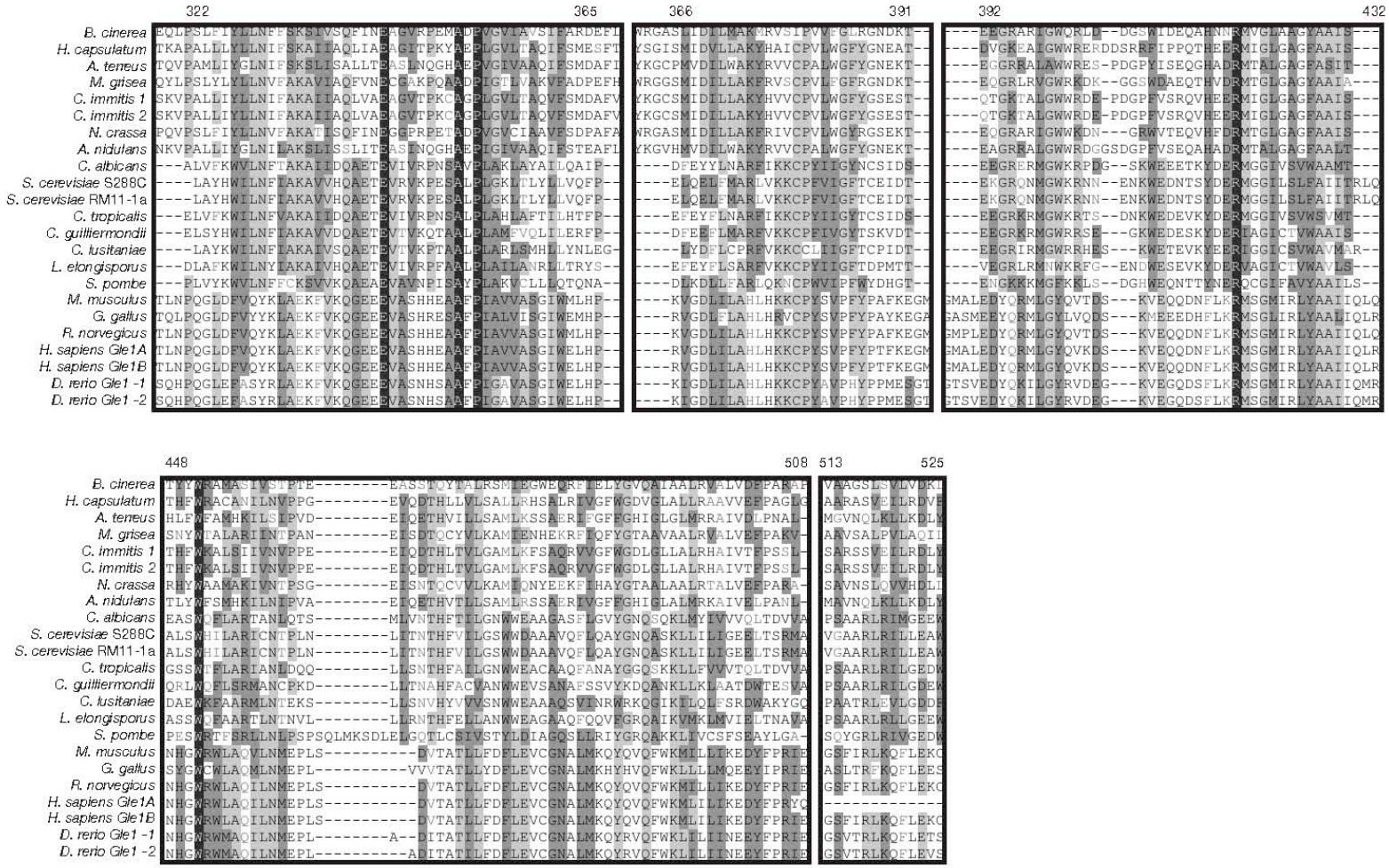

Figure B3: Sequence alignment of Gle1 from fungal and metazoan species. Species are noted on the left margin. Amino acid number for the scGle1 sequence is place above the alignment as a guide. Areas of high homology have a darker background, where as areas of low homology have a white background.

C. Yeast strains used in this study

Strain name	Mat	Genotype	Source
SWY3096	a	*nup42::HIS3 ipk1::KAN ura3 his3 leu2 ade2 LYS2 trp1*	This study
SWY3097	@	*nup42::HIS3 ipk1::KAN ura3 his3 leu2 ade2 lys2 TRP1*	This study
SWY3098	a	*nup42::HIS3 ipk1::KAN ura3 his3 leu2 ade2 LYS2 trp1*	This study
SWY3099	a	*nup42::HIS3 ipk1::KAN ura3 his3 leu2 ade2 lys2 TRP1*	This study
SWY3101	a	*nup42::HIS3 ipk1::KAN ade3::HISG ura3 his3 leu2 ade2 LYS2 trp1*	This study
SWY3376	@	*ipk1-5 ade2 ADE3 leu2 ura3 his3 trp1 LYS2*	This study
SWY3377	a	*ipk1-5 ade2 ADE3 leu2 ura3 his3 trp1 LYS2*	This study
SWY3413	@	*ipk1-5 ade2-1 ADE3 ura3-1 his3-11,15 trp1-1 leu2-3, LYS2 can1-100?*	This study
SWY3414	a	*ipk1-5 ade2-1 ade3 ura3-1 his3-11,15 trp1-1 leu2-3, LYS2 can1-100?*	This study
SWY3415	@	*ipk1-5 ade2-1 ade3 ura3-1 his3-11,15 trp1-1 leu2-3, LYS2 can1-100?*	This study
SWY3416	@	*ipk1-5 nup42::HIS3 ade2 ADE3 leu2 ura3 his3 lys2 TRP1*	This study
SWY3417	@	*ipk1-5 nup42::HIS3 ade2 ADE3 leu2 ura3 his3 lys2 TRP1*	This study
SWY3418	@	*ipk1-5 nup42::HIS3 ade2 ADE3 leu2 ura3 his3 lys2 TRP1*	This study
SWY3419	a	*ipk1-5 nup42::HIS3 ade2 ADE3 leu2 ura3 his3 lys2 TRP1*	This study
SWY3463	a	*DHH1-GFP:HIS ipk1::KAN nup42::HIS ade2 ADE3 ura3 his3 leu2 can1-100? lys2 TRP1*	This study
SWY3464	@	*DHH1-GFP:HIS ipk1::KAN nup42::HIS ade2 ADE3 ura3 his3 leu2 can1-100? LYS2 trp1*	This study
SWY3465	@	*DHH1-GFP:HIS ipk1::KAN nup42::HIS ade2 ADE3 ura3 his3 leu2 can1-100? LYS2 TRP1*	This study
SWY3466	@	*DHH1-GFP:HIS ipk1::KAN nup42::HIS ade2 ADE3 ura3 his3 leu2 can1-100? lys2 TRP1*	This study
SWY3467	a	*gle1-2 kcs1::HIS ade2 ADE3 ura3 his3 leu2 can1-100? LYS2 trp1*	This study
SWY3468	@	*DHH1-GFP:HIS ipk1::KAN nup42::HIS ade2 ADE3 ura3 his3 leu2 can1-100? lys2 trp1*	This study
SWY3469	a	*DHH1-GFP:HIS nup42::HIS ade2 ADE3 ura3 his3 leu2 can1-100? LYS2 TRP1*	This study
SWY3470	@	*DHH1-GFP:HIS nup42::HIS ade2 ADE3 ura3 his3 leu2 can1-100? lys2 TRP1*	This study
SWY3471	@	*gle1-2 kcs1::HIS3 ade2 ade3 ura3 his3 leu2 can1-100? LYS2 trp1*	This study
SWY3472	@	*gle1-2 kcs1::HIS3 ade2 ADE3 ura3 his3 leu2 can1-100? LYS2 trp1*	This study
SWY3473	a	*gle1-2 kcs1::HIS3 ade2 ADE3 ura3 his3 leu2 can1-100? LYS2 trp1*	This study
SWY3474	a	*dbp5-2 kcs1::HIS3 ipk1::KAN ADE2 ADE3 ura3 his3 leu2 trp1 LYS2 can1-100? + CP3009*	This study
SWY3475	@	*dbp5-2 kcs1::HIS3 ipk1::KAN ade2 ADE3 ura3 his3 leu2 trp1 LYS2 can1-100? + CP3009*	This study
SWY3476	a	*dbp5-2 kcs1::HIS3 ipk1::KAN ade2 ADE3 ura3 his3 leu2 trp1 LYS2 can1-100? + CP3009*	This study
SWY3477	a	*kcs1::HIS3 ipk1::KAN ADE2 ADE3 ura3 his3 leu2 trp1 LYS2 can1-100? + CP3009*	This study
SWY3478	@	*kcs1::HIS3 ipk1::KAN ADE2 ADE3 ura3 his3 leu2 trp1 LYS2 can1-100?*	This study
SWY3479	a	*kcs1::HIS3 ipk1::KAN ade2 ADE3 ura3 his3 leu2 trp1 LYS2 can1-100? + CP3009*	This study
SWY3480	a	*kcs1::HIS3 ipk1::KAN ade2 ADE3 ura3 his3 leu2 trp1 LYS2 can1-100?*	This study
SWY3486	@	*ipk1::KAN DHH1-GFP ade2-1 ade3::HISG leu2-3 ura3-1 his3-11,15 can1-100? lys2 trp1-1*	This study
SWY3487	a	*ipk1::KAN DHH1-GFP ade2-1 ade3::HISG leu2-3 ura3-1 his3-11,15 can1-100? LYS2 trp1-1*	This study
SWY3547	?	*dhh1::KANr ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100*	This study
SWY3548	?	*dhh1::KANr ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100*	This study
SWY3549	a	*tad1::KANr gle1-2 may or may not contain pGLE1::URA*	This study
SWY3550	@	*tad1::KANr gle1-2 may or may not contain pGLE1::URA*	This study
SWY3551	a	*tad1::KANr gle1-2 may or may not contain pGLE1::URA*	This study
SWY3552	@	*tad1::KANr gle1-2 may or may not contain pGLE1::URA*	This study
SWY3553	a	*ipk1::KAN rip1(nup42)::HIS3 leu2 ura3 his3 ade2-1can1-100? dsRED-HDAL:TRP1*	This study
SWY3554	a	*ipk1::KAN rip1(nup42)::HIS3 leu2 ura3 his3 ade2-1can1-100? dsRED-HDAL:TRP1*	This study
SWY3563	@	*ipk1::KAN GLE1-TAP:HIS3 ADE2 ade3? ura3 his3 leu2 TRP1 lys2? met15?*	This study
SWY3564	a	*ipk1::KAN GLE1-TAP:HIS3 ade2 ADE3 ura3 his3 leu2 TRP1 lys2? met15?*	This study

C. Yeast strains used in this study continued

SWY3565	@	*ipk1::KAN DBP5-TAP:HIS3 ADE2 ade3? ura3 his3 leu2 TRP1 lys2? met15?*	This study
SWY3566	a	*ipk1::KAN GFD1-TAP:HIS3 ADE2 ade3? ura3 his3 leu2 TRP1 LYS2 met15?*	This study
SWY3567	a	*ipk1::KAN GFD1-TAP:HIS3 ADE2 ade3? ura3 his3 leu2 TRP1 lys2 met15?*	This study
SWY3568	a	*ipk1::KAN NAB2-TAP:HIS3 ADE2 ade3? ura3 his3 leu2 TRP1 lys2 met15?*	This study
SWY3569	@	*ipk1::KAN NAB2-TAP:HIS3 ADE2 ade3? ura3 his3 leu2 TRP1 LYS2 met15?*	This study
SWY3608	a	*ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3,112 dhh1::KAN ipk1::KAN*	This study
SWY3612	@	*Dbp5-GFP:HIS5 gle1-2 ura3 -1 his3-11,15 leu2-3,112 LYS1 TRP1*	This study
SWY3613	@	*Dbp5-GFP:HIS5 gle1-2 ura3 -1 his3-11,15 leu2-3,112 lys1 TRP1*	This study
SWY3614	a	*Dbp5-GFP:HIS5 gle1-2 ura3 -1 his3-11,15 leu2-3,112 lys1 trp1*	This study
SWY3615	@	*Dbp5-TAP:HIS5 ipk1::KAN ADE2 ADE3 ura3 leu2 his3 TRP1 LYS1 met?*	This study
SWY3616	a	*Dbp5-TAP:HIS5 ipk1::KAN ADE2 ADE3 ura3 leu2 his3 TRP1 lys1 met?*	This study
SWY3617	@	*Dbp5-TAP:HIS5 ipk1::KAN ADE2 ADE3 ura3 leu2 his3 TRP1 lys1 met?*	This study
SWY3642	a	*ura3-1 his3-11,15 leu2-3,112 can1-100 ? trp1-1 LYS2 ade2-1 ipk1-3*	This study
SWY3643	@	*ura3-1 his3-11,15 leu2-3,112 can1-100 ? trp1-1 lys2 ade2-1 ade3 ipk1-3*	This study
SWY3644	a	*ura3-1 his3-11,15 leu2-3,112 can1-100 ? trp1-1 LYS2 ade2-1 ade3 ipk1-3*	This study
SWY3645	@	*ura3-1 his3-11,15 leu2-3,112 can1-100 ? trp1-1 LYS2 ade2-1 ade3 ipk1-3*	This study
SWY3646	?	*ura3 his3 leu2 trp1-1 LYS2 can1? ade2-1 ade3 dbp5-2 ipk1-5*	This study
SWY3647	?	*ura3 his3 leu2 trp1-1 LYS2 can1? ade2-1 ADE3 dbp5-2 ipk1-5*	This study
SWY3648	?	*ura3 his3 leu2 trp1-1 LYS2 can1? ade2-1 ade3 dbp5-2 ipk1*	This study
SWY3649	?	*ura3 his3 leu2 trp1-1 LYS2 can1? ade2-1 ADE3 dbp5-2 ipk1-5*	This study
SWY3650	?	*ura3 his3 leu2 trp1-1 LYS2 can1? ade2-1 ade3 dbp5-2 ipk1-5*	This study
SWY3651	?	*ura3 his3 leu2 trp1-1 LYS2 can1? ade2-1 ade3 dbp5-2 ipk1-5*	This study
SWY3652	?	*ura3 his3 leu2 trp1-1 LYS2 can1? ade2-1 ADE3 dbp5-2 ipk1-5*	This study
SWY3653	?	*ura3 his3 leu2 trp1-1 LYS2 can1? ade2-1 ade3 dbp5-2 ipk1-5*	This study
SWY3682	a	*trp1Δ63 leu2Δ1 ura3-52 his3Δ200 dbp5::HIS3 + DBP5:URA3/CEN (CP3009)*	This study
SWY3766	@	*gle1::HIS3 ipk1::KAN ade2-1 ade3::HISG ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100? LYS2 + pSW410*	This study
SWY3767	a	*gle1::HIS3 ipk1::KAN ade2-1 ade3::HISG ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100? LYS2 + pSW410*	This study
SWY3768	a	*gle1::HIS3 ipk1::KAN ade2-1 ADE3 ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100? LYS2 + pSW410*	This study
SWY3769	a	*gle1::HIS3 ipk1::KAN ade2-1 ADE3 ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100? LYS2 + pSW410*	This study
SWY3770	a	*gle1::HIS3 ipk1::KAN ade2-1 ADE3ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100? LYS2 + pSW410*	This study
SWY3812	@	*gle1::HIS3 ipk1::KAN nup42::HIS3 ade2 ADE3 ura3 leu2 his3 LYS2 + pGLE1/CEN/URA3 TRP1 Spore 1A*	This study
SWY3813	@	*gle1::HIS3 ipk1::KAN nup42::HIS3 ade2 ADE3 ura3 leu2 his3 LYS2 + pGLE1/CEN/URA3 trp1 Spore 1C*	This study
SWY3814	a	*gle1::HIS3 ipk1::KAN nup42::HIS3 ade2 ADE3 ura3 leu2 his3 LYS2 + pGLE1/CEN/URA3 trp1 Spore 2B*	This study
SWY3815	a	*gle1::HIS3 ipk1::KAN nup42::HIS3 ade2 ADE3 ura3 leu2 his3 LYS2 + pGLE1/CEN/URA3 TRP1 Spore 4B*	This study
SWY3823	@	*gle1::HIS3 ade2 ADE3 ura3 his3 leu2 trp1 LYS2 can1 + pSW3343 (gle1-K377Q:LEU2/CEN)*	This study
SWY3824	@	*gle1::HIS3 ade2 ADE3 ura3 his3 leu2 trp1 LYS2 can1 + pSW3344 (gle1-K494Q:LEU2/CEN)*	This study
SWY3825	@	*gle1::HIS3 ade2 ADE3 ura3 his3 leu2 trp1 LYS2 can1 + pSW3345 (gle1-K377Q/K378Q:LEU2/CEN)*	This study
SWY3826	@	*gle1::HIS3 ade2 ADE3 ura3 his3 leu2 trp1 LYS2 can1 + pSW399 (GLE1:LEU2/CEN)*	This study
SWY3827	@	*gle1::HIS3 ipk1::KAN nup42::HIS3 ade2 ADE3 ura3 leu2 his3 LYS2 TRP1 + pSW3343*	This study
SWY3828	@	*gle1::HIS3 ipk1::KAN nup42::HIS3 ade2 ADE3 ura3 leu2 his3 LYS2 TRP1 + pSW3344*	This study
SWY3829	@	*gle1::HIS3 ipk1::KAN nup42::HIS3 ade2 ADE3 ura3 leu2 his3 LYS2 TRP1 + pSW399*	This study
SWY3831	@	*gle1::HIS3 ipk1::KAN nup42::HIS3 ade2 ADE3 ura3 leu2 his3 LYS2 TRP1 + pSW3345 +pSW410*	This study
SWY3832	a	*gle1::HIS3 ipk1::KAN ade2-1 ADE3 ura3-1 leu2-3,112 his3-11,15 LYS2 trp1-1 can1-100? + pSW3343*	This study
SWY3833	a	*gle1::HIS3 ipk1::KAN ade2-1 ADE3 ura3-1 leu2-3,112 his3-11,15 LYS2 trp1-1 can1-100?+ pSW3344*	This study
SWY3834	a	*gle1::HIS3 ipk1::KAN ade2-1 ADE3 ura3-1 leu2-3,112 his3-11,15 LYS2 trp1-1 can1-100?+ pSW3345*	This study
SWY3835	a	*gle1::HIS3 ipk1::KAN ade2-1 ADE3 ura3-1 leu2-3,112 his3-11,15 LYS2 trp1-1 can1-100? + pSW399*	This study
SWY3836	@	*gle1::HIS3 ipk1::KAN nup42::HIS3 ade2 ADE3 ura3 leu2 his3 LYS2 TRP1+ pSW3343 + pSW1157*	This study
SWY3837	@	*gle1::HIS3 ipk1::KAN nup42::HIS3 ade2 ADE3 ura3 leu2 his3 LYS2 TRP1 + pSW3343 + pSW1272*	This study

C. Yeast strains used in this study continued

SWY3838	@	*gle1::HIS3 ipk1::KAN nup42::HIS3 ade2 ADE3 ura3 leu2 his3 LYS2 TRP1+ pSW3344 + pSW1157*	This study
SWY3839	@	*gle1::HIS3 ipk1::KAN nup42::HIS3 ade2 ADE3 ura3 leu2 his3 LYS2 TRP1 + pSW3344 + pSW1272*	This study
SWY3840	@	*gle1::HIS3 ipk1::KAN nup42::HIS3 ade2 ADE3 ura3 leu2 his3 LYS2 TRP1 + pSW399 + pSW1157*	This study
SWY3841	@	*gle1::HIS3 ipk1::KAN nup42::HIS3 ade2 ADE3 ura3 leu2 his3 LYS2 TRP1+ pSW399 + pSW1272*	This study
SWY3968	a/@	*K699 pho85::LEU2 trp1::PHO85-F82G pho3Δ ade2-1 trp1-1 can1-100 leu2,3,112 his3-11,15 ura3 GAL+*	This study
SWY3969	a	*ipk1::KAN trp1:PHO85-F82G pho85::LEU2 pho3Δ ade2-1 trp1-1 can1-100 leu2,3,112 his3-11,15 ura3*	This study

D. Plasmids used in this study

Plasmid Name	Description		Source
pSW3174	*SSD1*	2μ plasmid	This Study
pSW3175	*DHH1*	2μ plasmid	This Study
pSW3176	*PDE2*	2μ plasmid	This Study
pSW3177	*DHH1*	2μ plasmid	This Study
pSW3178	*DHH1*	deletion of C' half (no stop codon)	This Study
pSW3179	*DHH1*	dhh1 E196Q	This Study
pSW3180	*DHH1*	dhh1 E196Q	This Study
pSW3181	*PDE2*	2μ plasmid	This Study
pSW3209	*DHH1*	BD-DHH1	This Study
pSW3210	*DHH1*	BD-DHH1	This Study
pSW3211	*DHH1*	Under GPD promoter	This Study
pSW3212	*DHH1*	Under the GPD promoter	This Study
pSW3213	*MSS4*	AD-Mss4	This Study
pSW3214	*MSS4*	BD-Mss4	This Study
pSW3215	*MSS4*	BD-Mss4	This Study
pSW3239	*DHH1*	in pRS426	This Study
pSW3240	*DHH1*	DHH1 fuse to Gal4 AD	This Study
pSW3251	*dbp5*	dbp5-E240Q	This Study
pSW3252	*dbp5*	dbp5-E240Q	This Study
pSW3256	RNA-B-AA1		This Study
pSW3291	GLE1	Point mutation-K377Q	This Study
pSW3292	GLE1	Point mutation-K494Q	This Study
pSW3293	GLE1	Point mutation-K377Q/K378Q	This Study
pSW3294	GLE1	Point mutation-K377Q/K378Q/R417Q	This Study
pSW3295	GLE1	Point mutation-R417Q	This Study
pSW3296	GLE1	Point mutation-K377Q	This Study
pSW3297	Gle1	Point mutation-K494Q	This Study
pSW3300	GLE1	Overexpression	This Study
pSW3301	GLE1	Overexpreesion	This Study
pSW3315	Gle1	aa244-538	This Study
pSW3319	dbp5	gst	This Study
pSW3325	scGle1	2-538 bacterial expression	This Study
pSW3326	scGle1	2-538 bacterial expression	This Study
pSW3327	scGle1	241-538 bacterial expression	This Study
pSW3328	scGle1	241-538 bacterial expression	This Study
pSW3329	scGle1	244-538 bacterial expression	This Study
pSW3330	scGle1	244-538 bacterial expression	This Study
pSW3331	scGle1	272-538 bacterial expression	This Study
pSW3332	scGle1	272-538 bacterial expression	This Study
pSW3333	scGle1	435-538 bacterial expression	This Study
pSW3334	scGle1	435-538 bacterial expression	This Study
pSW3335	scGle1	241-538 bacterial expression	This Study
pSW3336	scGle1	241-538 bacterial expression	This Study
pSW3337	scGle1	244-538 bacterial expression	This Study
pSW3338	scGle1	244-538 bacterial expression	This Study
pSW3339	scGle1	272-538 bacterial expression	This Study

D. Plasmids used in this study continued

pSW3340	scGle1	272-538 bacterial expression	This Study
pSW3341	scGle1	435-538 bacterial expression	This Study
pSW3342	scGle1	435-538 bacterial expression	This Study
pSW3343	Gle1	Gle1-k377Q	This Study
pSW3344	Gle1	Gle1-K494Q	This Study
pSW3345	Gle1	Gle1-KK377/78QQ	This Study
pSW3346	Dbp5	E240Q	This Study
pSW3370	Gle1	MBP-Gle1 tev 1-240	This Study
pSW3371	Gle1	MBP-Gle1 tev 1-240	This Study
pSW3372	Gle1	MBP-tev 1-434	This Study
pSW3373	Gle1	MBP-Tev-Gle1 1-434	This Study
pSW3391	spGLE1	NUS-tev-188-479	This Study
pSW3392	spGLE1	NUS-tev-193-479	This Study
pSW3393	spGLE1	NUS-188-479	This Study
pSW3394	spGLE1	NUS-193-479	This Study
pSW3395	ncGLE1	NUS-283-541	This Study
pSW3396	ncGLE1	NUS-320-541	This Study
pSW3397	ncGLE1	MBP-tev-283-541	This Study
pSW3398	ncGLE1	MBP-tev-320-541	This Study
pSW3399	anGLE1	NUS-225-481	This Study
pSW3400	anGLE1	NUS-260-481	This Study
pSW3401	anGLE1	MBP-tev-225-481	This Study
pSW3402	anGLE1	MBP-tev-260-481	This Study

E. Methods

^{3}H-IP_6 Binding Assays

General properties:

The total buffer concentration is:
16 mM HEPES pH 7.5
120 mM NaCl
16% Glycerol w/v
3 mM MgCl2
1mg/ml BSA

6μl 5X ATPase Buffer A
18μl Proteins in Buffer B
6ul Waters (^{3}H-IP_6/RNA/ATP/etc)
30ul

- Buffer B: 20 mM HEPES pH. 7.5; 150 mM NaCl; 20% Glycerol w/v
- 5X ATPase Buffer A: 20 mM HEPES pH 7.5/150 mM NaCl/20% Glycerol w/v/ 15 mM MgCl2/1mg/ml BSA
- Use siliconized tubes (1.5ml)
- ^{3}H-IP_6 21.4 Ci mmol-1; PerkinElmer LAS
- 30% PEG 3350 filtered

1. – Calculate experiments for triplicates for each control and unknown

2. Make master in this order:

5X ATPase buffer
Waters (but no IP_6)
Buffer B
Proteins

3. Aliquot in however many tubes you need. Add to each tube Buffer B and then your protein to make a final sample prior to addition of ^{3}H-IP_6

4.- Mix well by softly flicking a couple of times and then spinning them down in the nanofuge for a few seconds

5.- Add ^{3}H-IP_6 (and/or non-label IP_6 and or competitors).

Add IP_6 with siliconized tip. Mix all samples at the end by passing them 3x times through rack, then mix by soft flicking a couple of times and spin in nanofuge.

6.- Let equilibrate for 10min at room temperature (or longer on the protein)

7.- Precipitate by addition of 20ul 30% PEG 3350 (w/v) for a final concentration of 12% PEG.
Mix well by flicking till you cannot see any changes in PEG and sample (usually a couple of seconds of soft flicking)

8.- Place in centrifuge carefully so that the back of the tube is align with the well. Close centrifuge and let it reach 4 ˚C with samples inside (about 10') – then spin for 25'

9.- Extremely carefully extract all unbound IP_6. Be careful not to touch the side of the tube where the invisible pellet would be.

10.- Optional steps:

Wash with100ul Buffer B or spin again for 20 sec. and re-aspirate the 1-3 μl remaining

11.- Add 300ul 1% SDS. Incubate overnight at room temperature

12.- Resuspend the invisible pellet. Mix and finally pour all 300 μl into a scintillation vial with 4ml scintillation fluid

13.- Mix well and count

ATPase assay

Keep reaction on Ice. I have seen that Dbp5's ATPase activity on ice is very low, but it has some. You start you rxn by addition of Mix3. Time it and stop by putting back in ICE and applying STOP MIX as soon as possible. This is the recipe for 1rxn of 10ul. A time course can be done as well just by doing one tube with a grater volume.

Reaction components:
6 μl Protein
2 μl Waters
2 μl 5X ATPase buffer

1 Reation

Master Mix1:

5xATPase Buffer	0.3 μl
Superasin 20 U/ul	0.5 μl
H20	0.2 μl
RNA 20μM	0.5 μl
	1.5 μl

Master Mix2:

5xATPase Buffer	1.0 μl
DTT 100mM	0.1 μl
Buffer B (protein)	3.4 μl
IP_6 (water)	0.5 μl
	5.0 μl

Master Mix3:

5xATPase Buffer	0.7 μl
Buffer B (protein)	2.6 μl
ATP 100mM	0.1 μl
ATP ^{32}P	0.1 μl
	3.5 μl

Aliquot and Mix 1 and Mix2 in order shown.
Mix well and spin down.
Start reaction with addition of Mix3 (if dealing with one sample) or by quickly adding Mix3 to a number of samples (in plastic float) and simultaneously passing them all from ICE to the 30 ˚C water bath and starting a timer.

Reactions are carried out at 30 ˚C or 37 ˚C.

Linear ATPase activity lasts for about 13 or 15 minuntes when using 500 nM Dbp5 and 250 nM Gle1.

At the end of reaction stop with 2ul stop buffer: (50 mM Tris pH 7.4; 5 mM EDTA; 1.5% SDS; 2mg/ml Proteinase K (Ambion))

Incubate at 37 ˚C x 30min
Place on ice.

Label Baker Flex Cellulose PEI thin layer chromatography plates (J. T. Baker). Spot 2ul of stopped reaction on TLC plates (1 inch from bottom and at least 1.25cm from each other).

Make up a solution of 0.6 M potassium phosphate pH 3.4. Fill TLC tank with 330ml. Run TLC. Note - Make sure TLC sheet is dry and buffer in the wall of the TLC tank does not touch TLC sheet.

Run chromatography for ~5 hrs. Dry and expose for 30 min. or 1 hr. (Depending of the strength of signal.

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