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Roles of Npl3 phosphorylation in mRNA export

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Roles of Npl3 Phosphorylation in mRNA Export

by

Wendy V. Gilbert

DISSERTATION

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of the

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For my teachers

Roles of Npl3 Phosphorylation in mRNA Export

ABSTRACT

Eukaryotic gene expression demands bridging the distance that separates mRNA synthesis in the nucleus from mRNA translation in the cytoplasm. To avoid reimporting mRNA in a futile cycle, the cell must distinguish between nuclear and cytoplasmic compartments. It is not known how directionality of mRNA export is achieved. mRNA export is mediated by Mex67p:Mtr2p/NXF1:p15, a conserved heterodimeric export receptor which is thought to bind mRNAs through RNA-binding adaptor proteins. The best candidates for adaptor proteins are members of two classes of shuttling RNA-binding proteins: hnRNP proteins and SR (serine-arginine rich) proteins. Npl3p has sequence similarity to both hnRNP and SR proteins, and is required for mRNA export in *S. cerevisiae*. Phosphorylation on serine residues within the RS-domain is a defining characteristic of SR proteins, but the physiological role(s) of RS-domain phosphorylation have remained uncertain.

Here we demonstrate that Npl3p is phosphorylated on serine in vivo by cytoplasmic Sky1p, the yeast homologue of the cytoplasmic mammalian SR protein kinase SRPK1. We show that phosphorylation of Npl3p by Sky1p requires a single C-terminal RS dipeptide, Ser411, and this specific phosphorylation is required for proper nuclear localization of Npl3p and for mRNA export. The cytoplasmic accumulation of Npl3p in the absence of Sky1-mediated phosphorylation is accompanied by increased association with poly(A)$^+$ RNA, suggesting that phosphorylation of Npl3p is required for efficient release of mRNA upon termination of export. We show that expressing Sky1p

in the nucleus strongly inhibits growth and mRNA export, demonstrating the functional significance of Sky1p's observed nuclear exclusion and raising the possibility that the asymmetric distribution of Sky1/SRPK1 in the cell could promote directionality in mRNA export by specifically disassembling exported mRNP particles in the cytoplasm. This model predicts that there will be a nuclear Npl3p phosphatase required for the assembly of export-competent mRNPs.

In support of this model, we present evidence that the essential, nuclear phosphatase Glc7p is required for dephosphorylation of mRNP-associated Npl3p in vivo and that Glc7p function is required for mRNA export. Specifically, a Glc7p-dependent event is required for efficient recruitment of the mRNA export receptor Mex67p to polyadenylated RNPs. This event is likely to be dephosphorylation of Npl3p: We show that recombinant (unphosphorylated) Npl3p binds directly to Mex67p in vitro, and a mutation that eliminates phosphorylation of Npl3p increases association of Mex67p with mRNA in vivo. We present a model for directional mRNA export governed by mRNP-remodeling events catalyzed by compartment-specific post-translational modification of Npl3p.

TABLE OF CONTENTS

LIST OF FIGURES

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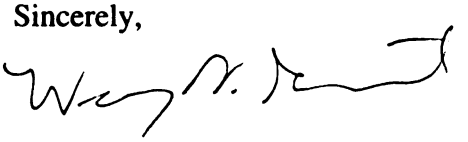

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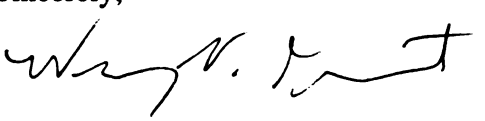

Wendy V. Gilbert

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First and foremost, I wish to thank my mentor, Christine Guthrie. She is a peerless advisor who has guided me in life as well as in science. She supported and encouraged me through two very dark chapters, and has deepened my joy in this last wonderful year by sharing it with me. I am so grateful that life led me to her lab.

The Guthrie Lab is a remarkable community of scholars and friends. It is love to remember the details of your labmates' experiments, to hold her pet hypotheses in mind when you are looking for your own information in the literature, to rejoice in another's success. Anne de Bruyn Kops, Mette Lund, Karen Kim, Pascal Preker, Alan Kutach, Gregg Whitworth, Megan Bergkessel, Tamara Brenner, Jeff Pleiss, Maki Inada, and Tommaso Villa are the finest colleagues in the world. Thank-you, dear friends, for sharing this adventure with me.

Finally, I wish to thank my family – my parents John and Susan Gilbert, and my brother Peter. You were my first, and most important teachers.

Wendy Gilbert

April 11, 2004

Not less because in purple I descended
The western day through what you called
The loneliest air, not less was I myself.

What was the ointment sprinkled on my beard?
What were the hymns that buzzed beside my ears?
What was the sea whose tide swept through me there?

Out of my mind the golden ointment rained,
And my ears made the blowing hymns they heard.
I was myself the compass of that sea:

I was the world in which I walked, and what I saw
Or heard or felt came not but from myself;
And there I found myself more truly and more strange.

-Wallace Stevens

If you have built castles in the air, that is where they should be.
Now go and put the foundations under them.

-Susan Gilbert

PROLOGUE

When I first started in the Guthrie lab, I wanted to understand the molecular requirements for an mRNP to be 'export-competent'. At this point in time, the mRNA export field was in its infancy. Genetic screens had identified a handful of factors with roles in mRNA export, principally of two classes – shuttling RNA-binding proteins such as Npl3, and components of the nuclear pore complex (NPC) through which the mRNP traveled. It was not known how mRNPs were targeted to the NPC. I proposed to apply a classical biochemical approach to this problem. Although a true in vitro mRNA export assay was considered out of reach, it was known for protein import that an NPC-docking step preceded translocation through the NPC. It was anticipated that export would similarly proceed via a two-step docking and translocation mechanism. I proposed to establish an in vitro mRNP-docking assay using in vitro-assembled mRNPs and purified nuclear pore complexes, using a protocol that had just been published by Mike Rout.

After five months of gradients that "just didn't, you know, whatever", Christine proposed that I briefly turn my attention to a project initiated by Chris Siebel, a study of the role of Sky1 in promoting nuclear localization of Npl3. Sky1 is the *S. cerevisiae* homologue of SRPK1 and 2, cytoplasmic kinases that phosphorylate SR (Serine/Arginine-rich) RNA binding proteins. SR proteins were first characterized in higher eukaryotes as essential splicing factors that could also influence splice site selection. Based on sequence homology, Npl3 is the best candidate for a yeast SR protein. Chris had hoped to exploit yeast molecular genetics and biochemistry to understand the role of SR proteins and SRPKs in splicing. Chris determined that Npl3p was indeed a substrate for Sky1p both in vitro and in vivo. However, initial efforts to

uncover a role for Npl3p and Sky1p in pre-mRNA splicing proved frustrating. In the meantime, other labs had identified Npl3p as a shuttling protein required for mRNA export (Kadowaki et al., 1994; Lee et al., 1996). The mechanisms responsible for Npl3p's nucleo-cytoplasmic shuttling were unknown, but presumed to have important functions in Npl3-dependent mRNA export. Chris' observations suggested a role for Sky1-dependent phosphorylation in promoting Npl3 shuttling, which I set out to characterize.

As I describe in Chapter 1 (Gilbert et al., 2001), Sky1 promotes nuclear localization of Npl3p by promoting dissociation of Npl3p from poly(A)$^+$ RNA in the cytoplasm. Because Sky1 is a strictly cytoplasmic protein, I imagined that phosphorylation of Npl3p by Sky1p could potentially provide a mechanism for imparting directionality to the export process, by acting as a molecular switch signaling the disassembly of mRNA-protein particles after export. This would be conceptually similar to the mechanism underlying directionality in karyopherin-mediated protein transport, which is basically a matter of compartment-specific assembly and disassembly of transport-competent complexes achieved by coupling receptor-cargo complex formation to the asymmetric distribution of RanGTP in the cell.

During the course of my graduate career, the nuclear pore associated protein, Mex67, was determined to be the elusive mRNA export receptor. Surprisingly, unlike karyopherins (the conserved family of protein transport receptors), Mex67p does not bind to Ran. In fact, mRNA export was definitively shown to be resistant to perturbations of the RanGTP gradient (Clouse et al., 2001), and thus the source of directionality in mRNA export remained a fundamental question, and an unsolved mystery, throughout most of

my graduate career. The favorite model (indeed the only model that got much air time) for directional mRNA export relied on specific disassembly of exported mRNPs by Dbp5, a cytoplasmic DEAD box protein required for mRNA export in yeast (Snay-Hodge et al., 1998; Tseng et al., 1998). The appealing features of this model were the likely enzymatic activity of Dbp5p as an RNP unwindase, and its concentration at the cytoplasmic face of the NPC. However, there is still no direct evidence for such a function. This model is further complicated by the recent discovery that Dbp5p associates with mRNPs in the nucleus (Zhao et al., 2002). This does not preclude a role for Dbp5 in cytoplasmic disassembly, but it necessitates another determinant of compartment-specificity, ie. a way to restrict Dbp5-dependent disassembly to the cytoplasm. (In the Epilogue, I suggest a model in which Sky1 performs such a function.)

My model that the asymmetric distribution of Sky1p in the cell promotes directionality in mRNA export by specifically disassembling exported mRNP particles in the cytoplasm predicts that there is a nuclear Npl3p phosphatase required for the assembly of export-competent mRNPs. In Chapter 2 (Gilbert and Guthrie, 2004), I show that wildtype poly(A)$^+$ RNPs contain predominantly unphosphorylated Npl3p, consistent with my model that phosphorylation promotes disassembly of these particles. I show that expressing the Sky1p kinase in the nucleus strongly inhibits growth and mRNA export, demonstrating the functional significance of Sky1p's observed nuclear exclusion. I present evidence that the essential, nuclear phosphatase Glc7p is required for dephosphorylation of mRNP-associated Npl3p in vivo and that Glc7p-function is required for mRNA export. Specifically, Glc7p promotes loading of the mRNA export receptor, Mex67p, onto poly(A)$^+$ RNPs and facilitates dissociation of polyadenylated

mRNA from the non-shuttling 3' end processing factor Rna15p. Finally, I show that a constitutively dephosphorylated Npl3p mutant, *npl3S411A*, increases association of Mex67p with mRNA in vivo, and recombinant Npl3p binds directly to Mex67p in vitro, consistent with the hypothesis that the phosphorylation state of Npl3p determines the transport-competence of the mRNP by regulating binding to the export receptor.

Taken together, the results presented in Chapters 1 and 2 support a coherent model for directional mRNA export governed by mRNP-remodeling events catalyzed by post-translational modification of Npl3p. All of the yeast factors described in Chapter 2 have homologues throughout eukarya, and the homologous proteins are similarly localized within the cell. Thus the mechanism we discovered in yeast is likely to be conserved. This is a supremely satisfying conclusion to my search for the molecular determinants of mRNP export-competence, and I am really delighted by the reception this work has received (Izaurralde, 2004).

Yet, at the end of the day, what I have really produced is an outline. A tremendous amount of work remains to make sense of the 'basic' process of messenger RNA export at a molecular level. Indeed, it looks like more work now than when I began. In the Epilogue, I discuss a few of the new questions raised by my thesis work and suggest future directions for experiments. Indeed, I found it very difficult to abandon this project, and so the Epilogue also contains some new data (and many models) that I hope may be of use to my colleagues in the near future.

Chapter 1

Phosphorylation by Sky1p promotes Npl3p shuttling and mRNA Dissociation

ABSTRACT

Mammalian SR proteins are currently thought to function in mRNA export as well as splicing. They contain multiple phosphorylated serine/arginine (RS/SR) dipeptides. Although SR domains can be phosphorylated by many kinases in vitro, the physiologically relevant kinase(s), and the role(s) of these modifications in vivo have remained unclear. Npl3 is a shuttling protein in budding yeast which we showed previously to be a substrate for the mammalian SR protein kinase, SRPK1, as well as the related yeast kinase, Sky1. Here we demonstrate that Sky1p phosphorylates only one of Npl3p's eight SR/RS dipeptides. Mutation of the C-terminal RS to RA, or deletion of SKY1, results in the cytoplasmic accumulation of Npl3p. The redistribution of Npl3p is accompanied by its increased association with poly(A)$^+$ RNA and decreased association with its import receptor, Mtr10p, in vivo. We propose that phosphorylation of Npl3p by the cytoplasmically localized Sky1p is required for efficient release of mRNA upon termination of export.

INTRODUCTION

Eukaryotic gene expression demands bridging the distance that separates mRNA synthesis in the nucleus from mRNA translation in the cytoplasm. Newly-synthesized pre-mRNAs must be processed in the nucleus via a number of covalent modifications, including splicing of noncoding intron sequences and polyadenylation of the 3' end. Mature mRNAs are then transported, via an active and specific export process, through the nuclear pore complex (NPC) to the cytoplasm(Stutz and Rosbash, 1998). The substrates for these reactions and for export are RNA-protein complexes (RNPs)(Daneholt, 1997; Dreyfuss et al., 1993).

The dynamic cycle of nuclear assembly and cytoplasmic disassembly of export RNPs raises enormous challenges for the orchestration of macromolecular transport through NPCs. Newly-translated RNP proteins must be transported into the nucleus to package nascent mRNAs. Subsequently, at least a subset of these same RNP proteins must be transported in the opposite direction as part of mRNA-protein particles that deliver mRNAs to the cytoplasm(Stutz and Rosbash, 1998). Finally, these particles must be disassembled in the cytoplasm, to release the mRNA for translation and to liberate the proteins for nuclear reimport and use in subsequent rounds of mRNA export. To avoid reimporting RNPs in a futile cycle, the cell must coordinate disassembly with the completion of export and distinguish the RNA binding proteins that are part of export RNPs from those that have been released.

The best candidates for mRNA export carriers are the shuttling RNA-binding proteins(Stutz and Rosbash, 1998). Two classes of RNA binding proteins known to shuttle in higher eukaryotes are the hnRNP (heterogeneous nuclear RNP) proteins and the SR proteins(Alzhanova-Ericsson et al., 1996; Caceres et al., 1998; Nakielny and Dreyfuss, 1997; Nakielny et al., 1997). Both contain one or more RRM-type RNA binding motifs in their N-termini. The C-terminal domain of hnRNP proteins is typically rich in arginine and glycine (RGG) and is the site of arginine dimethylation(Liu and Dreyfuss, 1995). The C-terminal domain of SR proteins contains multiple copies of serine-arginine (SR/RS) dipeptides, which are the sites of serine phosphorylation(Fu, 1995). Historically, hnRNP proteins have been studied as potential mRNA export factors. The prototype for this class is hnRNPA1, which accompanies mRNA from the nucleus to the cytoplasm(Daneholt, 1997; Visa et al., 1996). Export is terminated by the release of mRNA and re-importation of hnRNPA1 via its import receptor, Transportin(Pollard et al., 1996; Siomi et al., 1997). SR proteins were initially identified as essential splicing factors but the recent discovery that certain members of this family shuttle has suggested a broader role for these phosphoproteins in mRNA metabolism(Caceres et al., 1998). The role of post-translational modifications in the functions of these two classes of proteins is only poorly understood.

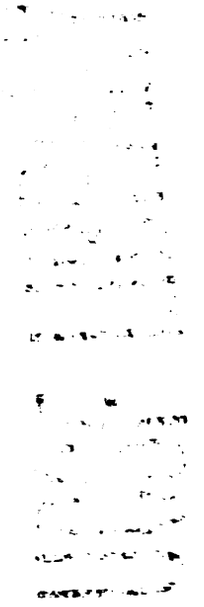
Yeast has a large number of RRM-containing proteins, many of which carry an RGG domain. The best-studied protein of this class is Npl3, which shuttles between the nucleus and the cytoplasm, and is required for bulk mRNA

export(Flach et al., 1994; Kadowaki et al., 1994; Lee et al., 1996). We and others have shown previously that the RGG domain of Npl3p is a substrate for the arginine dimethylase, Hmt1p(Henry and Silver, 1996; Siebel and Guthrie, 1996). On the other hand, the existence of SR proteins in budding yeast has been controversial; the genome contains no examples of the extensive stretches of SR/RS dipeptides which typify mammalian SR proteins(Birney et al., 1993). Interestingly, however, Npl3p contains eight such dipeptides within the C-terminal RGG domain. In support of the interpretation that Npl3 is a yeast SR-type protein, we demonstrated that Npl3p can be phosphorylated by the mammalian SR protein kinase, SRPK1(Siebel et al., 1999). Using this information, we went on to identify a protein with significant sequence homology; indeed, Sky1p (SR kinase in yeast) phosphorylates Npl3p in vitro(Siebel et al., 1999).

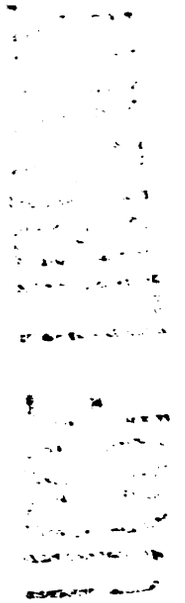
Phosphorylation on serine residues within the RS-domain is a defining characteristic of SR proteins, but the physiological role(s) of RS-domain phosphorylation have remained uncertain. Mammalian cells contain at least two families of SR protein kinases, the Clk/Sty dual-specificity kinases which are nuclear at steady-state, and the SRPK family of serine kinases which are predominantly cytoplasmic(Fu, 1995). SRPK1 was purified based on its ability to phosphorylate SC35 and other SR proteins(Gui et al., 1994). A closely related mammalian kinase, SKPK2, displays similar activity in vitro(Wang et al., 1998). Clk/Sty was found to interact in a yeast two-hybrid screen with members of the SR protein family and was subsequently shown to phosphorylate RS domains in

vitro(Colwill et al., 1996). In addition to these kinases, whose discovery depended on their interaction with SR proteins, various other proteins have been shown to phosphorylate RS domains in vitro, including protein kinases C and A(Colwill et al., 1996), p34cdc2, and DNA topoisomerase I(Rossi et al., 1996). Perhaps due to the repetitive nature of RS domain sequences, little attention has been focused on the potential for differential effects of phosphorylation at particular serines within these domains, despite the identification of such a wide variety of potential regulatory kinases.

Nonetheless, recent progress has been made in determining the site preferences of mammalian SRPK1,2. In vitro peptide selection experiments revealed sequence features in addition to RS dipeptides that contribute to recognition by this kinase(Wang et al., 1998). Importantly, these in vitro site preferences are consistent with in vivo binding studies using SF2/ASF and SRPK1,2 which showed that only a subset of SF2/ASF RS dipeptides were required for recognition by SRPK1,2 in vivo(Koizumi et al., 1999). Phosphorylation has been hypothesized to affect SR proteins' functions by modulating both protein-protein and protein-RNA interactions(Fu, 1995), and recent reports indicate that phosphorylation of metazoan SR proteins can influence their subcellular localization(Caceres et al., 1998; Koizumi et al., 1999; Misteli et al., 1998; Yeakley et al., 1999). There has been no evidence linking phosphorylation at a single RS dipeptide by a specific SR protein kinase to an in vivo function.



Here we demonstrate that Npl3p is phosphorylated on serine in vivo by Sky1p, further evidence that Npl3p is a bona fide yeast SR protein. We show that phosphorylation of Npl3p by Sky1p requires a single C-terminal RS dipeptide, and this specific phosphorylation is required for proper nuclear localization of Npl3p. The mislocalization of Npl3p in the absence of Sky1p-mediated phosphorylation is accompanied by Npl3p's increased association with poly(A)$^+$ RNA and decreased association with its import receptor, Mtr10p, in vivo. We discuss the possibility that phosphorylation of Npl3p by cytoplasmic Sky1p could play a role in distinguishing nuclear and cytoplasmic compartments, thereby imparting directionality to the export of Npl3p-containing RNPs.



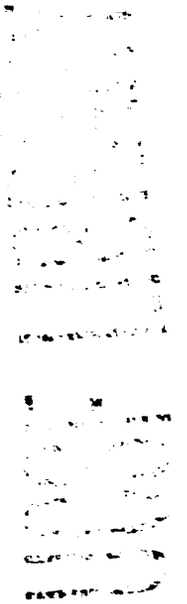
RESULTS

Npl3p is serine phosphorylated in vivo by Sky1p

Serine phosphorylation within the C-terminal RS domain is a hallmark of the SR protein family in metazoans. As described in the introduction, the Npl3 protein of budding yeast appears related to this family based on a number of criteria, including our observation that Npl3p is an efficient substrate for mammalian SRPK in vitro(Siebel et al., 1999). To determine whether this observation is biologically relevant, we asked whether Npl3p was phosphorylated in vivo. Crude cell extract was prepared from a yeast cell culture that had been labeled in vivo with 32P orthophosphate. Npl3p was purified from this extract by

immunoprecipitation. Anti-Npl3p antibodies specifically precipitated a radiolabeled protein of the size expected for Npl3p (Figure 1A, lane 2). Immunoblot experiments confirmed that this protein was in fact Npl3p (data not shown). As an additional control, pre-immune sera failed to immunoprecipitate the radiolabeled protein (Figure 1A, lane 1). These data demonstrate that Npl3p is a phosphoprotein.

To determine which amino acid(s) was phosphorylated, we performed phosphoaminoacid analysis. Npl3p that had been radiolabeled in vivo was immunoprecipitated, purified from a SDS gel and then hydrolyzed to yield individual amino acids, which were then separated using two-dimensional electrophoresis. The radiolabel was detected exclusively on serine, and not on threonine or tyrosine (Figure 1B). Thus, like metazoan SR proteins, Npl3p is a serine phosphoprotein.

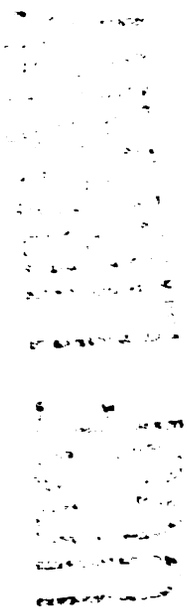


The similarities between Npl3p and metazoan SR proteins led us to ask whether Sky1p is responsible for Npl3p phosphorylation in vivo. We radiolabeled cultures of a strain in which the SKY1 gene had been precisely deleted and an otherwise isogenic wild-type strain. Npl3p was immunoprecipitated from extracts made from each culture, and the incorporation of radiolabel into Npl3p was quantitated using a phosphorimager. In the strain lacking SKY1, Npl3p radiolabeling was reduced to approximately 50% of that observed in the isogenic wild-type strain (Figure 1C, lower panel). Coomassie protein staining revealed that equal amounts of Npl3p were immunoprecipitated from the wild-type and Δsky1 extracts (Figure 1C, upper panel). Thus SKY1 is required to fully

phosphorylate Npl3p in vivo. The fact that the deletion of SKY1 does not eliminate Npl3p phosphorylation reveals the existence of at least one additional kinase capable of phosphorylating Npl3p in vivo.

In vitro phosphorylation of Npl3p by Sky1p requires RS8

We have previously shown that Sky1p phosphorylates purified recombinant HisNpl3p in vitro(Siebel et al., 1999). We have now tested the activity of a C-terminally truncated rHisNpl3p substrate that lacks all eight RS/SR dipeptides (rHisNpl3ΔRS) (Figure 2A). As we predicted for an SR kinase, protein A-tagged Sky1p purified from yeast failed to phosphorylate rHisNpl3ΔRS (Figure 2B, lane 4), but was active against a wild-type rHisNpl3p substrate (Figure 2B, lane2). rGSTSky1p purified from *E. coli* displayed identical substrate specificity (data not shown). Mammalian SR protein kinases phosphorylate multiple serine residues within RS/SR dipeptides in the C-terminal RS domains of their substrates. Peptide selection experiments with human SRPK2 have identified additional sequence requirements for residues flanking the RS dipeptide(Wang et al., 1998). According to these rules, only the final C-terminal RS dipeptide in Npl3p fits the mammalian consensus. To test the prediction that Sky1p phosphorylates this site, we prepared a mutant substrate in which the final serine was changed to an alanine (rHisNpl3RA8p) (Figure 2A). Strikingly, Sky1p is completely unable to phosphorylate rNpl3RA8p (Figure 2B, lane 3). The Npl3RA8 mutant protein is fully active in an RNA binding assay (data not



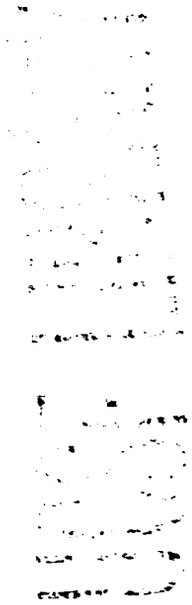
shown), therefore its inactivity as a Sky1p substrate cannot be explained by gross misfolding of the protein.Thus the single serine in Npl3p's RS domain that is found within a mammalian SRPK consensus sequence is essential for phosphorylation by Sky1p in vitro.

Phosphorylation by Sky1p is required for proper nuclear localization of Npl3p

The shuttling of Npl3p between the nucleus and cytoplasm is a key feature of its proposed function as an mRNA carrier. Recent studies in mammals have suggested a role for phosphorylation in regulating the sub-cellular localization of shuttling SR proteins(Caceres et al., 1998). To test whether phosphorylation of Npl3p is important for its localization, we inserted the coding sequence for the green fluorescent protein (GFP) at the 5' end of the NPL3 coding sequence under control of the NPL3 promoter. This construct fully complements an NPL3 deletion (data not shown). Wild-type cells expressing this GFP-NPL3 reporter displayed a bright fluorescent signal that localized exclusively to nuclei (Figure 3A), consistent with previously published experiments(Lee et al., 1996). In contrast, GFP-Npl3p was readily detectable in both the cytoplasm and nucleus of cells lacking SKY1 (Figure 3B). Immunoprecipitation and immunoblot experiments using anti-Npl3 antibodies demonstrate that GFP-Npl3p levels were not detectably altered in strains lacking SKY1 (Figure 5A, and data not shown). Therefore, the increase in the cytoplasmic levels of GFP-Npl3p in cells lacking

SKY1 suggests that Sky1p-catalyzed phosphorylation of Npl3p is important for proper nuclear localization of Npl3p.

To test directly whether the Sky1p phosphorylation site in Npl3p is required for proper nuclear localization, we constructed a GFP-reporter in which the final serine was changed to alanine, the same mutation that abolished Sky1p-catalyzed phosphorylation of Npl3p in vitro (Figure 2B). This mutant GFP-Npl3RA8p was observed in the cytoplasm of wild-type cells (Figure 3C), mimicking the localization pattern observed for wild-type GFP-Npl3p in cells lacking SKY1. Other serine to alanine point mutations in the RS domain of Npl3p had no effect on GFP-Npl3p localization (data not shown). Taken together, our data support a model in which Sky1-catalyzed phosphorylation at RS8 is important for the nuclear localization of Npl3p.

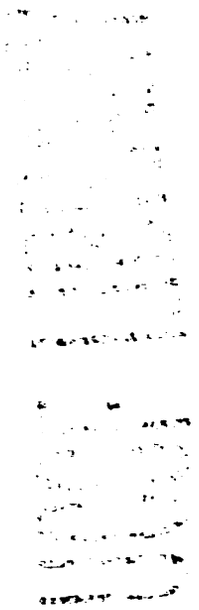


Phosphorylation by Sky1p enhances binding of Npl3p to its import receptor Mtr10 in vivo

Phosphorylation could facilitate the steady-state nuclear localization of Npl3p by decreasing the rate of Npl3p export, increasing the rate of Npl3p import, or both. The fact that Sky1p is cytoplasmic is consistent with a model in which cytoplasmic phosphorylation of Npl3p actively promotes its import. We examined whether Sky1-catalyzed phosphorylation promoted Npl3p import by enhancing Npl3p's binding to its import receptor. Previous studies have demonstrated that Mtr10p, a member of the importinß-family of nuclear transport receptors,

functions as an import receptor for Npl3p in yeast. Specifically, mutations in MTR10 lead to accumulation of Npl3p in the cytoplasm(Pemberton et al., 1997; Senger et al., 1998). Moreover, Npl3p co-purifies with protein A-tagged Mtr10 on IgG affinity resin, indicating that a fraction of Npl3p and Mtr10p exist in a complex together in vivo(Pemberton et al., 1997; Senger et al., 1998).

We exploited this affinity purification scheme to ask whether phosphorylation of RS8 by Sky1 is important for the Npl3p-Mtr10p interaction in vivo. Wild-type and Δsky1 strains were transformed with a plasmid expressing a protein A-Mtr10p fusion or an untagged Mtr10p as a negative control. Extracts were prepared from exponentially-growing cultures of each strain, and protein A-Mtr10p was purified by binding to an IgG resin. ProtA-Mtr10p and interacting proteins were eluted and Npl3p binding was assessed by immunoblotting with an anti-Npl3p polyclonal antibody. Consistent with previous results, a fraction of wild-type Npl3p bound to the IgG resin in the presence of protein A-tagged Mtr10p (Figure 4, lane 4), but not in extracts from the untagged strain (data not shown) (Pemberton et al., 1997; Senger et al., 1998). In extracts from a strain lacking SKY1, the binding of Npl3p to protein A-Mtr10p was reduced (Figure 4, lane 3). Mutation of the Sky1p phosphorylation site in Npl3p to alanine similarly reduced association of Npl3p with protein A-Mtr10p in vivo (data not shown). These results are particularly striking given the fact that loss of Sky1p-catalyzed phosphorylation increases the cytoplasmic pool of Npl3p (Figure 3B,C), thereby increasing the fraction available to bind Mtr10p, which is predominantly cytoplasmic at steady-state(Pemberton et al., 1997; Senger et al., 1998). These



data show that phosphorylation of Npl3p at RS8 by Sky1p is required for efficient binding of Npl3p to Mtr10p in vivo.

Phosphorylation of Npl3p decreases its association with mRNA in vivo

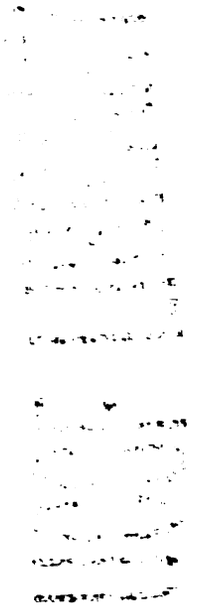
We considered two hypotheses to explain the loss of Npl3p binding to Mtr10p in a Δsky1 strain: 1)unphosphorylated Npl3p is sequestered in a different complex, inaccessible to Mtr10p, and 2)loss of phosphorylation directly reduces Npl3p's binding affinity for Mtr10p. These mechanisms are not mutually exclusive. To test the first hypothesis, we guessed at the identity of a competitive binding partner. mRNA was an obvious candidate for the following reasons: 1)Npl3p displays a high level of non sequence-specific RNA binding activity in vitro (W. Gilbert and C. Guthrie, unpublished observations) and is bound to poly(A)$^+$ in vivo(Krebber et al., 1999; Russell and Tollervey, 1995); 2)poly(A)$^+$ RNA is a very abundant potential competitor for binding to Mtr10p in the cytoplasm(Groner and Phillips, 1975); and 3)phosphorylation has been shown to decrease the non sequence-specific RNA-binding activity of some metazoan RS proteins in vitro(Tacke et al., 1997). We therefore set out to test the hypothesis that phosphorylation of Npl3p by Sky1p would act to dissociate Npl3p from mRNA in vivo.

To isolate Npl3p-poly(A)$^+$ RNA complexes formed in vivo, we first cross-linked proteins to RNA by irradiating living cells with UV and then purified the RNPs on oligo dT cellulose. We loaded equal A260 units of RNPs purified from

wild-type and Δsky1 strains on a gel, and analyzed the Npl3p content by Western blotting with anti-Npl3p polyclonal antibodies. As shown by previous work, Npl3p cross-links to poly(A)$^+$ RNA in wild-type cells (Figure 5A, lane 4)(Krebber et al., 1999; Russell and Tollervey, 1995). The amount of Npl3p cross-linked to poly(A)$^+$ RNA in Δsky1 cells was strikingly increased ~3-5 fold compared to wild-type (Figure 5A, compare lane 5 to lane 4). GFP-Npl3p behaved identically to endogenous Npl3p in this assay (Figure 5A), thus we have made use of our plasmid-borne GFP-Npl3 constructs to examine the effects of various mutations on Npl3p crosslinking. Mutation of the Sky1p phosphorylation site in Npl3p from serine to alanine increased cross-linking of Npl3p to poly(A)$^+$ RNA ~5 fold (Figure 5B, compare lane 4 to lane 3), duplicating the effect of deletion of SKY1 on wild-type Npl3p (Figure 5A). Thus we conclude that loss of Sky1p-catalyzed phosphorylation increases the association of Npl3p with poly(A)$^+$ RNA in living cells.

We have observed three effects of loss of phosphorylation on Npl3p: 1)mislocalization to the cytoplasm, 2)decreased binding to Mtr10p, and 3)increased binding to poly(A)$^+$ RNA. Any of these might be a secondary consequence of one of the other defects. To distinguish effects on localization and/or binding to Mtr10p from effects on RNA binding per se, we made use of a yeast strain in which the MTR10 gene was deleted. Wild-type GFP-Npl3p and mutant GFP-Npl3RA8p display identical localization in a Δmtr10 strain; both proteins are distributed throughout the cell (data not shown). If increased RNA binding were simply a secondary effect of cytoplasmic accumulation, we predict

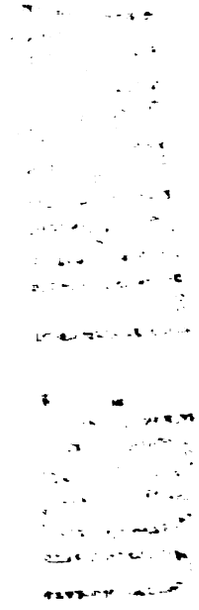
that the RA8 mutation would have no effect on Npl3p-RNA crosslinking in this strain background. Similarly, if increased RNA binding were an indirect effect of decreased binding to Mtr10p, deletion of MTR10 would abolish the difference between wild-type Npl3p and Npl3RA8p. Mutation of the Sky1p phosphorylation site clearly increased cross-linking of GFP-Npl3RA8p to poly(A)$^+$ RNA compared to wild-type GFP-Npl3p in a Δmtr10 background (Figure 5C, compare lanes 7-8 to lanes 5-6). These results show that Sky1p-catalyzed phosphorylation decreases the binding of Npl3p to poly(A)$^+$ RNA in vivo, independent of effects on localization and independent of Mtr10p.



However, phosphorylation of Npl3p is probably not the sole determinant of the extent of RNA binding in vivo. Deletion of MTR10 dramatically increased RNA crosslinking of wild-type (phosphorylatable) Npl3p (Figure 6A, compare lanes 3 and 4). mtr10-7ts, a mutant strain that localizes GFP-Npl3p to the nucleus and the cytoplasm at 25°C (data not shown, and(Senger et al., 1998)), similar to the localization of GFP-Npl3RA8p observed in wild-type cells (Figure 3C) also shows increased crosslinking of Npl3p (Figure 6B, compare lanes 4 and 5). We observed a modest increase in the total level of Npl3p protein in the mtr10 mutants (Figure 6A and B, compare lanes 1 and 2). These crosslinking data are consistent with the hypothesis that binding of Mtr10p to Npl3p promotes dissociation of Npl3p from RNA, but do not rule out the possibility that simply increasing the cytoplasmic pool of Npl3 protein increases binding to poly(A)$^+$ RNA, the bulk of which is cytoplasmic in rapidly-growing yeast cells(Groner and Phillips, 1975).

Phosphorylation by Sky1p does not affect binding of Npl3p to Mtr10p in a purified system

To test the hypothesis that Sky1p-catalyzed phosphorylation of Npl3p directly stimulates binding to Mtr10p, we performed in vitro binding studies using purified proteins. Full-length Sky1p tagged with protein A was purified from yeast by affinity purification on IgG Sepharose. In vitro kinase reactions were performed on these beads using recombinant HisNpl3p purified from *E. coli* as the substrate (Figure 2B, lane 2). The rHisNpl3p used in subsequent binding reactions was greater than ninety percent phosphorylated (see Materials and Methods). Mock-phosphorylated rHisNpl3p was prepared by incubating the substrate with a mock-purification from a Δsky1 yeast strain (Figure 2B, lane 1). Protein A-Mtr10p was purified from yeast by IgG-affinity chromatography and extensive washing with $MgCl_2$ (Figure 7A). Similar results were obtained using recombinant Protein A-Mtr10p purified from *E. coli* (data not shown).

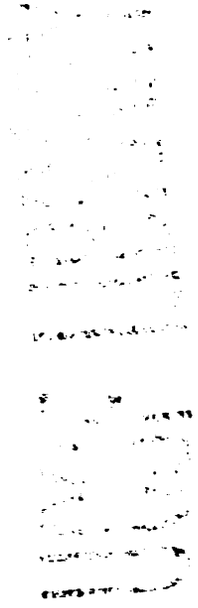


Purified ProtA-Mtr10p bound to phosphorylated and mock-phosphorylated rHisNpl3p with equal affinity (Figure 7B, compare lanes 3 and 4). In an effort to recapitulate the in vivo conditions in which phosphorylation of Npl3p enhanced binding to Mtr10p, we added yeast extract to our in vitro binding reactions. By increasing the concentration of yeast extract in the reaction, we were able to compete the binding of rHisNpl3p to ProtA-Mtr10p (Figure 7C). Strikingly, there was no difference in the responses of phosphorylated and mock-

phosphorylated rHisNpl3p to competition from yeast extract (Figure 7C, compare lanes 1-3 to lanes 4-6). Thus although phosphorylation of Npl3p clearly increases its association with Mtr10p in vivo, this does not appear to be due to a direct effect on the binding affinity of Npl3p for Mtr10p.

DISCUSSION

We have presented evidence that phosphorylation of Npl3p, a yeast shuttling SR protein, functions to promote the dissociation of Npl3p-RNA complexes in vivo. Sky1p, the yeast SRPK homologue, catalyzes Npl3p phosphorylation in vitro and in vivo, at a unique serine within Npl3p's RS domain. Loss of phosphorylation by Sky1p causes the mislocalization of Npl3p to the cytoplasm, and reduced association of Npl3p with its import receptor Mtr10p. Below we propose that phosphorylation of Npl3p by cytoplasmic Sky1p might serve as a molecular switch signaling the disassembly of mRNA-protein particles after export, thereby providing a potential mechanism for imparting directionality to the export process.



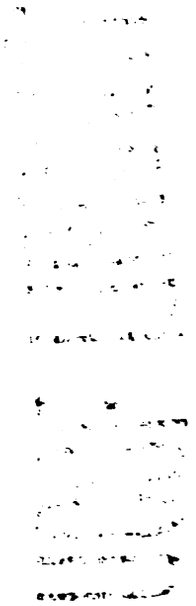
Mechanisms for dissociation of Npl3p-RNA complexes

Loss of phosphorylation by Sky1p resulted in a 3-5 fold increase in Npl3p's association with poly(A)$^+$ RNA in vivo and a decrease in Npl3p's association with Mtr10p in vivo. The inverse correlation between binding to RNA

and binding to Mtr10p in vivo suggests that formation of these Npl3p-containing complexes may be mutually exclusive. Previous studies of an Npl3p-Mtr10p complex purified from yeast extracts found that RNA could dissociate it in the presence of RanGTP, presumably reflecting a post-nuclear import event(Senger et al., 1998). Conversely, we have obtained preliminary data showing that purified Mtr10p can dissociate a pre-formed Npl3p-RNA complex in vitro (W. Gilbert and C. Guthrie, unpublished observations). Consistent with a model in which Mtr10p stimulates dissociation of Npl3p from RNA in the cytoplasm, we observe that mutations in MTR10 increase the amount of Npl3p that cross-links to poly(A)$^+$ RNA in vivo. Notably, however, the mechanism by which Sky1p-catalyzed phosphorylation of Npl3p dissociates Npl3p from poly(A)$^+$ RNA does not require the presence of Mtr10 protein; we observed a similar 3-5 fold effect of phosphorylation on RNA-binding in a Δmtr10 background. The converse is also true: mutation of MTR10 increases Npl3p-RNA crosslinking in the absence of phosphorylation by Sky1p. Thus our data suggest that at least two mechanisms exist to promote the dissociation of Npl3p from poly(A)$^+$ RNA in the cytoplasm: 1)phosphorylation by Sky1p, and 2)binding of Mtr10p (see Figure 8). The fact that deletion of the SKY1 gene is synthetically lethal with deletion of MTR10 is consistent with the interpretation that both mechanisms contribute to the same essential process, the termination of messenger RNA export in the cytoplasm. Npl3p, like most shuttling proteins described to date, is predominantly nuclear at steady-state. If in fact the bulk of Npl3p is continuously cycling between the nucleus and the cytoplasm, the release and re-import steps must be very rapid.

Direct coupling between release of Npl3p from RNA and binding to Mtr10p could increase the efficiency of re-import.

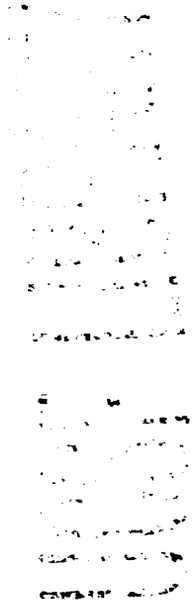
An important question for the future will be to determine where Npl3p normally dissociates from exported mRNA, and how phosphorylation affects the location and timing of this event. In a preliminary experiment to determine whether phosphorylation by Sky1p reduced the affinity of Npl3p for synthetic polynucleotides, phosphorylation at RS8 had no effect. The possibility remains that phosphorylation would influence binding to specific mRNA sequences. In addition, it seems likely that phosphorylation may influence RNA-binding via effects on protein-protein interactions within the RNP. A single Balbiani ring messenger RNP contains a variety of RNA-binding proteins, including cap-binding complex, hnRNP proteins, and shuttling SR proteins(Daneholt, 1997). Notably, the composition of this RNP changes several times during transit from the site of transcription to the ribosomes, with some shuttling proteins dissociating at the cytoplasmic face of the NPC while others remain associated with the mRNA in polysomes(Daneholt, 1997). We have obtained preliminary evidence that the presence of RA8 mutant Npl3p in a cell dominantly increases the crosslinking of Nab2p, another shuttling hnRNP protein(Duncan et al., 2000; Lee and Aitchison, 1999), to poly(A)$^+$ RNA in vivo (W. Gilbert and C. Guthrie, unpublished observations). This is consistent with a model in which phosphorylation of Npl3p by Sky1p functions as a trigger for cytoplasmic remodeling of mRNPs.



What are the physiological roles of phosphorylation of SR proteins by SRPKs? Both the yeast kinase and its mammalian homologues are predominantly cytoplasmic(Siebel et al., 1999; Wang et al., 1998). Studies of mammalian SRPKs have emphasized the potential effects of phosphorylation of SR proteins on nuclear pre-mRNA splicing, the process in which SR proteins' role is best appreciated. Our data do not exclude the possibility that cytoplasmic phosphorylation of SR proteins may be an important mechanism for regulating the nuclear concentration and/or activity of SR proteins involved in pre-mRNA splicing. However, our studies of yeast Sky1p raise the possibility that a conserved function of SRPKs may be to regulate the nucleo-cytoplasmic shutting of SR proteins. In recent studies of SF2/ASF, over-expression of an SRPK2 kinase-inactive mutant caused SF2/ASF to accumulate in the cytoplasm(Koizumi et al., 1999). It will be interesting to determine whether SRPK-mediated phosphorylation promotes the dissociation of mammalian SR proteins from RNA, as we have shown for Sky1p-phosphorylation of Npl3p.

A mammalian homologue of Mtr10p, TRN-SR, was recently identified as a cytosolic factor capable of mediating the import of recombinant RS-domain containing proteins in a permeabilized cell assay(Kataoka et al., 1999). The effects of phosphorylation were not determined in this paper. However, a recent study of TRN-SR2, which differs from TRN-SR by the absence of two short regions of ~30 amino acids, found that phosphorylation of RS domains was

required for efficient binding to the import receptor in vitro(Lai et al., 2000). Further experiments are needed to clarify the relationship between these two proteins, and to determine the effects of RS-domain phosphorylation on mammalian SR-protein import in vivo. Our in vitro binding studies with purified Mtr10 and Npl3 proteins indicate that phosphorylation of Npl3p by Sky1p does not significantly increase Npl3p's affinity for Mtr10p in vitro. This is consistent with a recent report from Fu and colleagues. However, in contrast to our results, these authors observed that phosphorylation of Npl3p did enhance binding to Mtr10p in the presence of yeast extract. They speculate that other Mtr10p cargos present in their extracts might preferentially compete the binding of unphosphorylated Npl3p. Alternatively, another Npl3p binding partner present in their extracts may be the phosphorylation-sensitive competitor. The likeliest explanation for our different results is some difference in the extracts used for competition.



Only 1 of 8 RS/SR dipeptides is required for phosphorylation by Sky1p

Npl3p was identified by our lab and others as a likely yeast SR protein based on the presence of eight SR/RS dipeptides in the C-terminal domain, in addition to homology within the RRM domains. In this report, we show that only one of Npl3p's SR/RS dipeptides is essential for phosphorylation by Sky1p in vitro. It is intriguing to note that six of the remaining seven RS/SR dipeptides in Npl3p which do not appear to be sites for Sky1p phosphorylation are in the

context SRGG. This sequence is predicted to be a site for arginine methylation by Hmt1p. Additionally, the serine that is required for phosphorylation by Sky1p in vitro is the only one found within the preferred sequence for human SRPK2(Wang et al., 1998). Our results clearly show that all RS/SR dipeptides are not equivalent and emphasize the importance of analyzing specific phosphorylation events when attempting to draw conclusions about the effects of RS-domain phosphorylation. This may be particularly important to consider in the interpretation of in vivo studies involving the overexpression of SR protein kinases; it will be important to determine that the sites phosphorylated under such conditions in fact reflect the normal sites of these kinases.

Implications

Whatever the specific mechanism by which Sky1p-mediated phosphorylation destabilizes Npl3p's association with RNA in vivo, this activity has the potential to perform two important functions. First it could be a signal to terminate mRNA export in the cytoplasm, thereby imparting directionality to the process. The model that cytoplasmic phosphorylation of Npl3p functions to terminate mRNA export predicts a nuclear Npl3p phosphatase, and a cycle of phosphorylation and dephosphorylation with each round of RNA export. We are currently investigating whether there is a correlation between Npl3p's phosphorylation state and its subcellular localization. Direct testing of this model awaits the identification of an Npl3p phosphatase. Mammalian PP1A phosphatase

has been shown to antagonize the activity of SRPKs in vitro(Fu, 1995); it will be interesting to see whether the yeast PP1A homologue GLC7 reverses Sky1p-catalyzed phosphorylation of Npl3p in vivo. A second function of Sky1p could be to prevent sequestering of newly-synthesized Npl3p protein in the cytoplasm by inhibiting inappropriate binding to abundant cytoplasmic poly(A)$^+$ RNA. This could be a role for SRPK-catalyzed phosphorylation of non-shuttling nuclear SR proteins such as SC35. Our discovery of a function for Sky1p unrelated to splicing invites further study of the functions of SR proteins and their regulators in all aspects of mRNA metabolism.

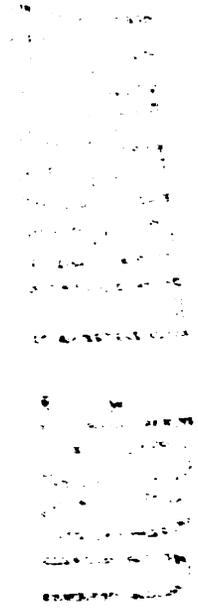
MATERIALS AND METHODS

Yeast strains and plasmids

Δsky1::TRP1 (YCS22) and the parent SKY1 strain (YCS19: Mata; ura3-52; his3-11,15; leu2-3,112; trp1-1; ade2-1; ade3; can1-100) were described previously(Siebel et al., 1999). MTR10 (RS453), mtr10-7, and Δmtr10::HIS3 were described previously(Senger et al., 1998).The SKY1-ProtA strain (YWG35) was generated by integrating the protein A coding sequence into YCS19 at the C-terminus of the SKY1 open reading frame using the method of Longtine et al.(Longtine et al., 1998). The integrating SKY1TEVProtA-kan plasmid was constructed by replacing the GFP coding sequence in pRA6a-GFP(S65T)-kanMX6 (Longtine et al., 1998) with a PCR product encoding protein A flanked

by PacI (5') and AscI (3') sites. The presence of the protein A tag was confirmed by whole-cell PCR and Western blotting.

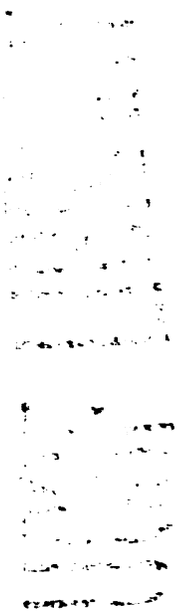
To visualize wild-type and mutant Npl3p in living cells, we inserted the GFP open reading frame at the 5' end of the NPL3 coding sequence within a 3880 bp HaeII/AflIII blunt-ended genomic fragment cloned into the pRS315 CEN, ARS vector previously cut with SmaI. DNA encoding GFP flanked by StuI sites was generated by PCR and cloned into an StuI site that was generated at the ATG of NPL3 by PCR. This pRS315-GFP-NPL3 fusion (pCS38) complemented a Δnpl3 strain for growth at all temperatures tested from 16°C-37°C. The RS->RA mutant reporter plasmid, pRS315-GFP-NPL3RA8 (pCS55), was generated by PCR of the C-terminus of NPL3 with a mutagenic oligo, followed by subcloning of the mutant fragment into pCS38. All PCR products were confirmed by sequencing. The protein A-tagged (pNOPPAMTR10) and untagged control (pUN100MTR10) plasmids for the production of ProtA-Mtr10p in yeast were described previously(Senger et al., 1998).



Expression and purification of E. coli recombinant proteins

The NPL3 gene (from ATG to stop codon) was inserted into pRSET,A (Invitrogen) by PCR amplification using BamHI (5') and DraI (3') primers and insertion of the BamHI-DraI fragment into pRSETA cut with BamHI and PvuII, to make pHisNpl3. pHisNpl3ΔRS was constructed by deleting the entire RS domain (see Figure 2A for domain boundaries) using two rounds of PCR.

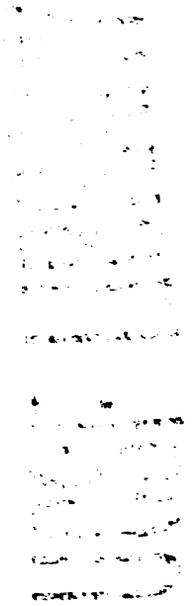
pHisNpl3RA8 was generated by inserting the HindIII fragment from pCS55 containing the RA8 mutation into pHisNpl3 cut with HindIII. All expression constructs were confirmed by sequencing. The vectors containing the wild-type and mutant NPL3 genes were transformed into E. coli BL21(DE3)plysS cells and 1 liter cultures were grown in Luria broth + ampicillin (100 µg/ml) + CAM (25 µg/ml) at 30°C to OD 0.6 and induced by the addition of 1 mM isopropyl-ß-D-thiogalactopyranoside (IPTG) in the presence of 1 mM PMSF for 3.5 hours. The bacterial cell pellet was lysed by sonication in 12.5 ml of 50 mM NaH_2PO4 pH 8, 300 mM NaCl, 0.05% TritonX-100, 10 mM imidazole. The lysate was cleared by centrifugation at 10,000 x g for 20 minutes, and bound in batch to 1.25 ml Ni-NTA Agarose (Qiagen) at 4°C for 1 hour. The resin was poured into a column and washed extensively with 80 mM imidazole in lysis buffer. Bound proteins were eluted with 250 mM imidazole in lysis buffer and dialyzed against 20 mM HEPES, 25 mM KCl, 10% glycerol, 0.5 mM DTT, pH 7.9.



Affinity purification of ProtA-fusions from yeast

The vectors containing the protein A-tagged (pNOPPAMTR10) and untagged (pUN100MTR10) genes were transformed into YCS22 cells and 1 liter cultures were grown in SD-Leu at 30°C to OD 1.0. Cells were lysed in IgG buffer [50 mM Tris-HCl, 150 mM NaCl, 0.05% Tween 20, 5 mM $MgCl_2$, 2 mM ß-mercaptoethanol, 1 mM PMSF, 1 µg/ml pepstatin, 10 mM NaF, 10 mM ß-glycerophosphate-HCl, pH 7.5, and a cocktail of protease inhibitors (Boehringer

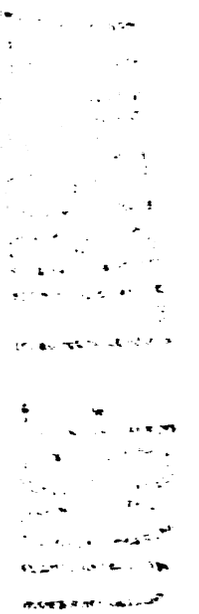
Mannheim)] by shaking with 0.5 mm glass beads in a multi bead-beater (Biospec) at 4°C for three 2-minute cycles with >2 min on ice in between cycles. The lysate was cleared by centrifugation and the supernatant (whole-cell extract) was mixed with 100 µl IgG-Sepharose beads (Pharmacia) and incubated at 4°C for 2 h. To check for associated Npl3p, 1% of the IgG-Sepharose bound lysate was washed in IgG buffer, boiled in SDS loading buffer, and analyzed by SDS-PAGE and Western blotting with anti-Npl3p polyclonal antibodies. ProtA-Mtr10p was purified from the remaining fraction by extensive washing with IgG buffer + 500 mM $MgCl_2$, followed by equilibration in 600 µl IgG buffer. To analyze the purified protein, 1/10th of the IgG-Sepharose beads were eluted with 80 µl of acetic acid (0.5 M, pH 3.4), neutralized with 20 µl 2 M Tris base, and boiled in 25 µl 4X SDS loading buffer. Twenty µl were analyzed by SDS-PAGE and silver staining.



Sky1-ProtA was purified as described above for ProtA-Mtr10p, with the following modifications: 1 liter cultures (YWG35 and YCS22) were grown in YPD at 30°C to OD 1.0. After binding in IgG buffer, the IgG-Sepharose bound protein was washed extensively in 1 X PBS + 0.1% NP-40, 10 mM NaF, 10 mM ß-glycerphosphate-HCl, pH 7.5 at 4°C, followed by equilibration in 1 ml kinase wash buffer [40 mM Tris-HCl, pH 7.4; 10 mM $MgCl_2$, 100 mM NaCl, 0.01% NP-40, 1 mM PMSF, 10 mM NaF, 10 mM ß-glycerophosphate-HCl, pH 7.5] at 22°C. 1 liter of starting culture yielded ~2 µg purified Sky1-ProtAp.

In vitro phosphorylation and binding

Recombinant Npl3p was purified from E. coli as described above. 500 µl in vitro kinase reactions contained 50 µg purified Npl3p substrate and ~1 µg Sky1-ProtAp (bound to IgG-Sepharose), or an equivalent fraction of IgG-Sepharose from a mock purification, in kinase reaction buffer [40 mM Tris-HCl, pH 7.4, 10 mM $MgCl_2$, 100 mM NaCl, 0.01% NP-40, 10 mM NaF, 10 mM ß-glycerophosphate-HCl, pH 7.5, 1 mM ATP] with 20 µCi gamma-32P-ATP. The reactions were incubated at 30°C with gentle mixing for 1 h. The extent of phosphorylation was determined by quantitating the incorporation of gamma 32P ATP into Npl3p on a phosphorimager and comparing the signal to a standard curve obtained by scanning a dilution series of the gamma32P ATP label. The phosphorylated or mock-phosphorylated Npl3p was recovered from the supernatant of the kinase reactions and frozen in liquid nitrogen in the presence of 20% v/v glycerol for use in subsequent experiments.



400 µl binding reactions containing ~3 µg ProtA-Mtr10p bound to IgG beads, 3 µg phosphorylated or mock-phosphorylated rHisNpl3p, and 0, 15, or 60 µg total yeast protein from whole-cell extract were incubated in IgG buffer +0.5 mg/ml BSA at 4°C for 4 h. The pellet and supernatant fractions were analyzed by SDS-PAGE and Western blotting with anti-Npl3 antibodies.

Purification of UV-crosslinked RNPs

1 liter yeast cultures were grown in YPD to OD 0.6-1.0. Cells were treated with UV light, as described(Anderson et al., 1993). Lysates in RNP lysis buffer [20 mM Tris-HCl, pH 7.5, 1 mM EDTA, 50 mM LiCl, 1% SDS, 1% ß-mercaptoethanol, 1 mg/ml heparin, 10 mM vanadyl adenosine, 1 mM PMSF, and a cocktail of protease inhibitors (Boehringer Mannheim)] were made by shaking with glass beads as described above. Subsequent purification of proteins UV-crosslinked to poly(A)+ RNA was performed essentially as described(Anderson et al., 1993). Resuspended poly(A)+ RNA samples were normalized by A260, treated with nuclease, and loaded for SDS-PAGE and immunoblotting with anti-Npl3p polyclonal antibodies followed by HRP-conjugated secondary antibody (Bio-Rad) and detection with Super Signal West Femto substrate (Pierce). 1 A260 unit of each eluate was loaded per gel lane. A detailed protocol is available at http://www.metazoa.com/upl2887.

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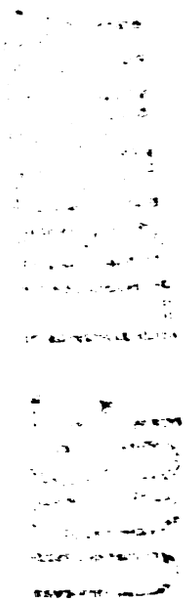
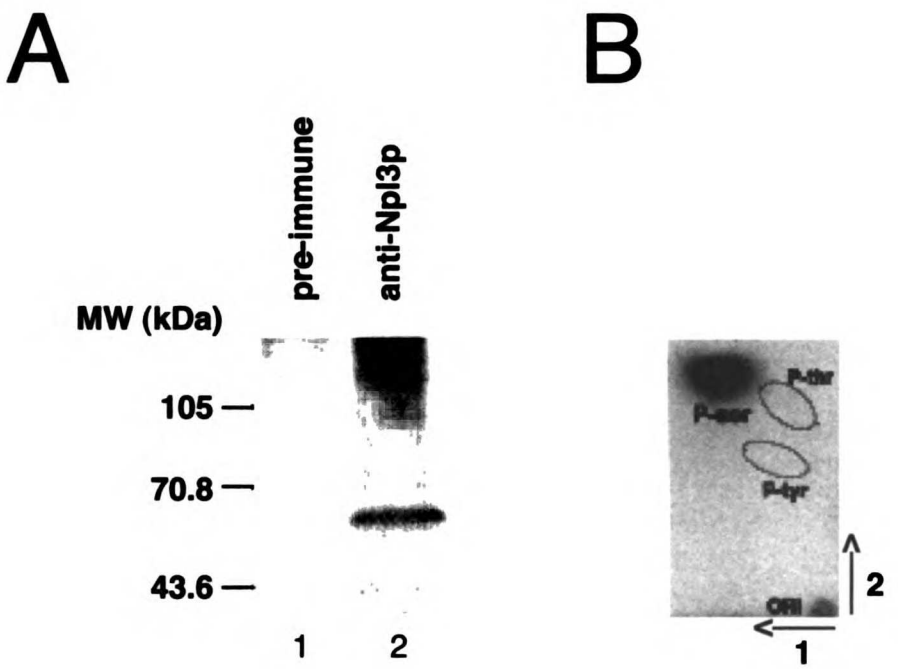
Figure 1 **SKY1-dependent Phosphorylation of Npl3p in vivo. a**, In vivo phosphate labeling. Whole cell extract was prepared from yeast grown in the presence of 32P orthophosphate. Protein was immunoprecipitated with anti-Npl3p polyclonal antibodies (lane 2) or pre-immune sera (lane 1) and analyzed by SDS-PAGE and autoradiography. b, Phosphoaminoacid analysis. Gel-purified radiolabeled Npl3p was hydrolyzed, and individual amino acids separated by two-dimensional electrophoresis. c, In vivo phosphate labeling of Npl3p in a Δsky1 strain (lane 2) compared to a wild-type strain (lane 1).




A
B
pre-immune
anti-Npl3p
MW (kDa)
105
70.8
43.6
1
2
P-thr
P-ser
P-tyr
ORI
2
1

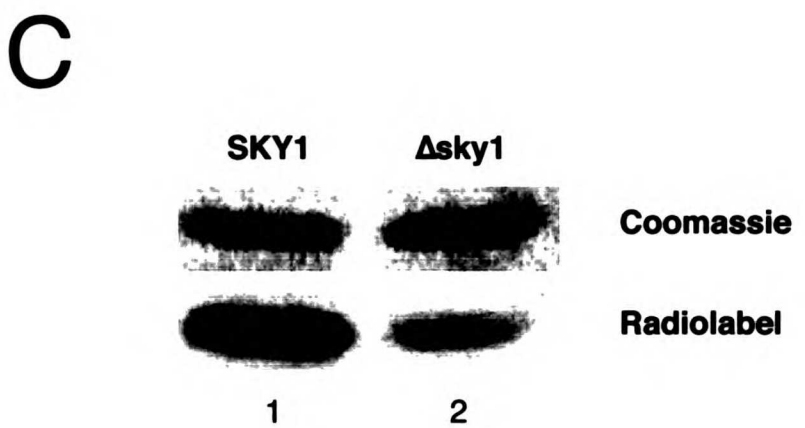
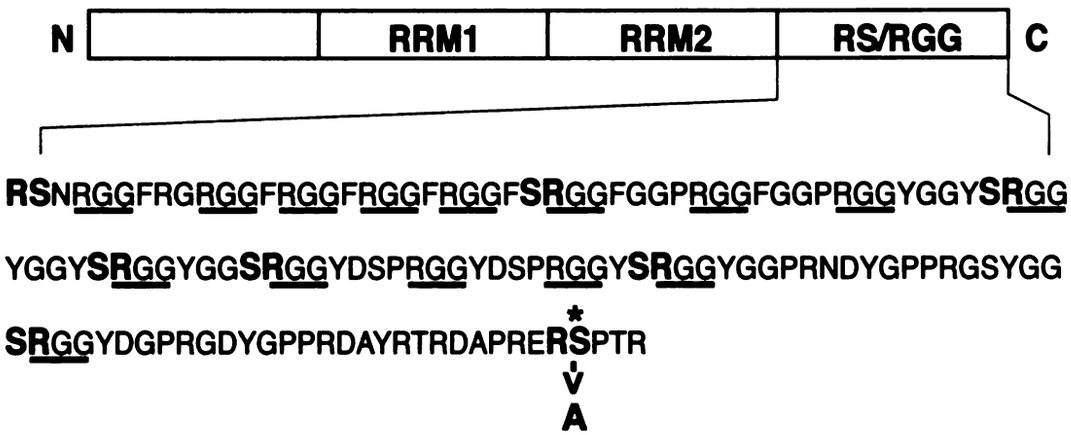

C
SKY1
Δsky1
Coomassie
Radiolabel
1
2

Figure 2 **Phosphorylation of Npl3p by Sky1p Requires RS8. a**, Domain structure and sequence of Npl3p. SR/RS dipeptides are in bold and RGG repeats are underlined. The site of the RA8 point mutation is indicated with an asterisk. **b**, In vitro kinase assays. Recombinant His-tagged substrates, wild-type (lanes 1 and 2), Npl3RA8 mutant (lane 3), and Npl3ΔRS mutant (lane 4), were purified from *E. coli* (Coomassie-stained gel, upper panel) and tested in kinase reactions with Sky1-ProtAp purified from yeast (lanes 2-4) or a mock-purified fraction from an untagged yeast strain (lane 1).

A


N
RRM1
RRM2
RS/RGG
C
RSNRGGFRGRGGFRGGFRGGFRGGFRGGFSRGGFGGPRGGFGGPRGGYGGYSRGG
YGGYSRGGYGGSRGGYDSPRGGYDSPRGGYSRGGYGGPRNDYGPPRGSYGG
SRGGYDGPRGDYGPPRDAYRTRDAPRERSPTR
*
V
A

B

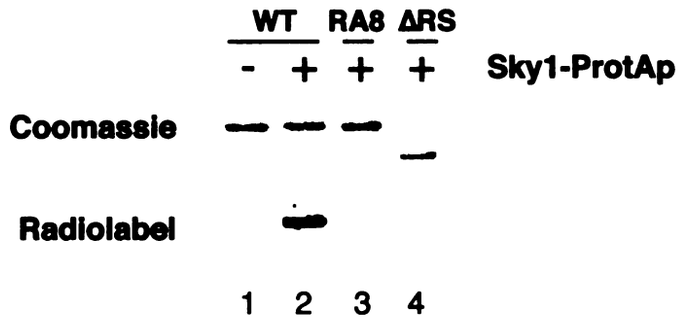
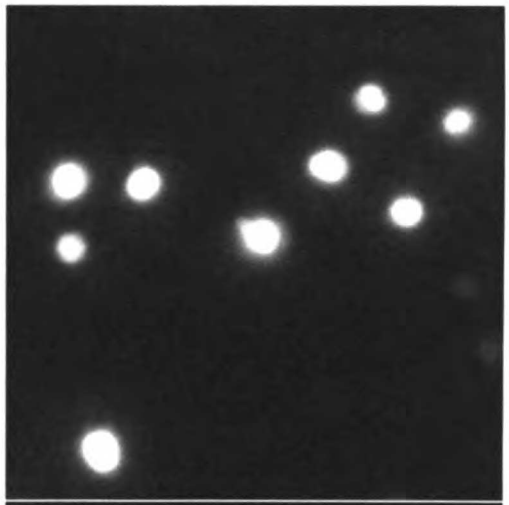
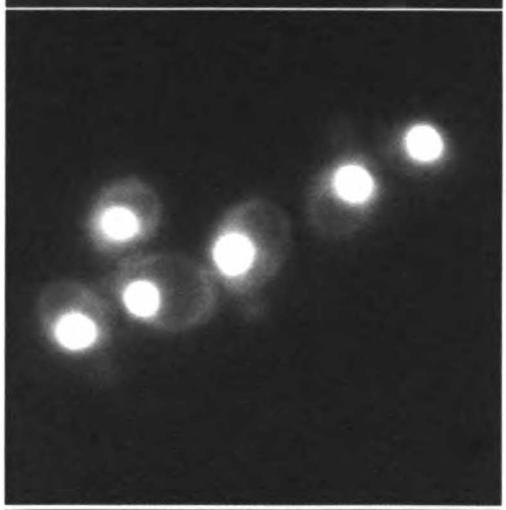

WT
RA8
ΔRS
- + + + Sky1-ProtAp
Coomassie
Radiolabel
1 2 3 4

Figure 3 Loss of Sky1p-mediated Phosphorylation Causes Cytoplasmic Accumulation of Npl3p. Localization of wild-type GFP-Npl3p (A,B) and mutant GFP-Npl3RA8p (C) in SKY1 (A,C) and Δsky1 (B) strains.

SKY1, GFP-Npl3



Δsky1, GFP-Npl3



SKY1, GFP-npl3S411A

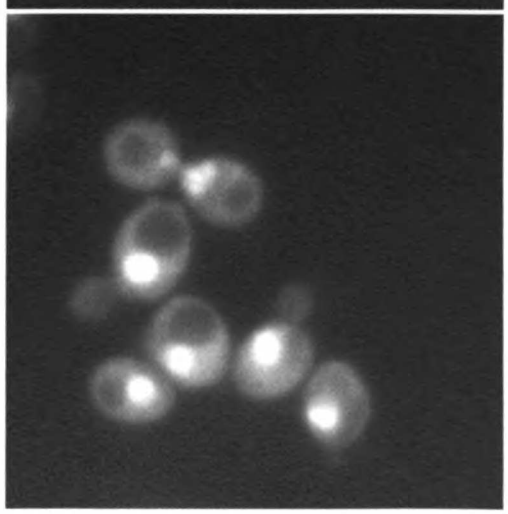


Figure 4 SKY1 Enhances Binding of Npl3p to Mtr10p in vivo. Whole cell extracts were prepared from wild-type (lane 2) and Δsky1 (lane 1) strains expressing ProtA-Mtr10p. Npl3p was co-immunoprecipitated with ProtA-Mtr10p by binding to IgG Sepharose. Proteins were detected by Western blotting with anti-Npl3p polyclonal antibodies. Note that the polyclonal antibodies also recognize the protein A tag on Mtr10p. Binding of Npl3p to ProtA-Mtr10p was reduced in a Δsky1 strain (lane 3) compared to WT (lane 4).

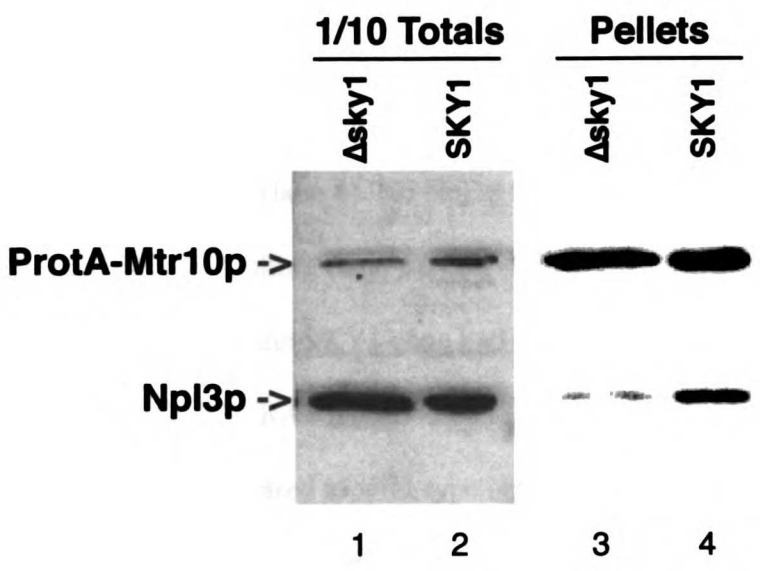
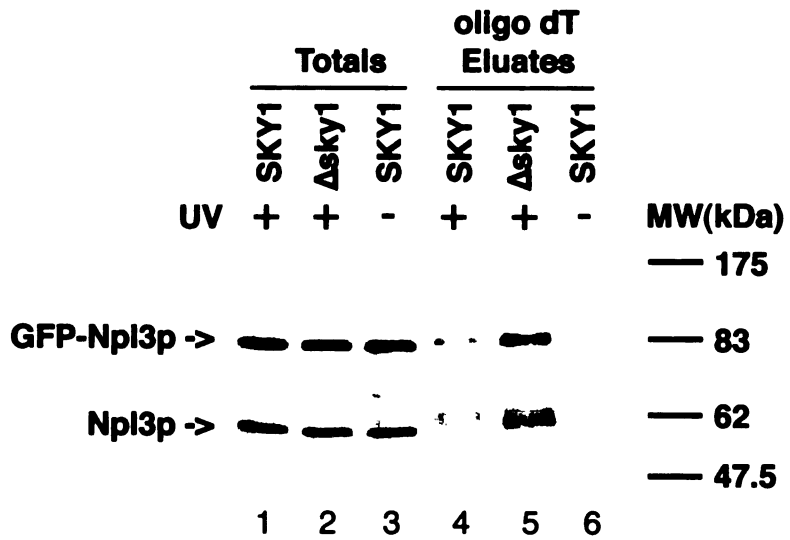
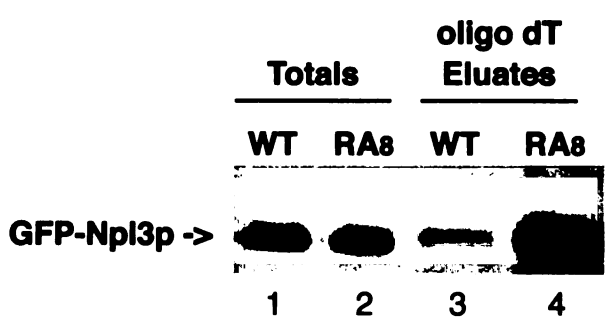

1/10 Totals
Pellets
Δsky1
SKY1
Δsky1
SKY1
ProtA-Mtr10p ->
Npl3p ->
1
2
3
4

Figure 5 Loss of Sky1p-mediated Phosphorylation Increases Binding of Npl3p to Poly(A)$^+$ RNA in vivo. Npl3p was cross-linked to RNA by mild UV-treatment of living cells, and poly(A)$^+$ RNA-protein complexes were purified on oligo dT cellulose. 1 A260 unit of purified RNPs was used for each eluate gel lane. Total lanes contain less than 0.1% of the starting material. **a**, Δsky1 vs. SKY1. Cross-linking to poly(A)$^+$ RNA was increased in a Δsky1 strain (lane 5) compared to SKY1 (lane 4). No Npl3p bound to the oligo dT cellulose in the absence of cross-linking (lane 6). GFP-Npl3p behaved identically to endogenous Npl3p. Deletion of the SKY1 gene had no effect total protein levels (compare lane 2 to lane 1). **b**, The RA8 mutation increases cross-linking of Npl3p to poly(A)$^+$ RNA (lane 4) compared to wild-type Npl3p (lane 3). The GFP-tagged wild-type (lane 1) and mutant (lane 2) proteins were present at similar levels in total cell extracts. **c**, Deletion of the MTR10 gene does not eliminate the effect of the RA8 mutation. RNPs were isolated from a Δmtr10 strain expressing either GFP-Npl3p or GFP-Npl3RA8p from a plasmid. The Npl3RA8 mutant protein was enriched in the oligo dT cellulose eluates (lanes 7 and 8) compared to wild-type GFP-Npl3p (lanes 5 and 6).

A


oligo dT
Totals
Eluates
SKY1
Δsky1
SKY1
SKY1
Δsky1
SKY1
UV + + - + + -
MW(kDa)
175
GFP-Npl3p ->
83
Npl3p ->
62
47.5
1 2 3 4 5 6

B


oligo dT
Totals
Eluates
WT
RA8
WT
RA8
GFP-Npl3p ->
1 2 3 4

C

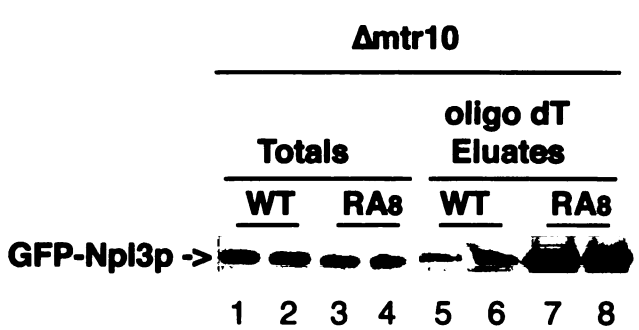
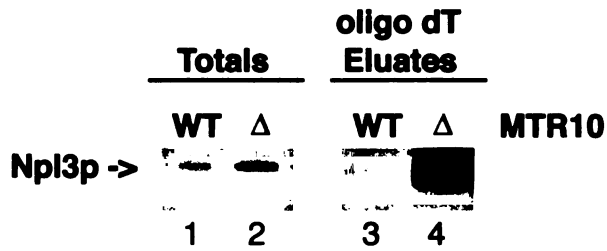

Δmtr10
oligo dT
Totals
Eluates
WT
RA8
WT
RA8
GFP-Npl3p ->
1 2 3 4 5 6 7 8

Figure 6 Mutations in MTR10 Increase Binding of Npl3p to Poly(A)$^+$ RNA in vivo. **a,** Crosslinking of Npl3p to poly(A)$^+$ RNA was increased in a Δmtr10 strain (lane 4) compared to WT (lane 3). Deletion of MTR10 resulted in a modest increase in total Npl3p protein levels (lane 2) compared to WT (lane 1). **b,** Crosslinking of Npl3p to poly(A)$^+$ RNA was increased in an mtr10-7 strain (lane 5) compared to WT (lane 4). No Npl3p bound to oligo dT cellulose in the absence of crosslinking (lane 6). Npl3p protein levels were slightly elevated in an mtr10-7 strain (lane 2) compared to WT (lane 1).

A


oligo dT
Totals
Eluates
WT
Δ
WT
Δ
MTR10
Npl3p ->
1
2
3
4

B

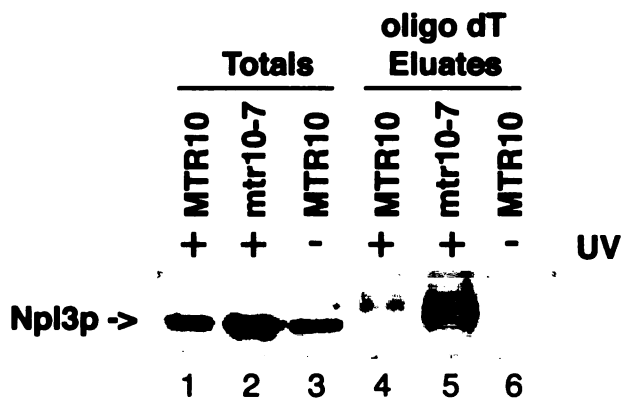
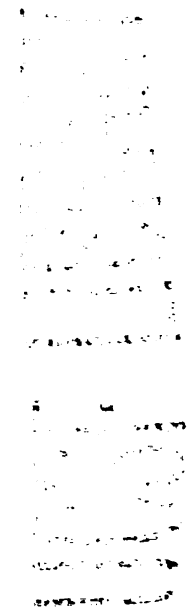
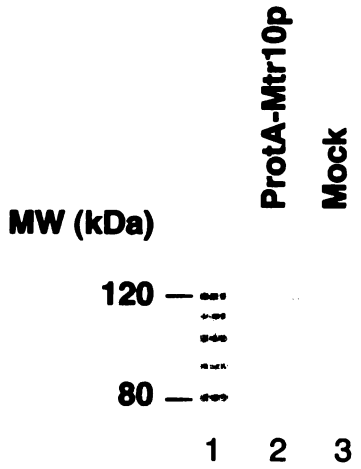

oligo dT
Totals
Eluates
+ MTR10
+ mtr10-7
- MTR10
+ MTR10
+ mtr10-7
- MTR10
UV
Npl3p ->
1
2
3
4
5
6

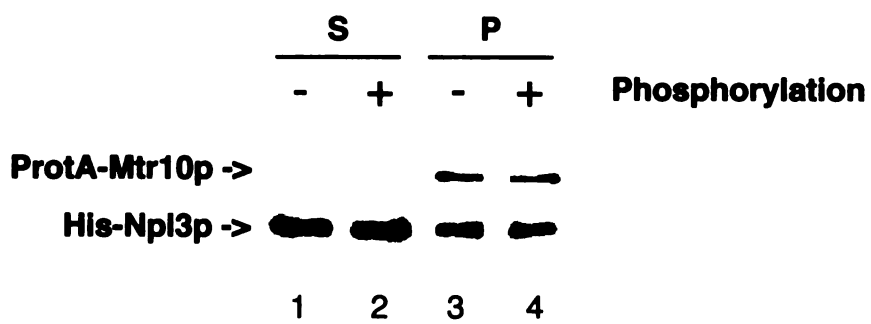
Figure 7 Phosphorylation of Npl3p Does Not Affect Binding to Mtr10p in vitro. a, ProtA-Mtr10p expressed from a low-copy plasmid was purified from yeast extract. A single band of ~120 kDa was detected on a silver-stained gel (lane 2). No band was detected in the same fraction purified from a yeast strain carrying an untagged MTR10 gene on a plasmid (lane 3). Molecular weight standards (10 kDa ladder) are resolved in lane 1. **b**, In vitro binding assay. Phosphorylated rHisNpl3p (lanes 2 and 4) and mock phosphorylated rHisNpl3p (lanes 1 and 3) were incubated with purified ProtA-Mtr10p at 4° C. Complexes were precipitated with IgG Sepharose, and the pellets (lanes 3 and 4) and supernatants (lanes 1 and 2) were analyzed by Western blotting with anti-Npl3p antibodies. **c**, Yeast extracts compete binding of ProtA-Mtr10p to phosphorylated and mock-phosphorylated rHisNpl3p alike. Addition of yeast protein from whole cell extract (15 μg, lanes 2 and 5; 60 μg, lanes 3 and 6) resulted in decreased binding of rHisNpl3p (compare lanes 2-3 to lane 1, lanes 5-6 to lane 2). Phosphorylated rHisNpl3p (lanes 1-3) and mock-phosphorylated rHisNpl3p (lanes 4-6) were competed equally.



A


ProtA-Mtr10p
Mock
MW (kDa)
120
80
1 2 3

B


S
P
- + - +
Phosphorylation
ProtA-Mtr10p ->
His-Npl3p ->
1 2 3 4

C

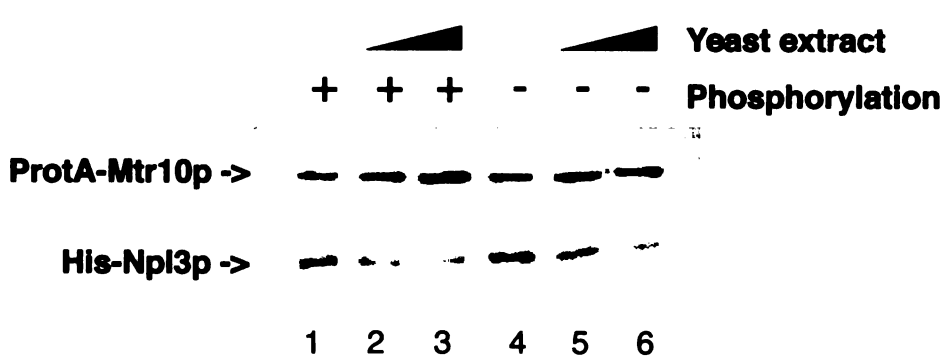
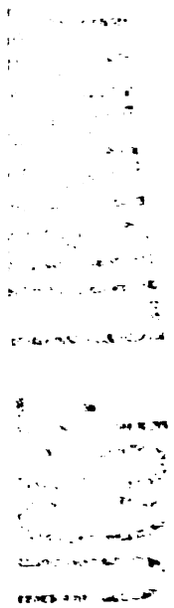
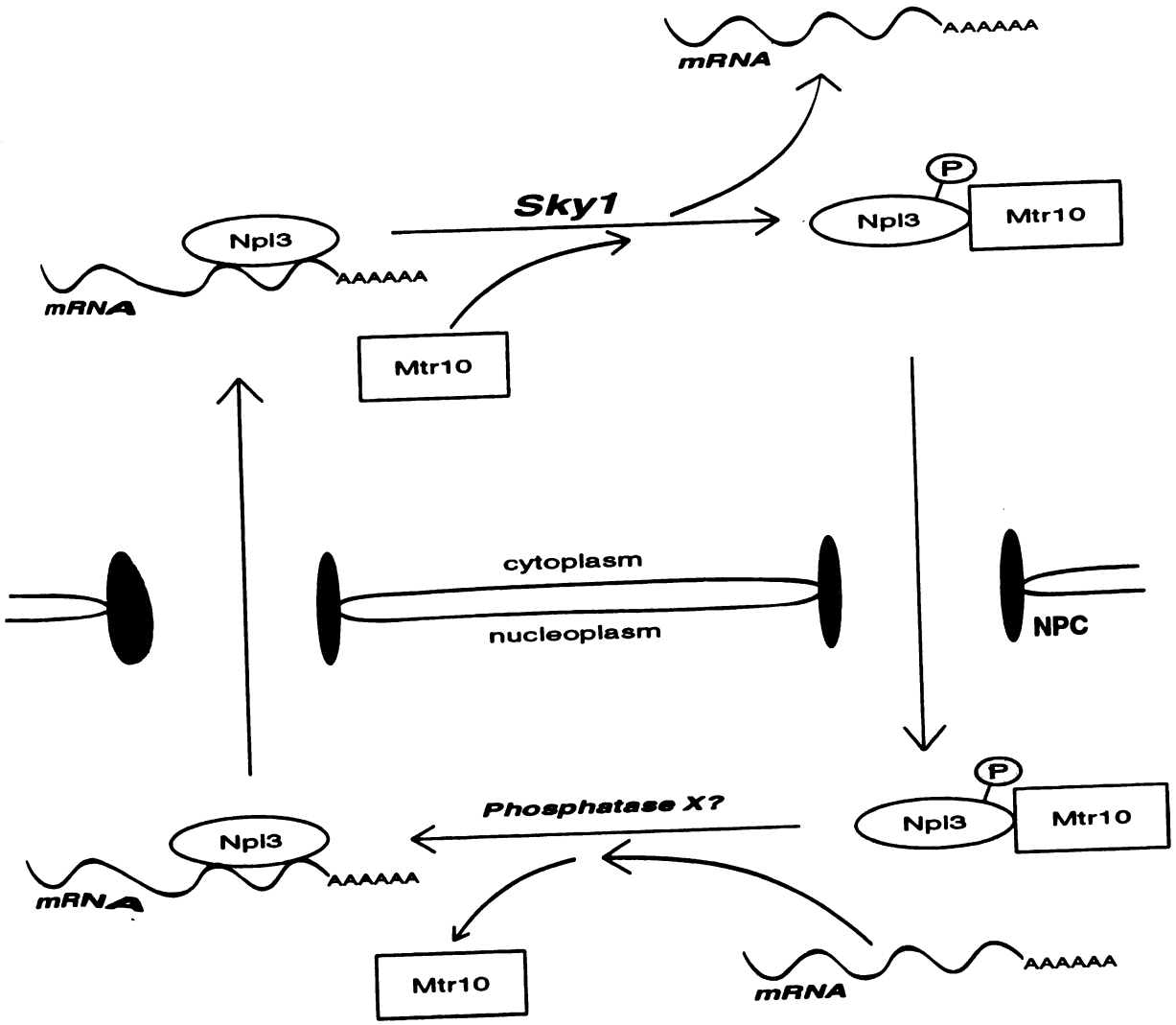

Yeast extract
+ + + - - -
Phosphorylation
ProtA-Mtr10p ->
His-Npl3p ->
1 2 3 4 5 6

Figure 8 Model: The Role of Phosphorylation in Npl3p's Nuclear Transport Cycle. In the cytoplasm, phosphorylation of Npl3p by Sky1p promotes the dissociation of Npl3p from exported RNPs. The disassembly of RNPs is facilitated by the binding of Mtr10p to Npl3p. Phosphorylated Npl3p is then targeted to the nucleus by Mtr10p. In the nucleus, Npl3p binds to newly-transcribed mRNA and dissociates from Mtr10p. This exchange of Npl3p's binding partners in the nucleus may be stimulated by dephosphorylation of Npl3p.




mRNA
AAAAAA
Sky1
P
Npl3
Mtr10
Npl3
AAAAAA
mRNA
Mtr10
cytoplasm
nucleoplasm
NPC
P
Npl3
Mtr10
Phosphatase X?
Npl3
AAAAAA
mRNA
Mtr10
mRNA
AAAAAA

Chapter 2

The Glc7p nuclear phosphatase promotes mRNA export by facilitating association of Mex67p with mRNA

SUMMARY

mRNA export is mediated by Mex67p:Mtr2p/NXF1:p15, a conserved heterodimeric export receptor which is thought to bind mRNAs through the RNA-binding adaptor protein Yra1p/REF. Recently, mammalian SR (serine/arginine-rich) proteins were shown to act as alternative adaptors for NXF1-dependent mRNA export. Npl3p is an SR-like protein required for mRNA export in S. cerevisiae. Like mammalian SR proteins, Npl3p is serine-phosphorylated by a cytoplasmic kinase. Here we report that this phosphorylation of Npl3p is required for efficient mRNA export. We further show that the mRNA-associated fraction of Npl3p is unphosphorylated, implying a subsequent nuclear dephosphorylation event. We present evidence that the essential, nuclear phosphatase Glc7p promotes dephosphorylation of Npl3p in vivo and that nuclear dephosphorylation of Npl3p is required for mRNA export. Specifically, recruitment of Mex67p to mRNA is Glc7p-dependent. We propose a model whereby a cycle of cytoplasmic phosphorylation and nuclear dephosphorylation of shuttling SR adaptor proteins regulates Mex67p:Mtr2p/NXF1:p15-dependent mRNA export.

INTRODUCTION

The compartmentalization of the eukaryotic cell requires macromolecular transport between the nucleus and cytoplasm. Messenger RNAs are synthesized in the nucleus and then exported to the cytoplasm through nuclear pore complexes (NPCs) that form aqueous channels spanning the nuclear envelope. mRNAs are generated from primary RNA polymerase II transcripts by multiple processing steps including 5' capping, splicing of introns, and cleavage and polyadenylation. Each of these processing steps results in addition of certain proteins to the mRNA-protein complex (mRNP) and removal of others (for review see(Dreyfuss et al., 2002)). Unlike small molecules that can diffuse freely through NPCs, mRNPs require association with specific NPC targeting proteins to facilitate their export.

A class of NPC targeting proteins known collectively as karyopherins directs most protein transport between the nucleus and cytoplasm. Karyopherins fulfill three necessary functions of transport receptors: 1) they bind cargo specifically; 2) they direct cargo through the NPC; and 3) they participate in mechanisms that dictate appropriate directionality of transport via coupling to the asymmetric distribution of RanGTP in the cell. The discovery of Xpo1/Crm1p, a karyopherin required for mRNA export in *S. cerevisiae* and for viral RNA export in HIV-infected cells, indicated that a similar mechanism could direct messenger RNA export (for review see (Fornerod and Ohno, 2002) and references therein). However, subsequent work suggests that the role of Xpo1/Crm1p in bulk mRNA export is limited (for review see (Fornerod and Ohno, 2002; Herold et al., 2003) and references therein).

An alternative model for the major mRNP export pathway has recently emerged, consisting of three key components conserved throughout eukarya: the Mex67p:Mtr2p/NXF1:p15 mRNA export receptor, Yra1p/ALY(REF1), an hnRNP-like adapter protein, and Sub2p/UAP56, a DECD-box putative RNA helicase (for review see (Reed and Hurt, 2002; Stutz and Izaurralde, 2003)). Although both Mex67p and NXF1 can be UV-crosslinked to poly(A)$^+$ RNA in vivo and bind RNA directly in vitro, NXF1 shows very low affinity for mRNA compared to CTE RNA and is thought to require RNA-binding adapter protein(s) to interact stably with mRNPs in vivo (Bachi et al., 2000; Braun et al., 1999; Katahira et al., 1999; Santos-Rosa et al., 1998). ALY/REF1 and its yeast counterpart Yra1p are thought to function as such adapters (Strasser and Hurt, 2000; Zenklusen et al., 2001) (Rodrigues et al., 2001; Stutz et al., 2000; Zhou et al., 2000) Recruitment of Yra1p and ALY/REF1 is thought to be promoted by the Sub2/UAP56 RNA-dependent ATPase and by specific transcription elongation factors (Luo et al., 2001; Strasser et al., 2002; Zenklusen et al., 2002).

Surprisingly, unlike NXF1 and UAP56, REF proteins are not essential for mRNA export in *Drosophila* cells or *C. elegans* (Gatfield and Izaurralde, 2002; Herold et al., 2003; Longman et al., 2003; MacMorris et al., 2003), suggesting that other adaptor proteins can chaperone mRNA through a NXF1-dependent export pathway. Some mutations in Yra1p that abolish binding to Mex67p in vitro reduce, but do not eliminate, association of Mex67p with mRNPs (Zenklusen et al., 2001), suggesting Yra1p-independent association of Mex67p with mRNPs may occur in yeast as well. Recent work by Steitz and colleagues showed that members of another class of abundant RNA-

binding proteins, the SR (serine/arginine-rich) family of splicing factors, can function as adapter proteins for NXF1-dependent mRNA export (Huang et al., 2003).

The discovery that certain SR proteins shuttle between the nucleus and cytoplasm raised the possibility of a role for this family of proteins in mRNA export (Caceres et al., 1998). Subsequently, two shuttling SR proteins, SRp20 and 9G8, were identified as proteins that specifically associate with a 22-nt RNA element found in the mouse histone H2a gene that is sufficient to promote export of intronless mRNAs in mammalian cells and Xenopus oocytes (Huang and Steitz, 2001). SRp20 and 9G8 can be UV cross-linked to polyadenylated RNAs in the nucleus and the cytoplasm (Huang and Steitz, 2001) and SRp20 and 9G8 bind directly to NXF1 (Huang et al., 2003), consistent with activity as general mRNA export adapter proteins.

Yeast mRNA export also requires an SR-like factor, Npl3p. Npl3p is an abundant RNA binding protein that is recruited cotranscriptionally to Pol II-transcribed genes and shuttles between the nucleus and the cytoplasm in association with polyadenylated mRNA ((Lee et al., 1996; Lei et al., 2001) and references therein). Npl3p has sequence similarity to mammalian SR proteins and, like related mammalian SR proteins, Npl3p is phosphorylated by a cytoplasmic SR protein kinase, Sky1p (Gilbert et al., 2001; Siebel et al., 1999; Yun and Fu, 2000). Cytoplasmic phosphorylation of Npl3p functions to promote the dissociation of Npl3p-RNA complexes in vivo: in the absence of Sky1p-mediated phosphorylation, Npl3p mislocalizes to the cytoplasm and accumulates bound to poly(A)$^+$ RNA (Gilbert et al., 2001). The asymmetric distribution of Sky1p in the cell could promote directionality in mRNA export by specifically disassembling exported

mRNP particles in the cytoplasm. This model predicts there will be a nuclear Npl3p phosphatase required for the assembly of export-competent mRNPs.

In this paper we report that wildtype poly(A)$^+$ RNPs contain predominantly unphosphorylated Npl3p, consistent with our model that phosphorylation promotes disassembly of these particles. We show that expressing the Sky1p kinase in the nucleus strongly inhibits growth and mRNA export, demonstrating the functional significance of Sky1p's observed nuclear exclusion. We present evidence that the essential, nuclear phosphatase Glc7p is required for dephosphorylation of mRNP-associated Npl3p in vivo and that Glc7p-function is required for mRNA export. Specifically, Glc7p promotes loading of Mex67p onto poly(A)$^+$ RNPs and facilitates dissociation of polyadenylated mRNA from the non-shuttling 3' end processing factor Rna15p. Finally, we show that a constitutively dephosphorylated Npl3p mutant increases association of Mex67p with mRNA in vivo, and recombinant Npl3p binds directly to Mex67p in vitro. We present a model for directional mRNA export governed by mRNP-remodeling events catalyzed by post-translational modification of Npl3p.

RESULTS

Npl3p is Unphosphorylated When Associated with Poly(A)$^+$ mRNA In Vivo

We have previously published evidence that phosphorylation of Npl3p by the cytoplasmic kinase Sky1p promotes dissociation of Npl3p from the exported mRNP, thereby providing one mechanism for efficiently terminating mRNA export, recycling

Npl3p, and promoting directional mRNA export via specific disassembly of the export particle in the cytoplasm (Gilbert et al., 2001). If phosphorylation by cytoplasmic Sky1p dissociates Npl3p from mRNA in vivo, poly(A)$^+$ mRNPs must contain unphosphorylated Npl3p. To assess the phosphorylation state of poly(A)$^+$-associated Npl3p in vivo, we isolated endogenous Npl3p-poly(A)$^+$ RNA complexes by UV-irradiating living cells, preparing total cell extracts under denaturing conditions, and purifying the crosslinked RNPs on oligo(dT) cellulose in the presence of phosphatase inhibitors. Purified RNPs were treated with RNase to liberate the protein components and analyzed by SDS-PAGE and Western blotting. Using an anti-Npl3p polyclonal antibody that recognizes both the phosphorylated and unphosphorylated forms of the protein, we detected Npl3p in both the total extract and the poly(A)$^+$-associated fraction (Fig. 1A, top panels). In contrast, using a polyclonal antiserum specific for the Ser411-phosphorylated form of Npl3p (Fig. 1B,C), we detected phosphorylated Npl3 protein in the total extract, but not in the poly(A)$^+$-associated fraction (Fig. 1A, lower panels). Thus Npl3p is unphosphorylated when bound to polyadenylated mRNA in wildtype cells in vivo.

Expressing Sky1p in the Nucleus Inhibits Growth and mRNA Export

If phosphorylation by Sky1p disassembles exported mRNPs, restriction of Sky1p to the cytoplasm should be important for cell growth and mRNA export. To test this prediction, we generated an inducible nuclear Sky1p by inserting the SV40 NLS at the amino terminus of the *SKY1* coding sequence under the control of the galactose-inducible GAL1 promoter. (Fig. 2A) As expected, the GAL1-NLS-Sky1p construct caused severe growth

inhibition on galactose media. GAL1 expression of cytoplasmic Sky1p had no effect on growth compared to the vector control. (Fig. 2B) Neither construct affected growth on glucose media, which represses expression from the GAL1 promoter (data not shown). Notably, the growth inhibition by NLS-Sky1p occurs in the presence of endogenous, cytoplasmic Sky1p. Thus the growth defect is due to a dominant effect of ectopically expressing Sky1p in the nucleus, and does not require depleting Sky1p from its normal site of function in the cytoplasm. We confirmed the nuclear localization of Sky1p in the GAL1-NLS-SKY1 strain and cytoplasmic localization of Sky1p in the GAL1-SKY1 strain by indirect immunofluorescence; immunoblotting showed equivalent expression of NLS-Sky1p and Sky1p in galactose media (data not shown). To determine whether mislocalization of Sky1p disrupts mRNA export, we examined poly(A)$^+$ RNA localization after growth for 6 hours in galactose media by in situ hybridization with an oligo(dT) probe. Expression of GAL1-NLS-SKY1 caused a striking accumulation of poly(A)+ RNA in the nuclei of >95% of cells (Fig. 2C). Thus, restriction of Sky1p activity to the cytoplasm is important for mRNA export and viability.

GLC7 is Required for Dephosphorylation of Npl3p In Vivo

Phosphorylation promotes nuclear import of Npl3p (Gilbert et al., 2001; Yun and Fu, 2000). The Npl3 protein that is subsequently associated with polyadenylated mRNA is unphosphorylated (Fig 1A); therefore, there should be a nuclear Npl3p phosphatase. In mammals, SR proteins can be dephosphorylated by PP1 phosphatase (Misteli and Spector, 1996). In *S. cerevisiae*, the essential gene *GLC7* encodes a PP1-type phosphatase that is predominantly nuclear (Bloecher and Tatchell, 2000; Feng et al., 1991).

Intriguingly, Glc7p co-purifies with a complex of proteins required for cleavage and polyadenylation of pre-mRNA (Gavin et al., 2002; Nedea et al., 2003; Walsh et al., 2002), but Glc7p is not required for processing of pre-mRNA transcripts in vitro (S. Roeck, M. Sadowski, B.Dichtl and W. Keller, personal communication). These characteristics prompted us to ask whether Glc7p could be an Npl3p phosphatase by examining the steady-state phosphorylation of Npl3 protein in *GLC7* wildtype and *glc7-5* temperature-sensitive mutants. Upon shift to 37°C, the *glc7-5* mutants showed increased levels of phosphorylated Npl3p compared to total Npl3p visualized by immunoblotting with anti-phospho Npl3p and anti-Npl3p respectively (Fig. 3A). Metabolic labeling of Npl3p in wildtype and *glc7* cells with ^{32}P corroborated these results (data not shown). Thus, we conclude that *GLC7* is required for efficient dephosphorylation of Npl3p in vivo.

Given the relatively modest effect of *glc7-5* on total Npl3p steady-state phosphorylation, we asked whether Glc7p activity specifically affected the phosphorylation state of the poly(A)$^+$ RNA-associated fraction of Npl3p. To address this question, we performed in vivo crosslinking experiments with wildtype and *glc7-5* cells. Cultures were grown to mid-log phase at 23°C followed by shift to 37°C for 30, 60, or 90 minutes before UV crosslinking and purification of poly(A)$^+$ RNA-associated protein on oligo(dT) cellulose as described above. We loaded equal poly(A)$^+$ RNA (normalized by OD_{260} units of purified RNPs) in each lane to permit quantitative comparison of the extent of protein-RNA association in wildtype and *glc7-5* mutant RNPs. Representative data from one 60-minute shift are shown in Fig. 3B; similar results were obtained after

30, 60, or 90 minutes at 37°C (data not shown). Two defects are evident in the *glc7-5* samples. First, we observed a quantitative increase in the amount of Npl3 protein associated with poly(A)$^+$ RNA (compare lanes 3 and 4, upper panels). Second, in striking contrast to the wildtype situation, most of the Npl3 protein bound to polyadenylated RNA in *glc7-5* cells was phosphorylated (compare lanes 3 and 4, lower panels to upper panels). Thus, *glc7-5* modestly increases total Npl3p phosphorylation (Fig. 3A) and dramatically increases phosphorylation of the poly(A)$^+$-associated fraction of Npl3p (Fig. 3B). These data suggest that Npl3p is normally dephosphorylated in a *GLC7*-dependent manner prior to, or coincident with, polyadenylation of the Npl3p-associated transcripts.

GLC7 is Required for mRNA Export

Accumulating evidence from many labs indicates a close coupling between 3' end processing and mRNA export (see Discussion). Glc7p is associated with the cleavage/polyadenylation machinery (Gavin et al., 2002; Nedea et al., 2003; Walsh et al., 2002) but is not required for transcript processing in vitro (S. Roeck, M. Sadowski, B.Dichtl and W. Keller, personal communication). This fact, combined with our observation that *glc7-5* mutants accumulate aberrant poly(A)$^+$ RNPs containing phosphorylated Npl3p, led us to examine the role of *GLC7* in mRNA export.

By in situ hybridization using an oligo(dT) probe, we compared the localization of poly(A)$^+$ RNA in wildtype and *glc7-5* mutants. In wildtype cells, the poly(A)$^+$ RNA is distributed throughout the cytoplasm (Fig. 3C, right panel). This is true for *glc7-5* cells grown at the permissive temperature of 23°C (Fig. 3C, left panel). Upon shift to 37°C,

glc7-5 cells rapidly accumulate poly(A)$^+$ RNA in punctate intranuclear foci (Fig. 3C, see inset). More than 50% of cells show nuclear accumulation of poly(A)$^+$ RNA after 15 minutes at 37°C. By 30 minutes, almost all cells show intranuclear accumulation and the intensity of the foci is increased compared to earlier times. The phenotype remains unchanged upon longer incubation at 37°C (data not shown).

This correlation between the accumulation of phosphorylated Npl3p bound to poly(A)$^+$ RNA and the inability to export mRNA is suggestive, but does not prove that failure to dephosphorylate Npl3p is solely responsible for the mRNA export defects of *glc7-5*. In an attempt to test this hypothesis directly, we asked whether preventing phosphorylation of Npl3p in the first place, by mutating serine 411 to alanine, could suppress the mRNA export defect of *glc7-5* mutants. We examined mRNA export in npl3S411A and *glc7-5* single and double mutants. Strikingly, mutation of the Npl3p phosphorylation site alone caused a strong constitutive mRNA export defect at all temperatures (Fig. 4A). Thus Npl3p phosphorylation is necessary not only for disassembly of the mRNP in the cytoplasm (Gilbert et al., 2001), it is also required for efficient mRNA export (see Discussion). The *glc7-5* npl3S411A double mutant showed nuclear accumulation of poly(A)$^+$ RNA in intranuclear dots indistinguishable from the *glc7-5* and npl3S411A single mutants (Fig. 4B). Thus our data clearly indicate a requirement for both Npl3p phosphorylation and Glc7p in mRNA export, but it remains an open question whether phosphorylated Npl3p is the only export-relevant target of Glc7p.

GLC7 is Required for Association of Mex67p with Poly(A)$^+$ RNA In Vivo

We sought to understand the nature of the *glc7-5* mRNA export defect by further characterizing the aberrant mRNPs that accumulate in *glc7-5* at the non-permissive temperature. A widely accepted model for mRNA export involves the sequential recruitment of three conserved factors to mRNAs: Sub2p/UAP56, Yra1p/ALY, and Mex67p/NXF1 (for review see (Reed and Hurt, 2002)). To further explore the molecular role of Glc7p in mRNA export, we examined the effects of *glc7-5* on the association of these essential mRNA export factors with poly(A)$^+$ mRNA in vivo. UV crosslinked poly(A)$^+$ mRNPs were purified from shifted cultures, and analyzed as described above. Equal OD_{260} units of purified RNPs were loaded in each lane to compare wildtype and *glc7-5* mutant cells. We were unable to detect Sub2p or Yra1p in the poly(A)$^+$ RNP fractions from either wildtype or *glc7-5* cells (data not shown), although these proteins accumulate with poly(A)$^+$ RNA in other export mutants (M. Lund, J. Pan, and C. Guthrie, unpublished observations). Mex67p has previously been shown to crosslink to poly(A)$^+$ RNA in vivo (Segref et al., 1997). When we examined crosslinking of Protein A-tagged Mex67p in *glc7-5* and *GLC7* strains, we observed a dramatic decrease in Mex67p crosslinking in *glc7-5* cells shifted to 37°C (Fig. 5A, lanes 3,4). This decrease in poly(A)$^+$- associated Mex67p was paralleled by a decrease in total Mex67 protein in the extract (Fig. 5A, lanes 1,2). These results show that loss of Glc7p activity causes both a loss of association of Mex67p with poly(A)$^+$ RNA in vivo, and a decrease in total Mex67

protein. In principle, either could be the primary defect, and be sufficient to explain the block to mRNA export we observed in *glc7-5* at 37°C.

To determine whether decreased Mex67 protein levels are sufficient to explain the reduced association of Mex67p with poly(A)$^+$ RNA and the block to mRNA export in *glc7-5* at 37°C, we transformed *glc7-5* and wildtype cells with a high-copy *MEX67-GFP* plasmid. The presence of 2μm *MEX67-GFP* resulted in identically high levels of tagged Mex67 protein in *glc7-5* and wildtype cells shifted to 37°C(Fig. 5B, compare lanes 1 and 2). We confirmed that high-copy Mex67-GFP localizes to the nuclear rim in both *GLC7* and *glc7-5* cells at 37°C (data not shown). Thus the presence of a high-copy *MEX67* plasmid is sufficient to overcome the loss of Mex67p in *glc7-5*. We then tested whether restoring Mex67p levels was sufficient to restore Mex67p crosslinking to poly(A)$^+$ RNA. Results shown in Figure 5B clearly show a dramatic defect in association of Mex67-GFP with poly(A)$^+$ RNA in *glc7-5*. Hrp1p crosslinking is unaffected by *glc7-5* (lower panel). Thus, *glc7-5* blocks Mex67p crosslinking independent of effects on Mex67p levels. Finally, we tested the effect of 2μm *MEX67* on mRNA export. As shown in Figure 5C, 2μm *MEX67* does not suppress the mRNA export defect of *glc7-5*. Thus we conclude that Glc7p is required for recruitment of Mex67p to poly(A)$^+$ RNA.

GLC7 is Required for Release of Poly(A)$^+$ RNA from Rna15p In Vivo

In principle, Glc7p could promote association of Mex67p with mRNPs directly, by stimulating binding, or indirectly, by releasing mRNPs from a tether that prevents their binding to Mex67p. One possible tether is the 3' processing machinery itself (see

Discussion). The complex of proteins required for cleavage and polyadenylation includes non-shuttling proteins (Hammell et al., 2002); it is not known how they are removed from the polyadenylated message prior to export. Of particular interest was the RNA-binding component of CF1A, Rna15p, which efficiently crosslinks to unprocessed substrate RNA in vitro but does not shuttle in vivo (Gross and Moore, 2001; Hammell et al., 2002). We examined association of Rna15p with poly(A)$^+$ RNA in vivo by UV crosslinking. In wildtype cells, Rna15p is barely detectable in the poly(A)$^+$ RNA fraction (Fig. 5D, lane 4), suggesting that mRNA is rapidly released from the 3' end processing machinery once polyadenylation is complete. In striking contrast to the wildtype scenario, *glc7-5* cells showed dramatically increased crosslinking of Rna15p to poly(A)$^+$ RNA at 37°C (Fig. 5D, lane 3).

Unphosphorylated Npl3p Binds to Mex67p In Vitro and Tethers Mex67p to mRNA In Vivo

The hypothesis that dephosphorylation of Npl3p by Glc7p directly stimulates binding of Mex67p to mRNPs predicts a direct protein-protein interaction between Npl3p and Mex67p. To test this, we purified Mex67p from yeast and incubated with recombinant His-Npl3p in the presence of BSA as a competitor. Mex67p bound to rHis-Npl3p and not to beads alone (Fig. 6A, compare lane 3 to lane 4). Thus Npl3p binds directly to Mex67p and could function as an adaptor protein linking Mex67p to RNA in vivo. This finding conflicts with earlier reports (Strasser and Hurt, 2000), as we discuss below.

Both Glc7p and Sky1p could have multiple targets with functions in mRNA export. Thus, the effects of *glc7-5* on association of Mex67p with poly(A)$^+$ RNA might not be directly related to its effects on the phosphorylation state of Npl3p. If it is dephosphorylation of Npl3p that promotes binding of Mex67p to RNA, then constitutive dephosphorylation of Npl3p in the npl3S411A mutant should also increase association of Mex67p with mRNA. To test this, we performed in vivo crosslinking experiments as described above. Mutation of Npl3p's phosphorylation site serine411 to alanine caused a striking increase in crosslinking of Mex67p to poly(A)$^+$ RNA (Fig. 6B, upper panel, compare lane 4 to 3) as well as an increase in crosslinking of Npl3p, as previously reported (Fig. 6B, middle panel (Gilbert et al., 2001)). Hrp1p crosslinking was unaffected (Fig. 6B, lower panel). These data show that association of Mex67p with mRNA is specifically affected by the phosphorylation state of Npl3p.

DISCUSSION

We showed previously that the cytoplasmic kinase Sky1p promotes disassembly of mRNPs by phosphorylating the shuttling SR/hnRNP protein Npl3p. This finding led us to propose a mechanism for enhancement of directional mRNA export via specific disassembly of the exported mRNP by phosphorylation of Npl3p in the cytoplasm (Gilbert et al.). In the present paper we show that exclusion of the Npl3p kinase Sky1p from the nucleus is important for mRNA export and cell growth, as expected for a cytoplasmic disassembly factor. We provide evidence that Npl3p is normally dephosphorylated in the nucleus, and that dephosphorylation of poly(A)$^+$ RNA-

associated Npl3p requires the essential nuclear phosphatase Glc7p. Furthermore, *GLC7* is required for mRNA export in yeast. Specifically, our data indicate that a Glc7p-dependent event is required for efficient recruitment of the mRNA export receptor Mex67p to polyadenylated mRNPs. This event is likely to be dephosphorylation of Npl3p: We show that unphosphorylated rNpl3p binds to purified Mex67p in vitro, and a mutation that eliminates phosphorylation of Npl3p increases association of Mex67p with mRNA in vivo.

Both Phosphorylation and Dephosphorylation of Npl3 are Required for mRNA Export

We found that, at steady-state, the majority of Npl3p associated with polyadenylated mRNA in wildtype cells is unphosphorylated. As Npl3p has been shown to associate with the 5' ends of actively transcribed genes (Lei et al., 2001), it is likely that Npl3p is incorporated into the nascent RNP prior to formation of the poly(A) tail. These data suggest that Npl3p is normally dephosphorylated prior to, or coincident with, polyadenylation of the Npl3-associated transcripts. Nuclear phosphorylation *per se* does not prevent association of Npl3p with mRNPs since, in *glc7-5* mutant cells, phosphorylated Npl3p accumulates in complexes with polyadenylated RNA. In fact, phosphorylation of Npl3p is required for efficient mRNA export. Mutation of serine 411 to alanine, which eliminates phosphorylation of Npl3p in vivo, causes a constitutive mRNA export defect. Previously we showed that cytoplasmic phosphorylation of Npl3p by Sky1p is important for release of Npl3p from exported mRNPs and recycling of Npl3p to the nucleus (Gilbert et al., 2001). However, we do not believe that depletion of Npl3p

from the nucleus due to slowed recycling is solely responsible for the mRNA export defect of npl3S411A. We examined the effect of Npl3p mislocalization, independent of any perturbation of Npl3p phosphorylation, using a temperature-sensitive mutation of the Npl3p import receptor, Mtr10p (Senger et al., 1998). The mtr10-7 mutation causes modest depletion of Npl3p from the nucleus at the permissive temperature of 25°C, comparable to the mislocalization seen with npl3S411A, yet mtr10-7 cells do not show mRNA export defects at this temperature (W. G. and C. G., unpublished observations). Thus, we suggest that efficient mRNA export requires the presence of phosphorylated Npl3p in the nucleus.

What could be the nuclear requirement for phosphorylated Npl3p? In mammals, phosphorylation of RS domains is required for recruitment of SR proteins to sites of active transcription (Misteli et al., 1998) and phosphorylated mammalian SR proteins bind to RNA pol II via the CTD of the large subunit independent of RNA (Kim et al., 1997). Perhaps the unphosphorylatable npl3S411A protein is inefficiently recruited to nascent transcripts, thereby causing inefficient mRNA export. Intriguingly, the npl3E409K mutation, which likely reduces Npl3p phosphorylation in vivo (Yun and Fu, 2000), is synthetically lethal with a mutation in TATA-binding protein and the double mutant has an mRNA export defect (Lei et al., 2001), consistent with the hypothesis that phosphorylation facilitates co-transcriptional recruitment of Npl3p.

Mechanisms Underlying Glc7p-dependent Recruitment of Mex67p

Glc7p is dispensable for the chemistry of cleavage and polyadenylation (S. Roeck, M. Sadowski, B.Dichtl and W. Keller, personal communication), yet it is bound to the 3' end

processing machinery in vivo (Gavin et al., 2002; Nedea et al., 2003; Walsh et al., 2002) and required for mRNA export. These properties make Glc7p an attractive candidate for a regulatory factor linking completion of 3' end processing to mRNA export.

Accumulating evidence from many labs supports the idea that 3' processing and export are closely coupled. Mutations in factors required for 3' end processing result in the accumulation of mRNA export reporter transcripts in the nucleus and prevent translation of heat-shock messages in the cytoplasm (Brodsky and Silver, 2000; Dower and Rosbash, 2002; Hilleren et al., 2001). Recently, special alleles of 3' processing factors (rna14, rna15, pcf11, pap1) were identified in yeast which, unlike canonical alleles, produce polyadenylated mRNA but are unable to export it (Hammell et al., 2002), consistent with additional roles for these factors in direct coupling between 3' pre-mRNA processing and mRNA export. Similarly, mutation of the *cis* signals for cleavage and polyadenylation cause nuclear retention of unprocessed transcripts in yeast and metazoans (Custodio et al., 1999; Hammell et al., 2002; Long et al., 1995). Notably, 3' end formation directed by either a T7 terminator or *cis*-ribozyme cleavage rather than the endogenous 3' processing machinery is not sufficient for transcript export (Dower and Rosbash, 2002; Huang and Carmichael, 1996), nor did insertion of a stretch of poly(A) immediately upstream of the ribozyme cleavage site promote export (Huang and Carmichael, 1996). This finding strongly suggests that, in addition to the modification of the pre-mRNA transcript, the 3' processing machinery produces changes in the RNP that are required for export. Based on our results, we propose that Npl3p is recruited to nascent transcripts in the phosphorylated state (as discussed in the preceding section) and dephosphorylated by Glc7p coincident with maturation of the transcript, thereby providing an attractive

mechanism for linking completion of 3' end processing to mRNA export. Important work for the future will be to determine precisely how the activity of Glc7p is regulated.

What is the Glc7p-dependent event in mRNA export? We observed decreased association of Mex67p with poly(A)$^+$ RNA in *glc7-5* cells at the non-permissive temperature. One straightforward hypothesis is that Npl3p directly tethers Mex67p to mRNPs, and this binding requires dephosphorylation of Npl3p. Consistent with this model, we observed direct binding of Mex67p to rNpl3p in vitro. This is in contrast to results reported by Hurt and colleagues (Strasser and Hurt, 2000). One possible explanation lies in the source of Npl3p: in their experiments, they used GST-Npl3p purified from *S. pombe*, whereas we used *E. coli*-expressed His-Npl3p. *S. pombe* contains a *SKY1* homologue, *DSK1* (Tang et al., 1998); the GST-Npl3p is likely to be phosphorylated. Our observation that mutation of the Npl3p phosphorylation site is sufficient to increase association of Mex67p with mRNA in vivo argues that Npl3p is a major export-relevant substrate of Glc7p. However, other targets may contribute to the export defect in *glc7-5*. In particular, Gpb2p is another shuttling SR-like phosphoprotein implicated in mRNA export (Windgassen and Krebber, 2003) whose activity may be regulated by Glc7p. Gpb2p binds to the TREX complex that is thought to couple transcription to Mex67p:Mtr2p-dependent export (Strasser et al., 2002).

Alternatively, Glc7p may be required for release of mRNPs from a nuclear tether that prevents association of Mex67p. Recent work suggests that the release of mRNPs from the site of transcription is a regulated event, sensitive to correct 3' processing of the pre-mRNA transcript (for review see (Jensen et al., 2003; Stutz and Izaurralde, 2003; Vasudevan and Peltz, 2003)). A possible Glc7p-sensitive nuclear tether could be the 3'

processing machinery itself. Components of the cleavage and polyadenylation machinery bind tightly and specifically to RNA elements upstream of the cleavage site (for review see (Zhao et al., 1999). Many of these proteins, including Rna14p, Rna15p, Clp1p, and Pcf11p, do not shuttle (Hammell et al., 2002) and must presumably be dissociated from the mature transcript for mRNP export to occur. The mechanism for this dissociation of factors is not known. However, based on the observation that phosphatase treatment of extract inhibits processing in vitro, it has been suggested that cycles of phosphorylation may be involved (Zhao et al., 1999). Intriguingly, we observed a dramatic increase in crosslinking of Rna15p to polyadenylated mRNA in *glc7-5*. Our data do not address whether this effect is downstream of failure to dephosphorylate Npl3p in *glc7-5*, but it is worth noting that a temperature-sensitive *rna15* mutant suppresses the mRNA export defect of *npl3-1* (Henry et al., 1996), suggesting that Rna15p and Npl3p may act antagonistically in mRNA export. In one appealing model, dephosphorylation of Npl3p would favor displacement of Rna15p from the mRNA, thereby releasing the mRNP to bind to Mex67p for export.

A Cycle of Localized Phosphorylation and Dephosphorylation of Shuttling SR Proteins Could Promote Directionality in mRNP Export

Compartment-specific assembly and disassembly of receptor-cargo complexes determines directionality of karyopherin-mediated protein transport. It is not known how directionality of mRNP export is achieved. Unlike karyopherins, Mex67p and NXF1 do not bind Ran and Mex67p/NXF1-dependent transport was presumed to be insensitive to the Ran gradient (see (Reed and Hurt, 2002), and references therein). Our results indicate

that a nuclear Npl3p phosphatase promotes formation of export-competent mRNPs and a cytoplasmic Npl3p kinase promotes their disassembly. The localization of competing mRNP-modifying enzymes to different subcellular compartments could function in a manner related to the asymmetric distribution of Ran effectors. A similar mechanism of localized phosphorylation/dephosphorylation has been shown to promote directionality in U snRNA export in higher eukaryotes (Ohno et al., 2000). While Npl3p has long been known as an important mediator of mRNA export in yeast, the general requirement for shuttling SR proteins in eukaryotic mRNA export has only recently been recognized. Because many of the kinases and phosphatases that modify SR proteins are conserved throughout eukarya, we expect that further analysis of the molecular consequences of localized phosphorylation and dephosphorylation of Npl3p will illuminate the conserved role of shuttling SR proteins in Mex67p/NXF1-dependent mRNA export.

EXPERIMENTAL PROCEDURES

Yeast Strains, Primers, and Plasmids

All *S. cerevisiae* strains are in the w303 background. *npl3S411A::kanMX6* mutants were generated by the method of (Longtine et al., 1998) using primers OWG63 5'-AGAACCAGAGATGCTCCACGTGAAAGAGCACCAACCAGGTGAGGCGCGCCACTTCTAAA-3' and OWG54 5'-TGTTTTCCTTTTTCATTTGTTCTCAGTCTCATATTTAAGGAATTCGAGCTCGTTTAAAC-3' resulting in integration of the S411A mutation at the *NPL3* locus, followed by the ADH1 terminator and the *kanMX6* module (Longtine et al., 1998). *NPL3::kanMX6*

control strains were created using the same OWG54 reverse primer and OWG62 5'-AGAACCAGAGATGCTCCACGTGAAAGATCACCAACCAGGTGAGGCGCGCCACTTCTAAA-3'. The GAL1-SKY1 plasmid was generated by PCR subcloning the SKY1 ORF into pEB1428 (pRS426-GAL1 promoter-TAP tag). pEB1428 was a gift from Erin O'Shea and will be described elsewhere. The NLS was introduced into GAL1 -SKY1 by PCR with OWG60 5'-CCGGGATCCATGGCTCCAAAGAAGAAGAGAAAGGTCGGTTCATCAATTACTATCCTGGG-3' and OWG61 5'-GGTTGCGACCCTGTGATAATTG-3'. *glc7-5* (w303 *MATa glc7::LEU2 trp1::glc7-5::TRP1*) and *GLC7* (w303 MATa *glc7::LEU2 trp1::GLC7::TRP1*) strains were obtained from Michael Stark (Andrews and Stark, 2000).

Isolation and Characterization of UV-crosslinked Poly(A)$^+$ RNPs

In vivo crosslinking, crosslinked extract preparation, and poly(A)$^+$ RNP purification were performed as described previously (Gilbert et al., 2001), with the addition of phosphatase inhibitors (160 mM β–glycerophosphate, pH 7.4, 10 mM NaF, 100 nM calyculin A) as indicated. Temperature shifts were performed by mixing 500 mls of 49°C media with 500 mls of 25°C yeast culture, OD_{600} 0.5-1.0, before transfer to a 37°C incubator. Shifted cultures were rapidly chilled in ice water for 10 min before UV treatment. Protein concentration in total extracts was determined using the BCA Protein Assay Kit (Pierce) according to manufacturer's instructions. Blots were stained with amido black to confirm equal protein loading in total lanes. Purified poly(A)$^+$ RNPs were analyzed by SDS-

PAGE and immunoblotting with the following antibodies: anti-Npl3p (1:2,000), anti-Phospho-Npl3p (1:1,000), rabbit anti-mouse IgG (1:1,000) (Pierce), anti-Mex67p (1:500) (Segref et al., 1997), anti-GFP (1:1,000) (Roche), anti-Hrp1 (1:1,000) (gift of M. Swanson) and anti-Rna15p rabbit polyclonals (1:10,000) (Amrani et al., 1997).

Preparation of Yeast Whole Cell Extracts and In Vitro Phosphorylation

For preparation of non-crosslinked whole-cell extracts, cells from10 mls of mid-log phase culture were lysed by vortexing with glass beads in HSB (10% glycerol, 25 mM Hepes, pH 7.9, 300 mM NaCl, 0.5 % NP-40, 2 mM EDTA, 10 mM NaF, 160 mM β–glycerophosphate, pH 7.4, 1 mM DTT, 2 mM PMSF, 2.5 mM Benzamidine, 1 μg/ml pepstatin, 1 μg/ml leupeptin, and 100 nM calyculin A). Preparation of recombinant Npl3p and in vitro phosphorylation with purified Sky1p was performed as described (Gilbert et al., 2001) except that soluble Sky1p was prepared from GAL1-SKY1-TAP expressing cells by tandem affinity purification as described by Seraphin and colleagues (Gavin et al., 2002): www-db.embl-heidelberg.de/jss/servlet/de.embl.bk.wwwTools.GroupLeftEMBL/ExternalInfo/seraphin/TAP.html.

Purification of Mex67p and In Vitro Binding to rNpl3p

Mex67p was purified from yeast expressing Mex67-TEV-ProteinA by binding to IgG Sepharose, followed by extensive washing, and elution with TEV protease as described by Seraphin and colleagues (see above). Mex67p was separated from His-tagged TEV protease by incubation with Ni-NTAgarose (Qiagen). The supernatant containing purified

Mex67p was concentrated in a centrifugal filter device (Millipore). Each 200 µl binding reaction contained ~ 0.1 µg purified Mex67p, 5 µl Ni-NTA beads, 2 µg BSA as a non-specific competitor, +/- 1 µg rHis-Npl3p in 200 µl binding buffer (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1% NP-40, 2 mM PMSF, 2.5 mM Benzamidine, 1 µg/ml pepstatin, 1 µg/ml leupeptin), incubated for 2 hours at 4°C. Beads were washed 4 times in binding buffer and bound proteins eluted with SDS sample buffer.

In Situ Hybridization

Cells were grown in YPD or appropriate dropout media at 25°C to an OD_{600} of 0.1-0.3. Where indicated, cultures were rapidly shifted to 37°C by immersion in a 37°C water bath before transfer to a 37°C incubator. Poly(A)$^+$ RNA was localized in fixed cells using a digoxygenin-tailed oligo(dT)50 probe and FITC-conjugated monoclonal anti-digoxygenin antibody (1:10) (Roche) as described (de Bruyn Kops and Guthrie, 2001).

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Figure 1. Poly(A)$^+$ RNA-associated Npl3p is Unphosphorylated

(**A**) Npl3p was crosslinked to RNA by UV irradiation of living cells and poly(A)$^+$ RNA-protein complexes were purified on oligo(dT) cellulose. Western blotting with anti-Npl3p antibodies (1:2,000) reveals association of Npl3p with poly(A)$^+$ RNA, whereas anti-Phospho-Npl3p (1:1,000) detects phosphorylated Npl3p in the total extract but not in the poly(A)-associated fraction.

(**B**) In vitro phosphorylation of recombinant Npl3p with purified Sky1p results in recognition by anti-Phospho-Npl3p on a Western blot (left lanes). Unphosphorylated rNpl3p is recognized by anti-Npl3p but not by anti-Phospho-Npl3p (right lanes).

(**C**) Mutation of Npl3p Ser411 to Ala abolishes phosphorylation of Npl3p in vivo and prevents recognition of Npl3p by anti-Phospho-Npl3p. Whole-cell extracts were prepared from wildtype and S411A mutants. Proteins were detected by Western blotting with anti-Npl3p (upper panels) or anti-Phospho-Npl3p (lower panels).

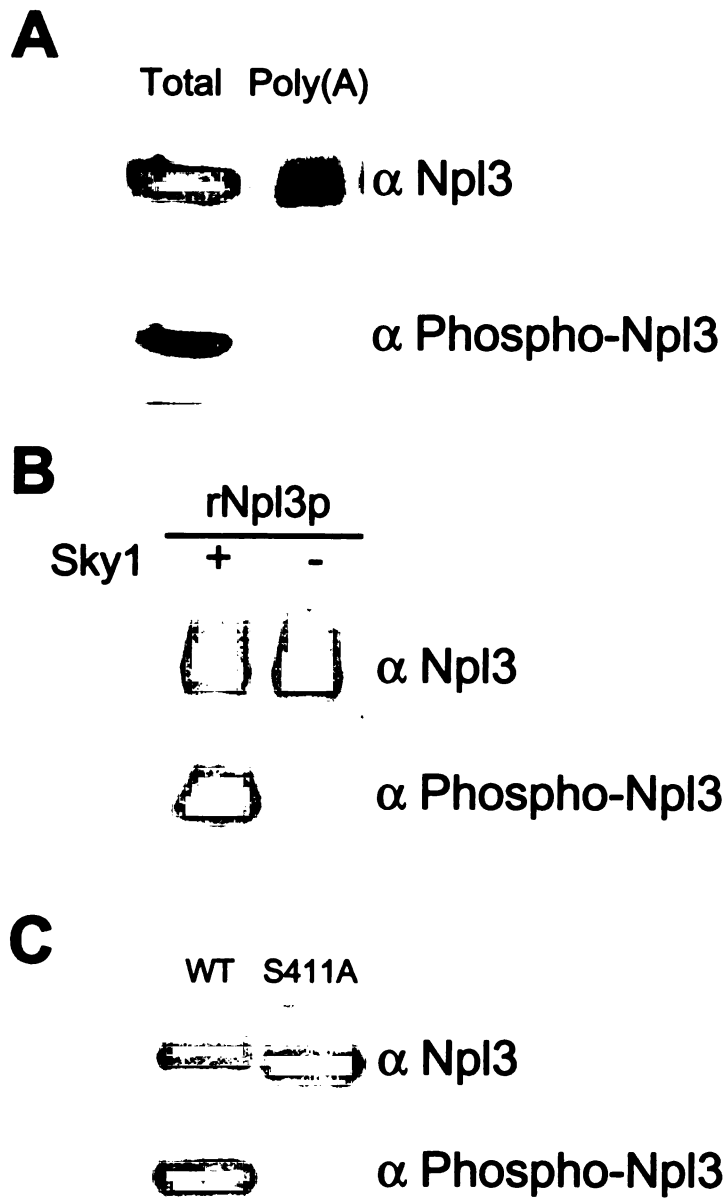

A
Total
Poly(A)
α Npl3
α Phospho-Npl3
B
rNpl3p
Sky1
+
-
α Npl3
α Phospho-Npl3
C
WT
S411A
α Npl3
α Phospho-Npl3

Figure 2. Nuclear-mislocalized Sky1p Inhibits Growth and mRNA Export

(**A**) Domain structure of the GAL1-(NLS)-SKY1 constructs.

(**B**) Two independent transformants of each construct (GAL1-vector, GAL1-SKY1, and GAL1-NLS-SKY1) were recovered on SD-Ura, then streaked for single colonies and grown on SGal-Ura for 3 days at 30°C.

(**C**) Poly(A)$^+$ RNA was visualized in galactose-grown cells transformed with GAL1-NLS-SKY1 or the GAL1-vector by in situ hybridization with an oligo(dT)$_{50}$ probe. The same cells stained with DAPI to visualize DNA are shown in the lower panels.

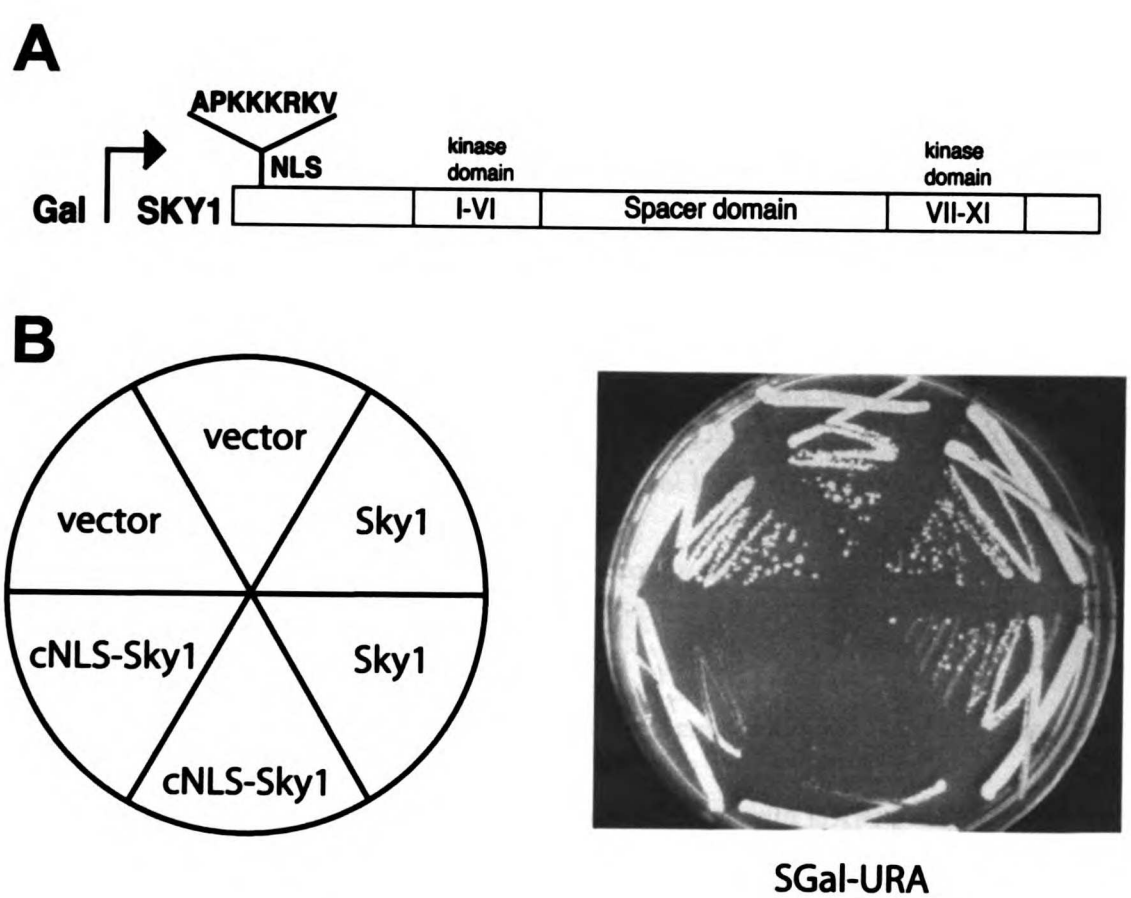

A
APKKKRKV
NLS
kinase domain
kinase domain
Gal
SKY1
I-VI
Spacer domain
VII-XI
B
vector
vector
Sky1
cNLS-Sky1
Sky1
cNLS-Sky1
SGal-URA
C
NLS-Sky1
vector
Poly(A)
DAPI

Figure 3. Glc7p Dephosphorylates Npl3p In Vivo and Promotes mRNA Export

(**A**) Immunoblotting with anti-Phospho-Npl3p (lower panels) and anti-Npl3p (upper panels) of total extracts prepared from wildtype and *glc7-5* cells shifted to 37°C for 60 min.

(**B**) Poly(A)$^+$ RNPs prepared from *glc7-5* and wildtype cells shifted to 37°C for 60 min were analyzed by immunoblotting with anti-Npl3p and anti-Phospho-Npl3p. Poly(A) lanes each contain 1.0 OD_{260} units of purified RNPs. Total lanes were normalized by extract volume; the *glc7-5* extract contains less total protein and therefore less phospho-Npl3p.

(**C**) Localization of poly(A)$^+$ RNA in wildtype and *glc7-5* cells was visualized by in situ hybridization with an oligo$(dT)_{50}$ probe. The same cells stained with DAPI to visualize DNA are shown in the lower panels. The insets are magnified 2.5X.

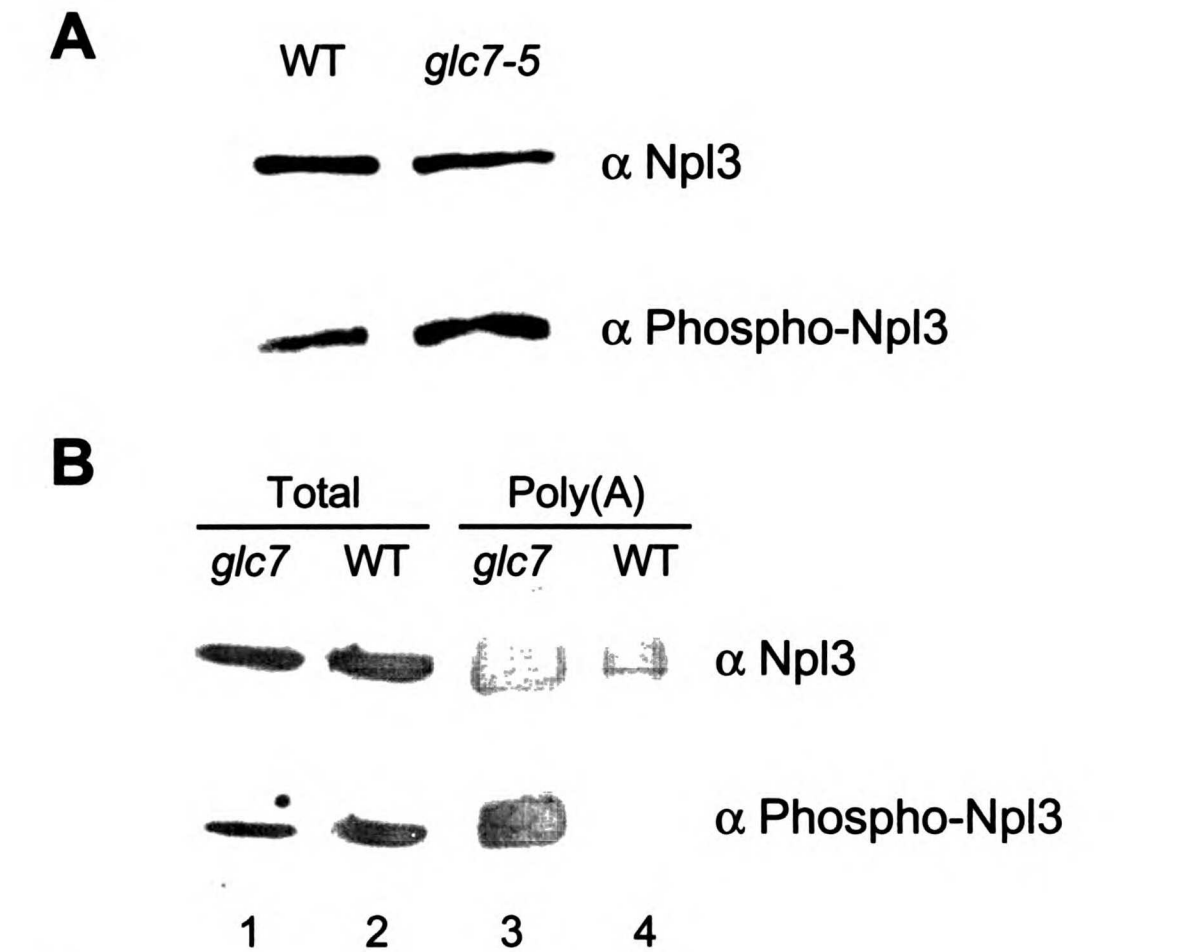

A
WT
glc7-5
α Npl3
α Phospho-Npl3
B
Total
Poly(A)
glc7
WT
glc7
WT
α Npl3
α Phospho-Npl3
1
2
3
4
C
glc7-5
Wild Type
23°C
37°C 15
37°C 30
37°C 30
Poly(A)
DAPI

Figure 4. Constitutive Dephosphorylation of Npl3p Inhibits mRNA Export

(**A**) Nuclear accumulation of poly(A)$^+$ RNA in *npl3S411A* cells was visualized by in situ hybridization with an oligo(dT)$_{50}$ probe.

(**B**) Nuclear accumulation of poly(A)$^+$ RNA in *npl3S411A*, *glc7-5*, and *glc7-5 npl3S411A* cells was visualized by in situ hybridization with an oligo(dT)$_{50}$ probe. DAPI staining is shown in the lower panels. Insets are magnified 3X.

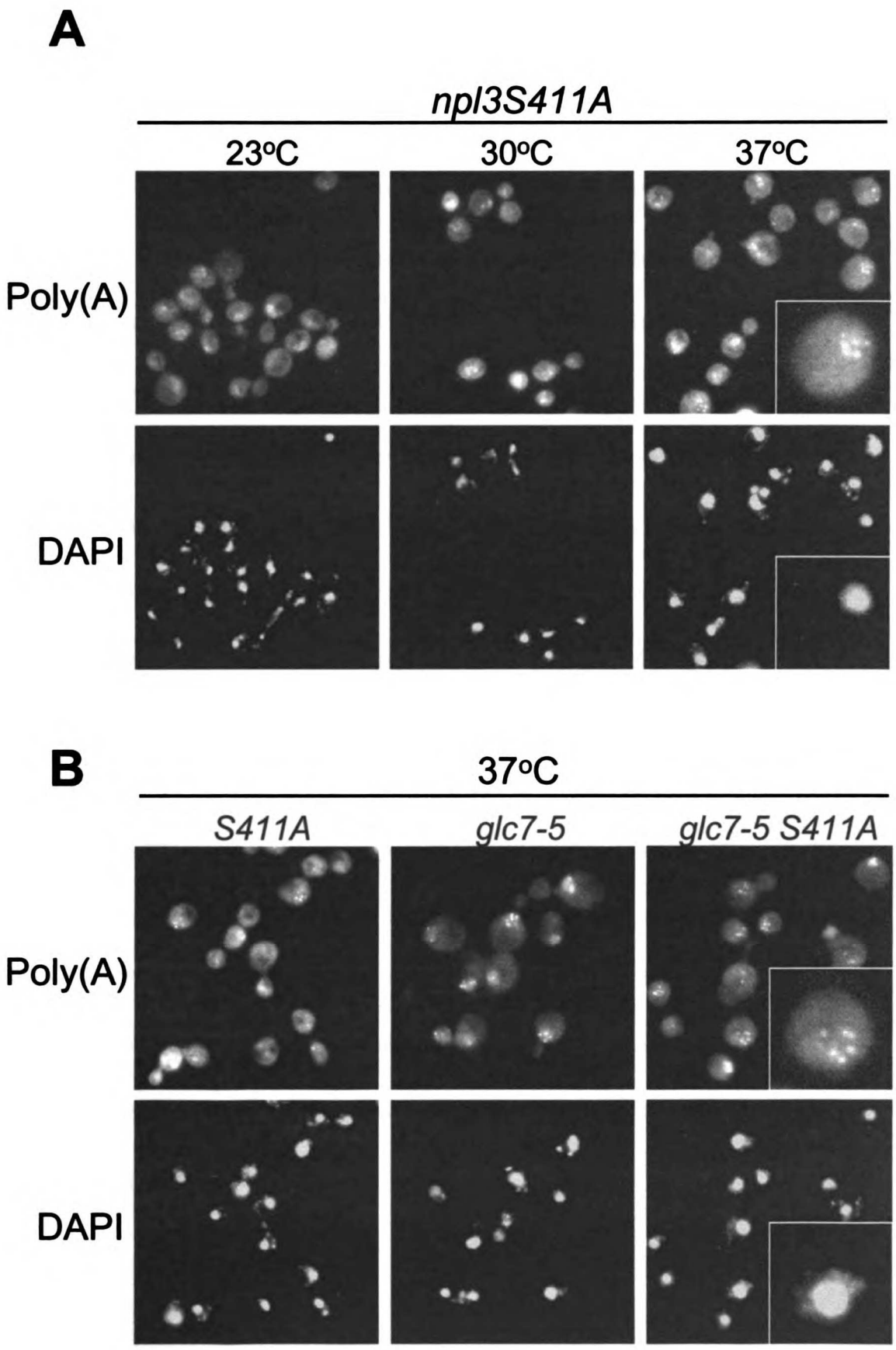

A
npl3S411A
23°C
30°C
37°C
Poly(A)
DAPI
B
37°C
S411A
glc7-5
glc7-5 S411A
Poly(A)
DAPI

Figure 5. Glc7p Promotes Association of poly(A)$^+$ RNA with Mex67p and Release from Rna15p

(A) Total extracts and poly(A)$^+$ RNPs were prepared from *glc7-5* and wildtype cells shifted to 37°C for 60 min. Total lanes contain equal total yeast protein and poly(A) lanes each contain 1.0 OD_{260} units of purified RNPs. Mex67-ProteinA was visualized by immunoblotting with rabbit IgG.

(B) Total extracts and poly(A)$^+$ RNPs were prepared from *glc7-5* and wildtype cells containing 2μm Mex67-GFP shifted to 37°C for 60 min. Total lanes contain equal total yeast protein and poly(A) lanes each contain 1.0 OD_{260} units of purified RNPs. Mex67-GFP was visualized with monoclonal anti-GFP antibody (1:1,000). Hrp1p was visualized with monoclonal anti-Hrp1p antibody (1:1,000).

(C) Localization of poly(A)$^+$ RNA was visualized by in situ hybridization with an oligo(dT)$_{50}$ probe. The same cells stained with DAPI to visualize DNA are shown in the lower panels.

(D) Total extracts and poly(A)$^+$ RNPs were prepared from *glc7-5* and wildtype cells shifted to 37°C for 60 min. Total lanes contain equal total yeast protein and poly(A) lanes each contain 1.0 OD_{260} units of purified RNPs. Rna15p was visualized with anti-Rna15p polyclonals (1:10,000).

A
Total
Poly(A)
glc7 WT glc7 WT
Mex67-ProtA
1 2 3 4
B
2µm MEX67-GFP
Total
Poly(A)
glc7 WT glc7 WT
Mex67GFP
Hrp1p
1 2 3 4
C
Wild Type
glc7-5
vector
2µm MEX67
vector
2µm MEX67
Poly(A)
DAPI
D
Total
Poly(A)
glc7 WT glc7 WT
Rna15p
1 2 3 4

Figure 6. Unphosphorylated Npl3p Binds to Mex67p In Vitro and Tethers Mex67p to mRNA In Vivo

(**A**) Mex67p purified from yeast was incubated with Ni-beads in the presence or absence of recombinant (*E. coli*-expressed) His-tagged Npl3p. Bound (lanes 3 and 4) and unbound (lanes 1 and 2) fractions were analyzed by Western blotting with anti-Mex67p polyclonals (1:1,000).

(**B**) Total extracts and poly(A)$^+$ RNPs were prepared from *npl3S411A* and wildtype cells grown at 30°C. Total lanes contain equal total yeast protein and poly(A) lanes each contain 0.5 OD_{260} units of purified RNPs. Proteins were visualized by Western blotting as described above.

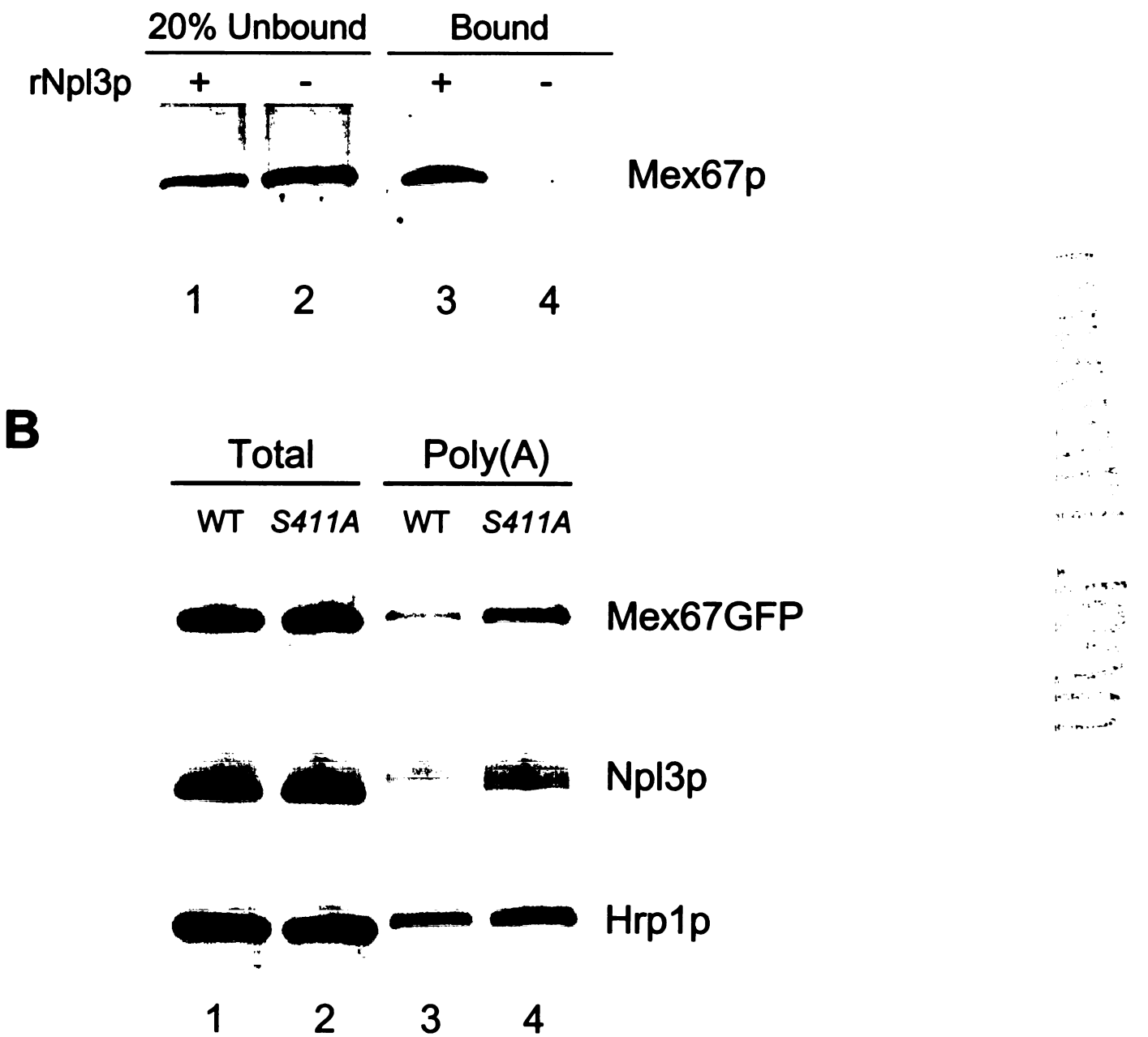
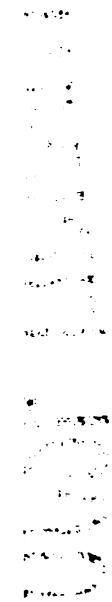
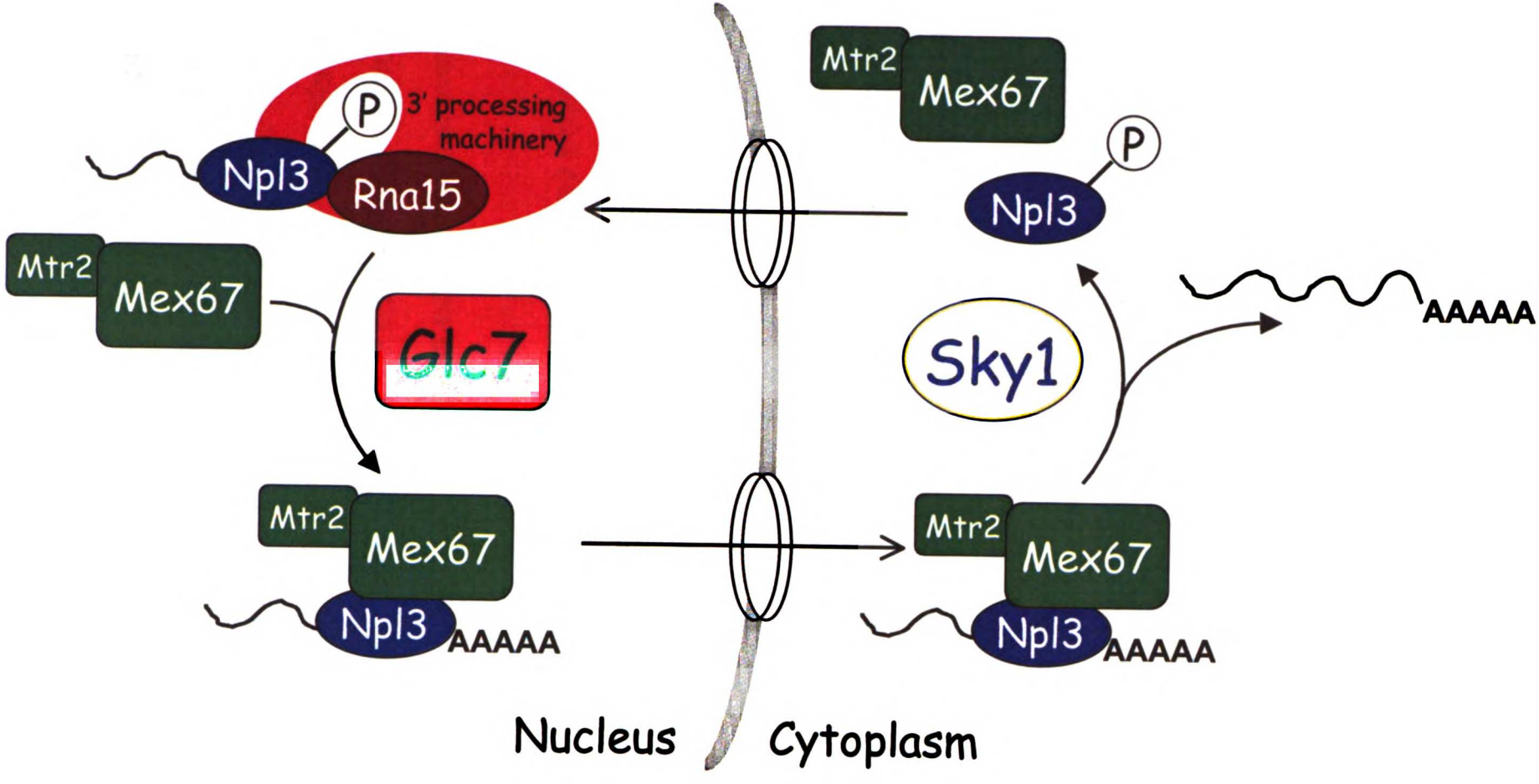

A
20% Unbound
Bound
rNpl3p
+
-
+
-
Mex67p
1
2
3
4
B
Total
Poly(A)
WT
S411A
WT
S411A
Mex67GFP
Npl3p
Hrp1p
1
2
3
4

Figure 7. Proposed Functions of Npl3p phosphorylation in mRNA Export

Phosphorylated Npl3p associates with nascent transcripts in the nucleus. Subsequently, 3' end processing of the transcript stimulates Glc7p to dephosphorylate Npl3p, resulting in release of the mRNP from the 3' processing machinery and association with Mex67p, thereby making the mRNP competent for export. In the cytoplasm, phosphorylation of Npl3p by Sky1p promotes the disassembly of exported mRNPs.





EPILOGUE

The results described in the preceding chapters provide an overview of the cycle of Npl3-dependent mRNA export, from the assembly of transport-competent mRNPs in the nucleus to their dissociation in the cytoplasm, and indicate crucial roles for localized phosphorylation/dephosphorylation of Npl3p in facilitating mRNP remodeling events. This sets the stage for a more detailed analysis of the molecular mechanisms underlying these phosphorylation-sensitive RNP rearrangements, and raises several questions for future investigations:

1) How does phosphorylation by Sky1p cause Npl3p to dissociate from mRNA?

2) What is the requirement for nuclear Npl3p phosphorylation in mRNA export?

3) How does Glc7p promote release of mRNPs from the 3' end processing machinery?

In this section I will briefly review some of the data from Chapters 1 and 2 in the context of the relevant literature to suggest experimental approaches to address these questions. I will also present new data that may contribute to these investigations.

Mechanisms of cytoplasmic mRNP disassembly

In Chapter 1, I found that loss of Npl3p phosphorylation resulted in increased cytoplasmic Npl3 protein and increased association of Npl3p with poly(A) RNA. How does phosphorylation cause the release of Npl3p from exported mRNA? One straightforward hypothesis is that phosphorylation significantly decreases Npl3p's affinity for RNA. This now seems unlikely, given that phosphorylated Npl3p

accumulates bound to poly(A) RNA in glc7-5 (Chapter 2). Nevertheless, I tested this hypothesis directly by assaying the in vitro RNA-binding affinity of recombinant Npl3p +/- phosphorylation with purified Sky1p. As shown in Figure 1, phosphorylation had no effect on RNA binding by Npl3p.

A second possibility is that Npl3p's association with RNA is stabilized by interaction with another protein, and phosphorylation destabilizes that interaction. A likely candidate is Mex67p. Loss of Npl3p phosphorylation increases crosslinking of Mex67p to poly(A) RNA (Chapter 2, Fig. 6B), consistent with a model of coupled cytoplasmic release. As shown in Chapter 2, Mex67p binds directly to unphosphorylated Npl3p in vitro (Fig. 6A). We predict, but have not yet shown, that phosphorylation of Npl3p will abolish binding by Mex67p. This could be tested using in vitro phosphorylated rNpl3p as described in Chapter 1 for binding studies with Mtr10p. A phosphorylation-sensitive protein-protein interaction would not be sufficient to explain phosphorylation-dependent RNA release. For this model to work, Mex67p must also stabilize Npl3p's association with RNA. This could be tested by adding purified Mex67p to the RNA gel-shift assay described in Figure 1. Given that phosphorylated Npl3p appears to be stably associated with mRNA in the nucleus when association of Mex67p is blocked (in glc7-5), one must postulate a nuclear factor to stabilize Npl3p's association with RNA until Glc7p triggers loading of Mex67p. This factor must not accompany the mRNP to the cytoplasm. Possible candidates include Rna15p and Rna14p, two non-shuttling RNA-binding components of the 3' end processing machinery that interact genetically with Npl3p (Henry et al., 1996).

A third, and not mutually exclusive, mechanism for phosphorylation of Npl3p to stimulate release from mRNPs is by creating an appropriate substrate for a trans-acting disassembly factor. An intriguing candidate for such a factor is Dbp5p. *DBP5* encodes a DEAD-box ATPase required for mRNA export (Schmitt et al., 1999; Snay-Hodge et al., 1998; Tseng et al., 1998). Dbp5p localizes to the cytoplasm, with concentration at the cytoplasmic side of NPCs mediated by interactions with components of the cytoplasmic fibrils of the NPC (Schmitt et al., 1999; Snay-Hodge et al., 1998; Tseng et al., 1998). Based on its localization and enzymatic activity, Dbp5p has often been suggested to function in disassembly of exported mRNPs, but to date there is no published demonstration of such a function. A strong prediction of this model is that inactivation of Dbp5 will prevent the removal of nuclear shuttle proteins from exported mRNPs. Mette Lund has promising data showing increased crosslinking of Npl3p and Mex67p to poly(A) RNA in conditional *dbp5* mutants at the non-permissive temperature.

Do Sky1p and Dbp5p act in concert? Intriguingly, Sky1p, which is exclusively cytoplasmic (Chapter 2), also concentrates at the nuclear rim (Fig. 2), similar to Dbp5p. This suggests that mRNP disassembly begins at the cytoplasmic face of the NPC, but does not tell us whether Sky1p and Dbp5p participate in one or two (distinct) reactions. Preliminary results are consistent with the hypothesis that phosphorylation by Sky1p stimulates, but is not absolutely required for, Dbp5p-dependent release of Npl3p and Mex67p from poly(A) RNA: overexpression of Dbp5p suppresses the increase in Npl3p and Mex67p crosslinking caused by *Δsky1* (M. Lund, unpublished observations). In support of the model that Dbp5p acts *after* Sky1p, *dbp5* mutants increase crosslinking of

phosphorylated Npl3p to poly(A) RNA (M. Lund, unpublished observations). Because *dbp5* mutants cause intranuclear accumulation of poly(A) RNA and hyperadenylation defects (Hilleren and Parker, 2001), it is not yet possible to conclude that the accumulation of phosphorylated Npl3p with mRNA results from a defect in cytoplasmic disassembly. Deletion of *RRP6* in *dbp5* mutants causes a shift in poly(A) localization from punctate intranuclear foci to the nuclear rim (M. Lund, unpublished observations). In this background it should be possible to determine whether cytoplasmic phosphorylation of Npl3p precedes Dbp5-dependent release by assaying the phosphorylation state of crosslinked Npl3p. A clear result of increased crosslinking of phosphorylated Npl3p to nuclear rim-arrested mRNPs in *Δrrp6 dbp5* would strongly support the hypothesis that Dbp5p is required for Sky1p phosphorylation to effect release of Npl3p and Mex67p.

A description of cytoplasmic mRNP disassembly would not be complete without incorporating a role for Mtr10p. As described in Chapter 1, mutation of *mtr10* results in a dramatic increase in cytoplasmic Npl3p, accompanied by a very dramatic increase in crosslinking of Npl3p to poly(A) RNA. We now know that *mtr10* mutants accumulate *phosphorylated* Npl3p in the cytoplasm (Gregg Whitworth and W. G., unpublished observations) and increase crosslinking of *phosphorylated* Npl3p to poly(A) RNA (Fig. 3, compare lanes 5 and 7), as well as increasing steady-state Npl3p phosphorylation (Fig. 3, compare lanes 1 and 3). This demonstrates that phosphorylation by Sky1p is not sufficient to completely remove Npl3p from cytoplasmic mRNPs, although it may be sufficient to stimulate removal of Mex67p (see below). The observed synthetic lethality

between *Δsky1* and *Δmtr10* (Yun and Fu, 2000) is consistent with functions for both proteins in cytoplasmic mRNP disassembly (see Discussion in Chapter 1). Furthermore, purified Mtr10p can dissociate preformed Npl3p-ssDNA complexes in vitro (Fig. 4), consistent with an active role for Mtr10p in dissociating Npl3p from cytoplasmic mRNPs in vivo. In Figure 5, I propose a model for step-wise disassembly of exported mRNPs that incorporates specific functions for Sky1p, Dbp5p, and Mtr10p, and makes a few testable predictions.

The model, in brief: Exported mRNPs contain dephosphorylated Npl3p bound to both mRNA and Mex67p, which likely contacts the RNA directly as well (Bachi et al., 2000; Braun et al., 2001; Katahira et al., 1999; Santos-Rosa et al., 1998). Phosphorylation of Npl3p by Sky1p disrupts the stabilizing protein-protein interaction between Npl3p and Mex67p, permitting Dbp5p to remove Mex67p from the mRNA. Removal of Mex67p exposes Npl3p's C-terminus, which constitutes the binding site for Mtr10p (Senger et al., 1998). Binding of Mtr10p completes the disassembly reaction by removing Npl3p from the mRNA. The Sky1p and Dbp5p-dependent removal of Mex67p is postulated to occur at the nuclear rim, consistent with the steady-state localization of these proteins. This model for step-wise disassembly of mRNPs is accessible to in vitro analysis using reagents and assays currently available in the lab (most described in this work): recombinant and/or purified Npl3p, Mex67p, Sky1p, Mtr10p, and Dbp5p (purified and shown to have ATPase activity in vitro, P. Preker, unpublished).

This model predicts synthetic genetic phenotypes between *dbp5* and *mtr10* mutants, analogous to the synthetic lethal interaction between *Δmtr10* and *Δsky1*. Like *Δsky1*, *dbp5* mutants should decrease the coimmunoprecipitation of Npl3p with Mtr10p

from yeast extract (see Chapter 1). The model also predicts that *mtr10* mutants should differ from *dbp5* and *sky1* (*npl3S411A*) mutants with respect to effects on Mex67p release. Whereas all three factors are required for release of Npl3p, only Dbp5p and Sky1p should affect Mex67p crosslinking. Furthermore, Mex67p should compete with Mtr10p for binding to Npl3p. Finally, rapid inactivation of Sky1p should mimic *Δrrp6 dbp5* double mutants in causing accumulation of poly(A) RNA at the nuclear rim.

Generation of conditional *sky1* alleles: purpose and strategy

To address the question of precisely where and when Sky1p-mediated mRNP disassembly occurs, it would be immensely useful to have a conditional *sky1* allele. Deletion of *SKY1* results in a 50% reduction in steady-state Npl3p phosphorylation in vivo (Chapter 1). In contrast to this relatively mild effect in vivo, *Δsky1* extracts are nearly 10-fold less active than *SKY1* extracts as a source of Npl3p kinase activity in vitro (Appendix 2). The in vitro reactions measure the rapid phosphorylation of added rNpl3p rather than the steady-state achieved in vivo over many generations. Thus, it is reasonable to imagine that rapid inactivation of Sky1p in vivo will cause a more dramatic defect in Npl3p phosphorylation. Even more importantly, a tight conditional *sky1* mutant would facilitate the cell biological and biochemical characterization of a homogeneous population of arrested mRNPs. I will briefly discuss three different strategies for obtaining conditional *sky1* alleles.

The Shokat lab has recently published a chemical genetic method for selectively inactivating a kinase of interest. Mutation of a single amino acid in the ATP binding pocket to a glycine residue rendered a variety of kinases sensitive to modified derivatives

of the PP1 kinase inhibitor (Bishop et al., 2000). A chromosomal inhibitor-sensitive allele can easily be generated in yeast by oligonucleotide-directed mutagenesis of the kinase gene of interest contained on an integrating plasmid. The chemical inhibitors are cell-permeable, and cause *rapid* (<30 minutes) and *reversible* inhibition of their targets in vivo (Bishop et al., 2000). This method has several advantages over the traditional approach of isolating temperature-sensitive conditional alleles. First and foremost, it avoids complications from temperature-induced changes in cell physiology. This is particularly desirable in the study of mRNA export. Rapid shift from 25°C to 37°C, the standard manipulation for inactivating temperature-sensitive mutants, induces a transient heat-shock response that has significant effects on mRNA export (Saavedra et al., 1996). Second, the chemical inhibitors can be washed out of the culture medium, resulting in a rapid and relatively synchronous reversal of the inhibitor-induced arrest (Bishop et al., 2000). This feature could be exploited to follow the cytoplasmic fate of exported mRNPs in vivo, an important and poorly understood step in gene expression. Finally, inhibitor-sensitive kinases exhibit dose-dependent responses in vivo (Bishop et al., 2000), which could be useful as a tool to identify synthetic genetic phenotypes between *sky1* and other mutants, such as *dbp5*. An inhibitor-sensitive *sky1* strain would also be invaluable in future biochemical experiments. It has proved difficult thus far to isolate mature mRNPs containing dephosphorylated Npl3p and Mex67p from yeast whole-cell extracts to permit the biochemical characterization of an export-competent mRNP (W. G., unpublished observations). This is likely due to Sky1p-mediated phosphorylation and disassembly upon disruption of the nuclear envelope, which could be prevented by the addition of the specific kinase inhibitor to the lysis buffer during extract preparation.

Clearly a 'Shokat allele" of *SKY1* would be a powerful tool. Nevertheless, it is important to consider alternative approaches for two reasons. First, the approach may simply fail to generate a functional, inhibitor-sensitive kinase. Even in the successful case of Cdc28, the necessary mutation resulted in a 6-fold decrease in k_{cat} (Bishop et al., 2000), and other kinases have been more severely crippled (Hiten Madhani, personal communication). A straightforward alternative approach to obtain a conditional *sky1* null allele is the insertion of a "ts-degron" at the amino terminus of Sky1p. The degron sequence targets the tagged protein for Ubr1-ubiquitylation-dependent degradation at 37°C (Labib et al., 2000). This approach generated temperature-sensitive alleles with a 60% success rate in a recent proteomic study (Kanemaki et al., 2003). Significantly, the degron can lead to very rapid inactivation of the target protein: depletion of the DNA-replication factor Mcm4 resulted in arrest in S phase within a single cell-cycle (Kanemaki et al., 2003). Function of a degron-Sky1p construct could be confirmed by assaying 1) temperature-sensitive loss of Npl3p phosphorylation, and 2) temperature-sensitive resistance to toxic concentrations of LiCl (see below).

There is also a compelling scientific reason to consider an alternative strategy for obtaining conditional *sky1* mutations: while an inhibitor-sensitive allele of *sky1* could reveal a great deal about the *role* of *SKY1*, it will tell us nothing about Sky1p itself. SRPKs comprise an unusual family of kinases with large 'spacer' domains inserted between conserved catalytic motifs. This spacer domain is required for nuclear exclusion of Sky1p (Siebel et al., 1999) and the *S. pombe* SRPK Dsk1p (Takeuchi and Yanagida, 1993). Given the functional significance of Sky1p nuclear exclusion (Chapter 2), the intriguing possibility that the spacer domain contains an NES should be explored. The

observation that overexpressed Sky1p accumulates at the nuclear rim (Fig. 2) raises the possibility that Sky1p may also contain a nuclear envelope/NPC targeting domain. Intriguingly, Sky1 is one of 68 yeast ORFs that contains a consensus sequence for N-terminal myristoylation (Ashrafi et al., 1998), a modification known to contribute to membrane anchoring of numerous proteins (Resh, 1999). These are just two examples of functional domains that might be identified and studied through the generation of conditional mutations in *SKY1*. PCR mutagenesis is an easy way of generating a relatively unbiased collection of point mutants. Loss of *sky1* function results in a dramatic resistance to high concentrations of LiCl (Appendix 1), permitting a simple selection scheme to identify conditional loss of function alleles: Li-resistant transformants would be selected at 37°C and then screened for candidates that displayed normal Li-sensitivity at 25°C. Such a pool of mutants could be used to answer questions about the functional domains required for Sky1p localization, substrate recognition, and possible regulation in response to changing environmental conditions.

Role(s) of nuclear Npl3p phosphorylation in mRNA export

In Chapter 2, I found that elimination of Npl3p phosphorylation (by mutation of Ser411 to Ala) resulted in a constitutive mRNA export defect, and argued against the model that slowed cytoplasmic release of Npl3p from poly(A) RNA is the cause of this defect. This argument rests on the observation that the *mtr10-7* mutation, which leads to constitutively increased cytoplasmic (poly(A)-associated) Npl3p, does not have a constitutive mRNA export defect. We can similarly rule out nuclear depletion of Mex67p

(due to delayed release from exported mRNA) as the cause of impaired mRNA export in *npl3S411A* because *Δsky1* similarly increases crosslinking of Mex67p (M.Lund, unpublished observations) but does not inhibit mRNA export (W.G., unpublished observations). While I cannot exclude the possibility that slowed cytoplasmic release results in depletion of another factor below the critical threshold, the most parsimonious explanation for impaired mRNA export in *npl3S411A*, and not in *Δsky1* or *mtr10-7* (at 25°C), is a requirement for phosphorylated Npl3p in the nucleus. Below I present two, not mutually exclusive models to explain this requirement.

Recent studies of mammalian SR proteins provide evidence that serine phosphorylation mediates their co-transcriptional recruitment, likely by enhancing binding to the CTD of the largest subunit of RNA pol II. In vivo recruitment of SF2/ASF to transcription sites requires a phosphorylatable RS-domain: SF2-RG mutant protein fails to colocalize with induced nascent transcripts whereas both wildtype SF2 and SF2-GS mutant protein, which is phosphorylated in vivo, accumulate at the site of transcription in more than 90% of cells (Misteli et al., 1998). Strikingly, truncation of the CTD of pol II abolishes colocalization of SR proteins with nascent transcripts (Misteli and Spector, 1999). Co-immunoprecipitation studies suggest a biochemical basis for these effects: a phospho-epitope specific anti-SR protein monoclonal antibody (mAb 104) coprecipitates pol II. Furthermore, this interaction is RNase-resistant and preferential for the hyperphosphorylated form of pol II (Kim et al., 1997).

Is phosphorylation of Npl3p similarly important for recruitment to transcription sites? Anti-Npl3p co-precipitates RNA pol II from yeast extracts, although this interaction is reported to be unaffected by the phosphorylation state of the CTD (Lei et

al., 2001). It is not known how the phosphorylation state of Npl3p influences binding to pol II. This could be examined by 1) determining whether anti-phospho Npl3p co-precipitates pol II, and 2) comparing the extent of co-precipitation in wild-type and *npl3S411A* cells. An in situ assay for phosphorylation-dependent co-transcriptional recruitment is probably not feasible in *S. cerevisiae* because the very bright and uniform anti-Npl3p signal would probably preclude detection of enrichment at an individual transcription site. An alternative approach is chromatin-IP, which can provide a quantitative measurement of transcription-dependent association of Npl3p with a gene in vivo (Lei et al., 2001). We predict that anti-phospho Npl3 will precipitate actively transcribed genes. A decrease in chromatin-IP by S411A mutant Npl3p compared to wild-type Npl3p would support the hypothesis that phosphorylation stimulates co-transcriptional recruitment. Proving that diminished recruitment of Npl3p is the cause of *npl3S411A*'s constitutive mRNA export defect is obviously a much more difficult problem.

Another possible role of nuclear Npl3p phosphorylation is facilitating the recruitment and /or proper assembly of other mRNA export factors into mRNPs. One intriguing candidate for an export-relevant interaction partner is the DECD-box mRNA export factor Sub2p. Previous reports identified *SUB2* in a 2-hybrid screen with the C-terminus of *NPL3* (Inoue et al., 2000), which contains the Sky1p phosphorylation site S411. Figure 6 shows that Sub2p co-purifies with Npl3p, and the *npl3S411A* mutation decreases this interaction. The hypothesis that Sub2p binds Npl3p directly, dependent on Npl3p's phosphorylation state, should be tested in vitro using recombinant proteins as described above (for Mex67p). Like Npl3p, Sub2p is recruited co-transcriptionally

(Zenklusen et al., 2002), but it is currently not known how Sub2p is recruited to nascent transcripts. The THO transcription elongation complex, which interacts physically with Sub2p (Strasser et al., 2002), stimulates recruitment of Sub2p to some genes (Zenklusen et al., 2002), but, unlike Sub2p, THO complex components are not essential, suggesting the existence of an alternative mechanism for Sub2p recruitment. Npl3p does not require the THO complex for export (Hacker and Krebber, 2004). If phosphorylated Npl3p forms a binding site for Sub2p on nascent transcripts, *npl3S411A* should decrease Sub2p's association with actively transcribed genes, as visualized by chromatin IP. A more interesting possibility is that the phosphorylation state of Npl3p, by affecting the affinity of the Sub2p-Npl3p interaction, regulates Sub2p's RNPase activity towards one or more targets in the maturing pre-mRNP, which is necessary for formation of export-competent mRNPs.

Mechanisms of Glc7-dependent nuclear mRNP remodeling

In Chapter 2, I presented evidence that Glc7p is required in vivo to release mature, polyadenylated mRNA from the 3' end processing factor Rna15p. Five polypeptides, Rna15p, Rna14p, Hrp1p, Pcf11p, and Clp1p, make up cleavage factor 1A (CF1A), which binds tightly and specifically, via RRM domains in Rna15p and Hrp1p, to RNA sequence elements upstream of the polyA site (for review, see (Zhao et al., 1999). Of these five proteins, only Hrp1p leaves the nucleus with polyadenylated mRNA (Hammell et al., 2002). The mechanisms underlying release of mature mRNA from the non-shuttling components of the 3' end processing machinery have not been explored. Below I propose mechanisms by which Glc7p-dependent dephosphorylation of Npl3p

could promote release of polyadenylated mRNA from the processing machinery prior to mRNP export, and outline experiments to test them in vitro and in vivo.

Dephosphorylation-dependent dissociation of nuclear mRNA from the 3' processing machinery is conceptually analogous to phosphorylation-dependent dissociation of cytoplasmic mRNA from the export complex. In each case, post-translational modification of Npl3p must alter the stability of protein-RNA and/or protein-protein interactions to permit replacement of one set of RNP factors by another. Above, I proposed a model for cytoplasmic disassembly in which phosphorylation of Npl3p functioned to create an appropriate RNP substrate for the DEAD-box protein Dbp5. Dephosphorylation of Npl3p could similarly activate a nuclear disassembly factor. Two attractive candidates for such a disassembly factor are Sub2p and Dbp2p, nuclear DEAD-box proteins known to interact (via 2-hybrid) with the SR/RGG-rich C-terminus of Npl3p (Inoue et al., 2000).

Intriguingly, Dbp2 is the yeast protein most related (32% identical) to human DDX1. DDX1 associates with CstF-64, the human homologue of Rna15p, based on co-immunoprecipitation from nuclear extracts and fluorescence resonance energy transfer (FRET) in HeLa cells (Bleoo et al., 2001). Recently, DDX1 was identified as a binding partner for human hnRNP K, and shown to bind via hnRNP K's RGG domain (Chen et al., 2002). The authors showed that DDX1 binds preferentially to poly(A) RNA in vitro, and that poly(A) but not poly(G) RNA stimulates DDX1 ATPase activity. Furthermore, immunoprecipitated (but not recombinant) DDX1 was shown to have double-stranded RNA unwinding activity in vitro, and preferred a substrate containing a single-stranded 3' poly(A) overhang. Binding of DDX1 to poly(A) RNA and hnRNP K was mutually

exclusive, raising the possibility that hnRNP K could regulate DDX1's poly(A)-dependent RNA helicase activity. Extrapolating from these observations, I propose a model in Figure 7 for Dbp2-dependent removal of Rna15p from mature, polyadenylated mRNA:

Dbp2p is recruited to unprocessed pre-mRNA by binding to the phosphorylated C-terminus of Npl3p. When bound to Npl3p, Dbp2p is inactive as an RNA-dependent ATPase (similar to the DDX1-hnRNP K interaction). Dephosphorylation of Npl3p by Glc7p, which is recruited via its association with the 3' processing machinery, releases Dbp2p from its inhibitory tether, allowing Dbp2p to bind to the newly-formed 3' poly(A) tail and translocate 3' to 5', using the energy of ATP hydrolysis to dislodge Rna15p from the mRNA, thereby releasing the polyadenylated transcript. Glc7p-dependent release of Dbp2p from Npl3p would thus play a dual role in coupling completion of 3' end processing to mRNA export by 1) stimulating dissociation of mRNA from the 3' processing machinery, and 2) exposing Mex67p's binding site on Npl3p.

This model makes several testable predictions. 1) The viable, cold-sensitive *Δdbp2* strain should have an mRNA export defect. 2) In the absence of Dbp2p, polyadenylated mRNA should be inefficiently released from the 3' processing machinery, giving rise to a strong Rna15p in vivo crosslink to poly(A) RNA, similar to the phenotype of *glc7-5*. The effect of *Δdbp2* on Mex67p crosslinking is difficult to predict, and should be explored. If release from the 3' end processing machinery is a prerequisite for Mex67p association with the mRNP, then *Δdbp2* should show decreased Mex67p association with poly(A) RNA in vivo. Alternatively, if Mex67p is able to associate with the mRNP while it remains bound to the 3' processing machinery,

provided that dephosphorylation of Npl3p has occurred, *Δdbp2* may result in a) an RNase-sensitive co-IP between Mex67p and Rna15p, b) redistribution of Mex67p from the nuclear rim to the nuclear interior, and c) a chromatin IP of the 3' ends of actively transcribed genes by anti-Mex67p. 4) Deletion of *dbp2* should exacerbate the growth and mRNA export defects of *glc7-5*, and overexpression of *DBP2* should suppress the increase in Rna15p crosslinking observed in glc7-5. 5) Like DDX1, Dbp2p should have poly(A) RNA-stimulated ATPase and RNPase/RNA unwindase activity, and be capable of removing pre-bound Rna15p from RNA. 6) Dbp2p and phosphorylated Npl3p should bind each other in vitro, and binding by Npl3p should attenuate Dbp2p's ATPase/RNA unwindase/RNPase activities.

Dbp2p is a particularly attractive candidate for a 3' processing complex disassembly factor because of its homology to DDX1. Nevertheless, Sub2p is also a candidate. Thus *sub2* mutants, particularly the *sub2S/L* allele (predicted to be deficient in coupling ATP hydrolysis to conformational rearrangements), should be examined for effects on Rna15p crosslinking in vivo.

An in vitro assay for release of polyadenylated mRNA from the 3' processing machinery would be an enormous aid to dissecting the molecular requirements for this step in gene expression. In vitro studies of the mechanisms underlying release of spliced mRNA from the spliceosome may provide a useful paradigm for developing such an assay for completion of 3' processing. The DEAD-box protein Prp22p was implicated in spliceosome release when Company and colleagues observed a novel in vitro splicing defect: in *prp22* mutant extracts, spliced mRNA remained associated with large spliceosomal complexes (Company et al., 1991). This release defect was visualized by

fractionating the complexes formed during in vitro splicing on native gels and/or glycerol gradients and following the positions of ^{32}P-labeled pre-mRNA and mRNA. In wild-type extracts, pre-mRNA is found in a high molecular weight complex containing snRNPs, and spliced mRNA is found in a much smaller RNP, 'complex D'. After splicing in *prp22* mutant extracts, complex D is absent and spliced mRNA is associated with a snRNP-containing particle (Company et al., 1991; Wagner et al., 1998). A well-characterized in vitro cleavage and polyadenylation reaction exists that is suitable for similar analysis of the requirements for release of polyadenylated mRNA from the 3' processing machinery post-catalysis. The specific complexes that form on synthetic polyadenylation substrates in extracts can be fractionated by native gel electrophoresis, glycerol gradient sedimentation, and gel-filtration (Gilmartin and Nevins, 1989; Humphrey et al., 1987; Veraldi et al., 2000). Pre-mRNA and cleaved intermediates are found in stable high molecular weight complexes from which polyadenylated mRNA is subsequently released (Humphrey et al., 1987). Nothing is currently known about the molecular requirements for this release.

The in vivo poly(A) RNA crosslinking results described in Chapter 2 showed that polyadenylated mRNPs formed in wild-type cells contain primarily dephosphorylated Npl3p, lack Rna15p, and are capable of associating with Mex67p, whereas inactivation of Glc7p leads to accumulation of polyadenylated mRNA in complexes containing phosphorylated Npl3p, Rna15p, and lacking Mex67p, which fail to be exported. To test whether the in vitro 3' end processing reaction recapitulates the in vivo situation, the association of each of these proteins with pre-mRNA and polyadenylated mRNA could be determined by UV-crosslinking and immunoprecipitation. Based on the in vivo

crosslinking results, anti-Npl3p is predicted to co-IP all three RNA species: precursor, cleaved intermediates, and mature polyadenylated mRNA, whereas anti-phosphoNpl3p and anti-Rna15p should preferentially co-IP the precursor and intermediates and anti-Mex67p should co-IP only the mature polyadenylated species. A transcript carrying a stretch of encoded A residues upstream of a self-cleaving ribozyme could be used to control for the possibility that the presence of a poly(A) tail *per se* determines the specificity of protein association, rather than passage through the steps of 3' end processing. This transcript, which is not exported in vivo (Huang and Carmichael, 1996), is predicted to associate with phosphorylated and dephosphorylated Npl3p indiscriminately. Mex67p should co-IP only the population bound to dephosphorylated Npl3p. Western blot analysis of precursor and product complexes fractionated by glycerol gradient or gel filtration could be used to confirm the expected patterns of association of Npl3p, phosphoNpl3p, Rna15p and Mex67p and to examine the relative distribution of other proteins of interest. These assays could be used to investigate the contributions of Glc7p, Npl3S411, Dbp2p, and ATP hydrolysis to release of polyadenylated mRNA from the 3' processing machinery and recruitment of Mex67p. These in vitro assays might illuminate the molecular basis for the mRNA export defects of polyadenylation-competent/export-deficient alleles of *rna14*, *rna15*, *pcf11* and *pap1* (Hammell et al., 2002), as well as the suppression of *npl3-1* by *rna15-101* (Henry et al., 1996). It will be particularly interesting to examine the effect of *npl3S411A* on the association of Rna15p and Mex67p with pre-mRNA. Constitutive dephosphorylation of Npl3p may destabilize the association of pre-mRNA with the 3' processing machinery and/or permit premature association of Mex67p with unprocessed precursor.

Is polyadenylation the only mRNA processing event that activates Npl3p dephosphorylation-dependent recruitment of Mex67p? Recent work from the Steitz lab determined that splicing-coupled dephosphorylation of mammalian SR proteins promotes preferential association of NXF1 with spliced mRNPs compared to unspliced pre-mRNPs both in vitro and in vivo (J. Steitz, personal communication). Npl3p associates with unspliced pre-mRNA, lariat intermediates, and spliced mRNA in vitro (Appendix 1). The phosphorylation state of Npl3p in each of these complexes has not yet been determined. The assays described above for characterizing 3' processing-coupled mRNP transitions in vitro can easily be applied to splicing reactions to address whether 1) splicing results in (Glc7p-dependent?) Npl3p dephosphorylation, and preferential association of mature mRNA with dephosphorylated Npl3p, 2) Mex67p preferentially associates with spliced mRNA over pre-mRNA, 3) Npl3p dephosphorylation affects the release activity of Prp22p, and 4) whether constitutive dephosphorylation of Npl3p (*npl3S411A*) is *sufficient* to promote association of Mex67p with unspliced pre-mRNA. Multiple mammalian SR proteins have been shown to bind to NXF1 directly and promote NXF1-dependent mRNA export (Huang et al., 2003). Two additional yeast SR-like proteins, Gbp2p and Hrb1p, are likely substrates for Sky1p (Hacker and Krebber, 2004; Hurt et al., 2004; Windgassen and Krebber, 2003) and have poorly-defined roles in mRNA export (Hurt et al., 2004; Windgassen and Krebber, 2003). They should be tested in parallel with Npl3p for processing-dependent dephosphorylation and for effects on Mex67p recruitment. To investigate the role of splicing in Mex67p recruitment in vivo, the RNA that co-IPs with Mex67p could be analyzed on splicing microarrays or by Q-PCR. Based on the results from mammalian cells, Mex67p should preferentially IP exon features compared to intron

features. If dephosphorylation of Npl3p is sufficient for Mex67p recruitment, than introns should be enriched in the Mex67p IP from *npl3S411A* cells compared to the Mex67p IP from *NPL3* wild-type. Premature association of Mex67p in *npl3S411A* might increase leakage of pre-mRNA to the cytoplasm, indicating a role for nuclear Npl3p phosphorylation in mRNA export quality-control.

Concluding Remarks

Although I diverged from my initial experimental approach, it is immensely gratifying that this work has led to significant progress on my original question: what are the molecular determinants of export-competence in mRNA export? In the process, I have also gained insight into the conserved role of SR proteins and their regulators. The Guthrie lab first began to study Npl3 and Sky1 hoping to exploit the power of yeast molecular genetics to dissect the function of SR proteins in splicing. One surprising conclusion of my work is that the most highly conserved function of SR proteins may be in coupling pre-mRNA processing to mRNA export. By far the sweetest and most unexpected fruit of my labors is the discovery of a coherent mechanism with the potential for determining directionality in mRNA export.

Given the ultimate success of this project, I think it bears considering for a moment how I arrived at this point. The experimentally productive phase of my thesis work began when I turned my attention to an apparently small, possibly peripheral question in mRNA export, namely, why does Npl3p mislocalize to the cytoplasm in the absence of Sky1-mediated phosphorylation? I anticipated a simple, commonplace explanation: that phosphorylation would be required for binding to Npl3p's import

receptor. This result, had it materialized, would have closed the book on a small inquiry. The consistent failure of phosphorylation to affect Npl3p's binding to Mtr10p in vitro is the first of two negative results that prompted major intellectual breakthroughs during the course of my thesis work. The need to reconcile this negative result in vitro with in vivo observations led to the hypothesis that the function of Sky1p is to dissociate Npl3p from exported mRNA, a hypothesis that proved to be the loose thread that ultimately allowed me to unravel the entire cycle.

The cycle that I first envisioned (outlined in Chapter 1) postulated a requirement for nuclear dephosphorylation to permit association of Npl3p with mRNA. My efforts to test this prediction brought me to the second pivotal 'negative' result: the robust failure of *glc7* mutants (which cause hyperphosphorylation of Npl3p) to inhibit association of Npl3p with poly(A)$^+$ RNA in vivo. Indeed, as presented in Chapter 2, *glc7* mutants cause a dramatic **increase** in association of *phosphorylated* Npl3p with mRNA. Grappling with this unexpected result led to the second breakthrough – the insight that 1) dephosphorylation of Npl3p occurred much later in nuclear mRNP biogenesis than I had supposed previously, and 2) de/phosphorylation of Npl3p was likely to affect association of Npl3p with RNPs by modulating protein-protein rather than Npl3-RNA interactions. From these realizations, it was only a short step to the identification of Mex67 and Rna15 as relevant players, which, in turn, culminated in molecular models both for conferring directionality to Mex67-dependent mRNA export and for coupling 3' end processing to mRNP export-competence.

My work can be fairly characterized as extremely model-driven. Most of my models have proved to be wrong. What is striking to me is that the incorrect models, and

my deep, if transient attachments to them, have been so useful. I leave behind a number of speculative models, rich in premature detail. I eagerly await the discoveries my successors will make in the process of disproving them.

Figure 1. Phosphorylation of S411A Does Not Affect Binding of Npl3p to RNA In Vitro

(**A**) ^{32}P labeled in vitro transcribed R2x2 RNA (74 nt) was incubated with increasing concentrations of recombinant Npl3p, phosphorylated in vitro with Sky1-ProtA purified from yeast or mock phosphorylated with a mock purification from an untagged yeast strain. The resulting RNA-protein complexes were resolved on native gels.

(**B**) As above, using RR-WT (52 nt) RNA. RNA sequences:

R2x2 –

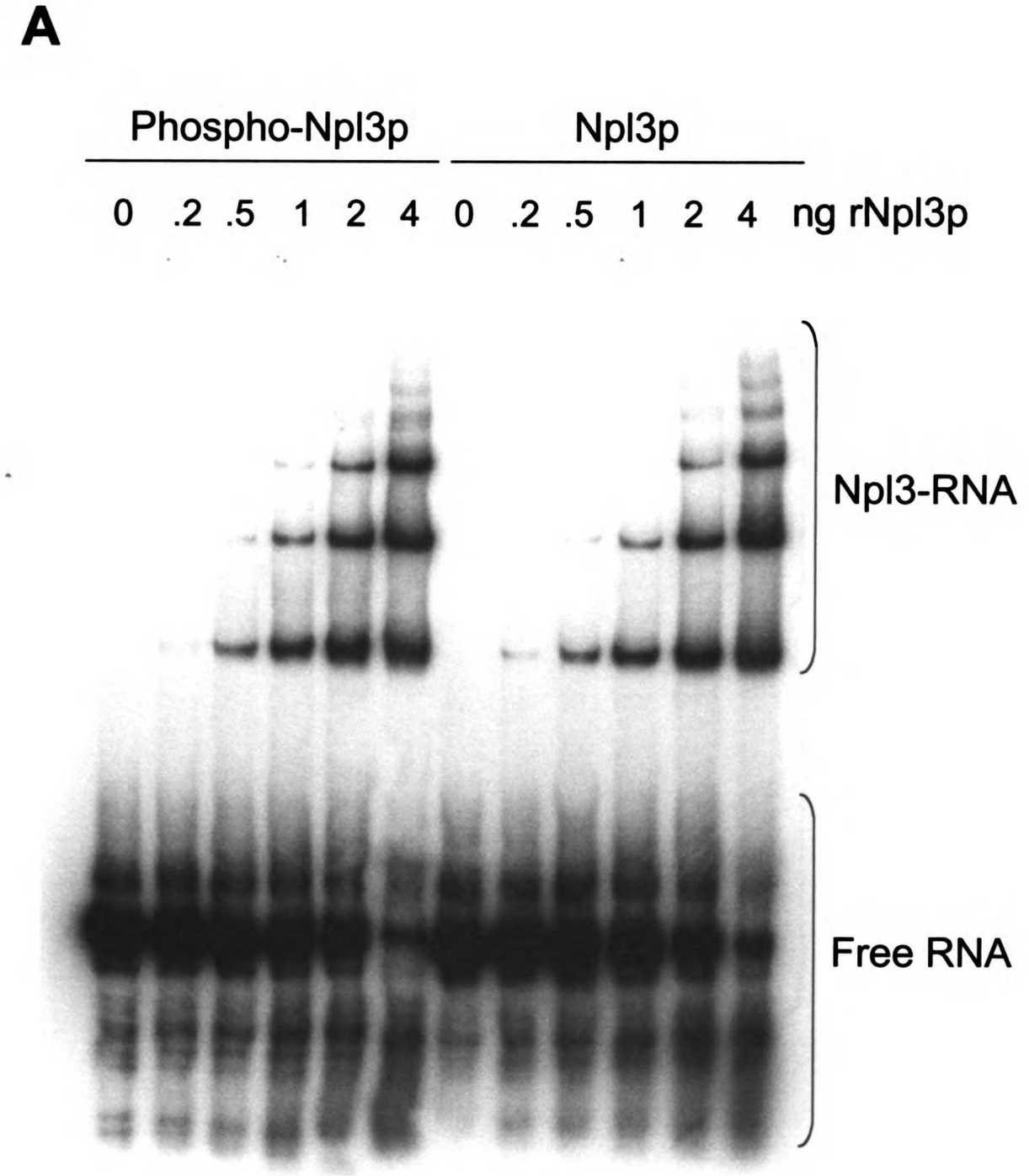
GGAUCCGUCUUCAAUCAACAUACGCGAGAUCCGUCUUCAAUCAACAUACGC

G (= 2 direct repeats of the doublesex regulatory element that binds SR proteins, plus flanking sequences, K. Lynch, personal communication);

RR-WT –

GGUGACCCCUUACCUACUCACACCACUGCAUUCUCACCCGCAAGCACCUUU

G (encompasses a branch point site and a polypyrimidine tract, K. Lynch, personal communication).


A
Phospho-Npl3p
Npl3p
0 .2 .5 1 2 4 0 .2 .5 1 2 4 ng rNpl3p
Npl3-RNA
Free RNA

B

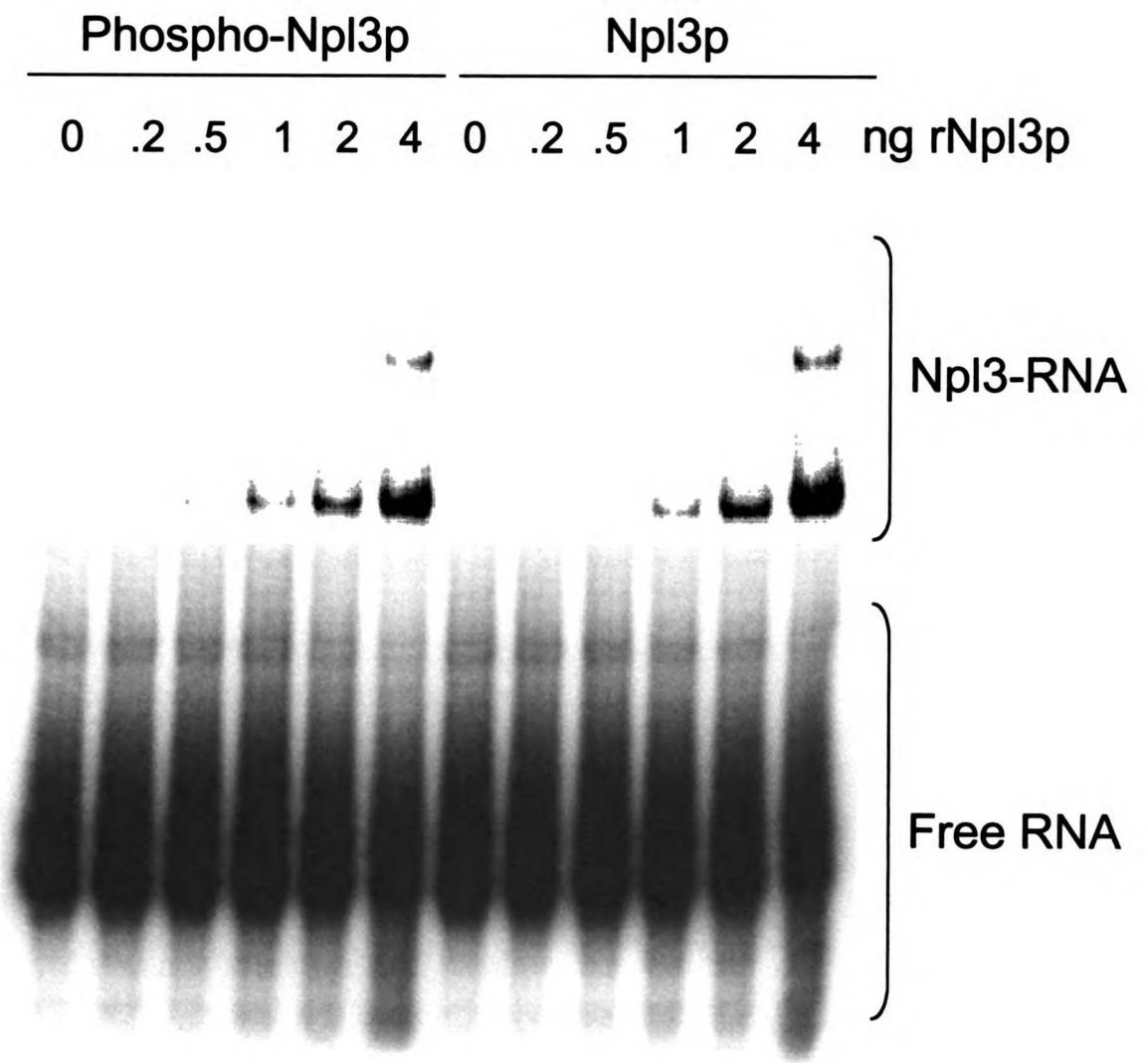

Phospho-Npl3p
Npl3p
0 .2 .5 1 2 4 0 .2 .5 1 2 4 ng rNpl3p
Npl3-RNA
Free RNA

Figure 2. Sky1p Localizes to the Cytoplasm with Concentration at the Nuclear Rim

Sky1-TAP was visualized in galactose-grown cells transformed with GAL1-SKY1-TAP by immunofluorescence using FITC-conjugated goat anti-rabbit antibodies, which bind to the protein A moiety of the TAP tag. The same cells stained with DAPI to visualize DNA are shown in the lower panels.

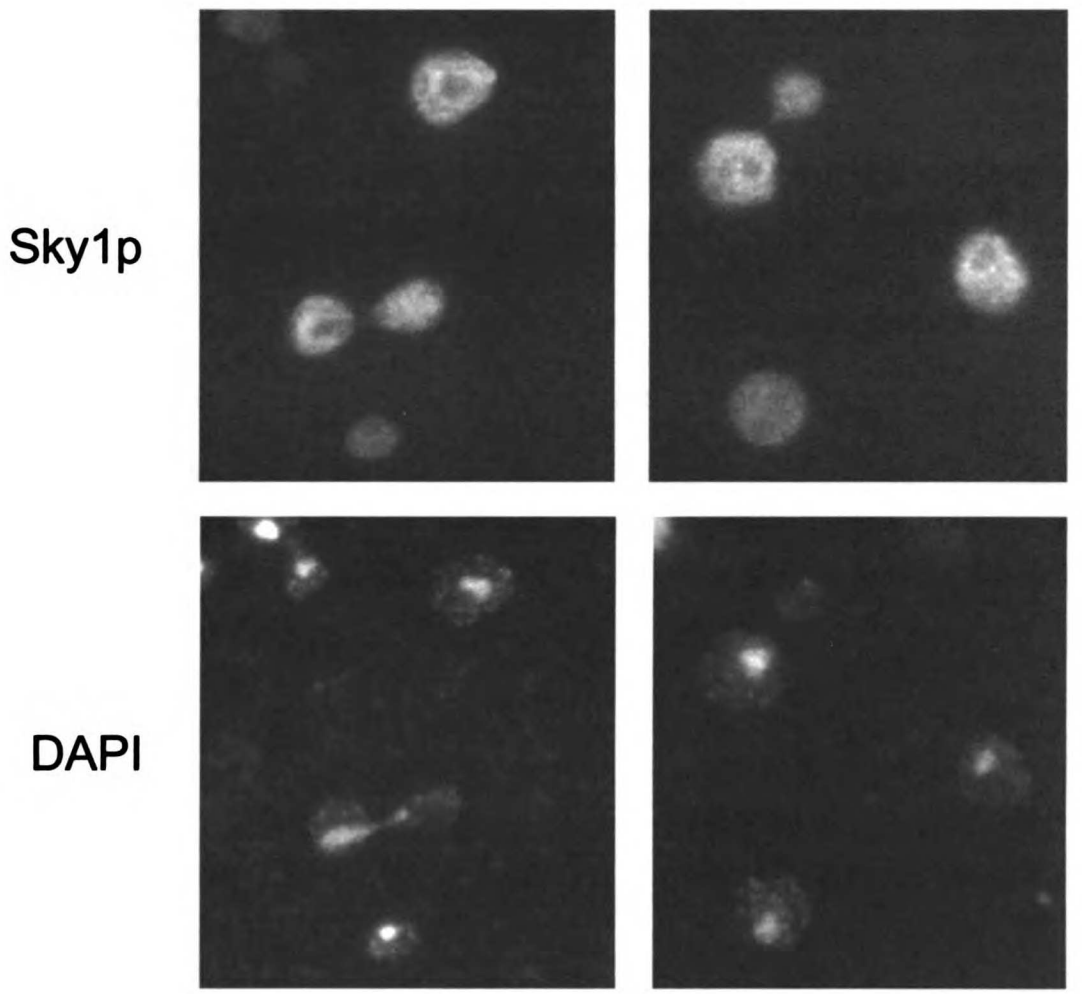

Sky1p
DAPI

Figure 3. Δmtr10 Increases Association of Phosphorylated Npl3p with Poly(A)$^+$ RNA In Vivo

Npl3p was cross-linked to RNA by mild UV-treatment of living cells, and poly(A)$^+$ RNA-protein complexes were purified on oligo dT cellulose. 1 A260 unit of purified RNPs was used for each eluate gel lane. Total lanes contain less than 0.1% of the starting material. The upper and lower panels show the same samples probed with anti-Npl3 and anti-Phospho-Npl3, respectively. Cross-linking of Npl3p to poly(A)$^+$ RNA was increased in a *Δmtr10* strain (lane 5) compared to *MTR10* (lane 7), and some of this protein is phosphorylated (lane 5, lower panel). *Δmtr10* increased steady-state phosphorylation of Npl3p (compare lanes 1 and 3).

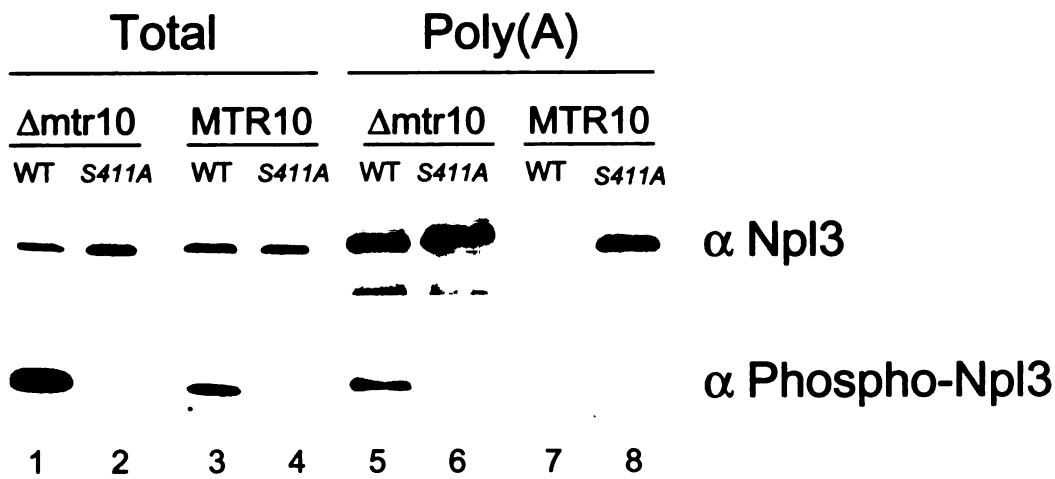

Total
Poly(A)
Δmtr10
MTR10
Δmtr10
MTR10
WT
S411A
WT
S411A
WT
S411A
WT
S411A
α Npl3
α Phospho-Npl3
1
2
3
4
5
6
7
8

Figure 4. Mtr10p Dissociates Npl3p from ssDNA In Vitro

rNpl3p (100 pmoles) was pre-bound to ssDNA cellulose before incubating with rMtr10p (200 pmoles) or buffer. The unbound (U) fraction (supernatant) represents the protein released from the resin upon incubation with rMtr10p or buffer. The remaining bound (B) fraction was eluted from the ssDNA cellulose beads by boiling in SDS sample buffer. Equal volumes of each fraction were separated by SDS-PAGE and probed for Npl3p with anti-Npl3 polyclonal antibodies (1:2,000).

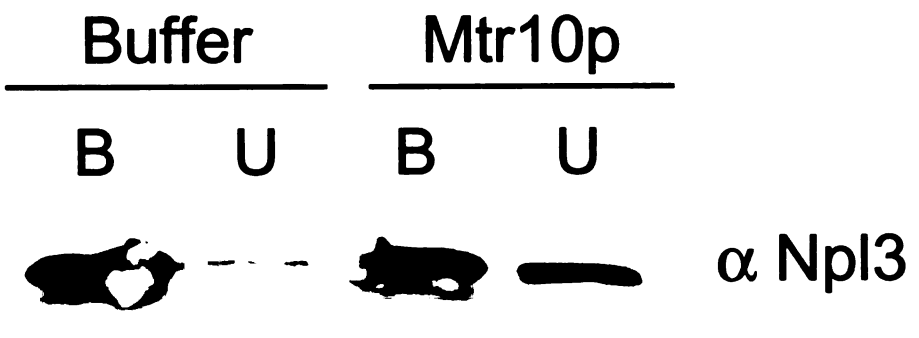

Buffer
Mtr10p
B
U
B
U
α Npl3

Figure 5. Model for Cytoplasmic Disassembly of Exported mRNPs

Exported mRNPs contain dephosphorylated Npl3p and Mex67p. Upon translocation to the cytoplasmic side of the NPC, the mRNP encounters both Sky1p* and Dbp5p. Phosphorylation of Npl3p by Sky1p disrupts a protein-protein interaction between Npl3p and Mex67p, thereby destabilizing Mex67p's association with the mRNP. Dbp5p subsequently removes Mex67p, exposing the Mtr10p binding site at the C-terminus of Npl3p. Binding of Mtr10p to Npl3p dissociates Npl3p from mRNA, and recycles Npl3p to the nucleus.

*Like Dbp5p, Sky1p is concentrated at the nuclear rim. Sky1 is one of 64 yeast ORFs containing a consensus sequence for N-myristoylation (Ashrafi et al., 1998), a modification that facilitates membrane tethering (Resh, 1999). Thus, Sky1p is drawn as a myristoylated protein anchored in the nuclear envelope.

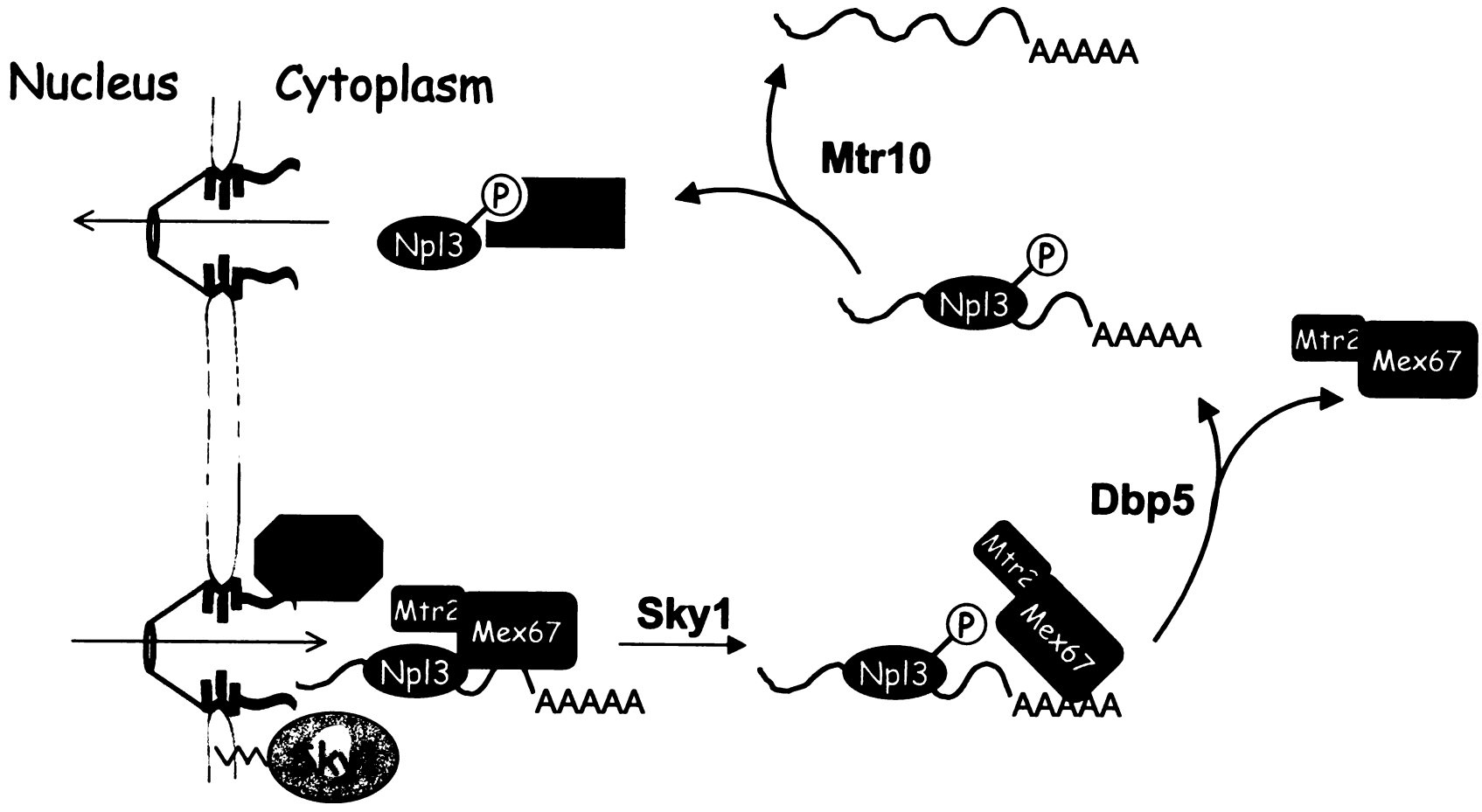

Nucleus
Cytoplasm
AAAAA
Mtr10
P
Npl3
P
Npl3
AAAAA
Mtr2
Mex67
Dbp5
Mtr2
Mex67
Npl3
AAAAA
Sky1
Mtr2
Mex67
P
Npl3
AAAAA

Figure 6. The S411A Mutation Decreases the Association of Sub2p with Npl3p In Vivo

Wild-type and *S411A* mutant Npl3p proteins were purified from yeast expressing Npl3-TEV-ProteinA by binding to IgG Sepharose, followed by extensive washing, and elution with TEV protease. Co-purifying Sub2p was visualized by Western blotting with anti-Sub2p polyclonals (upper panel, lanes 3 and 4). Mutation of the Npl3p phosphorylation site decreased association of Sub2p with Npl3p (upper panel, lane 3 vs. 4). The lower panels, blotted with anti-Npl3p polyclonals, show that equivalent amounts of Npl3 protein were purified from wild-type and *S411A* mutant extracts.

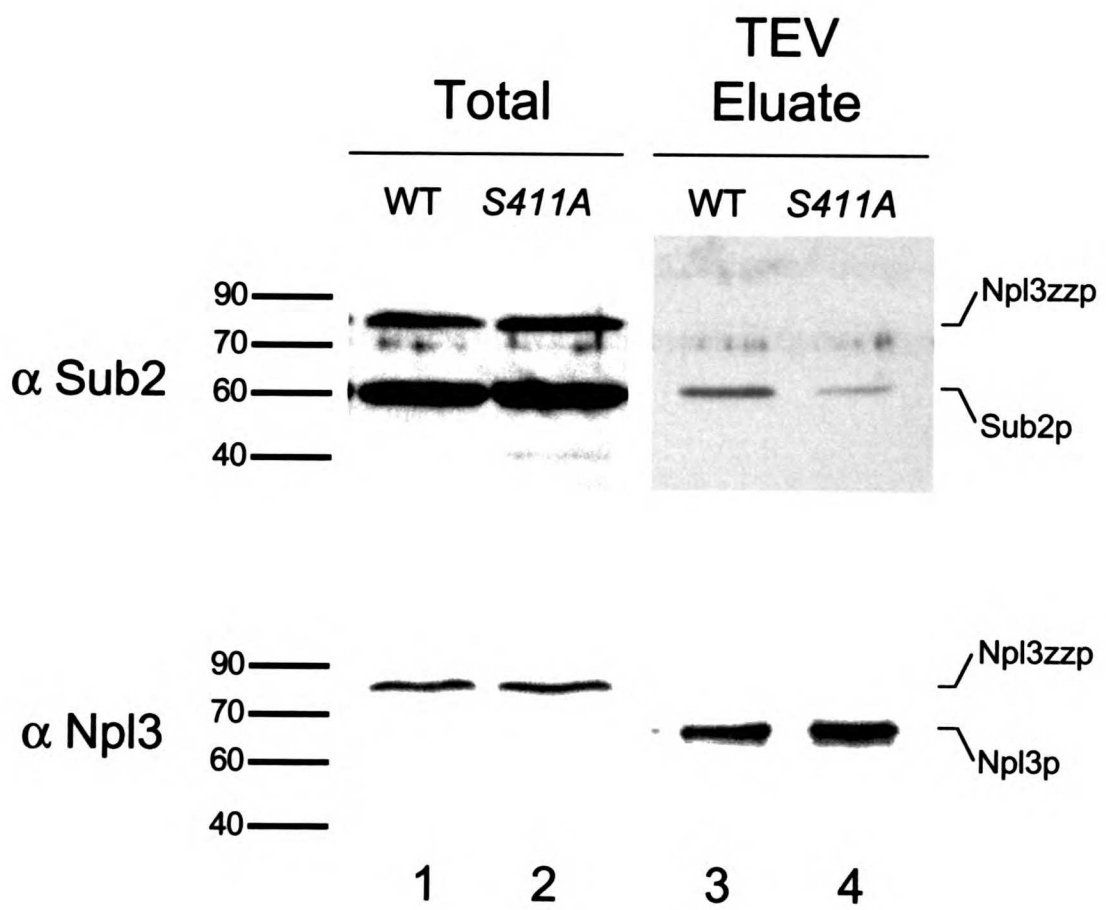
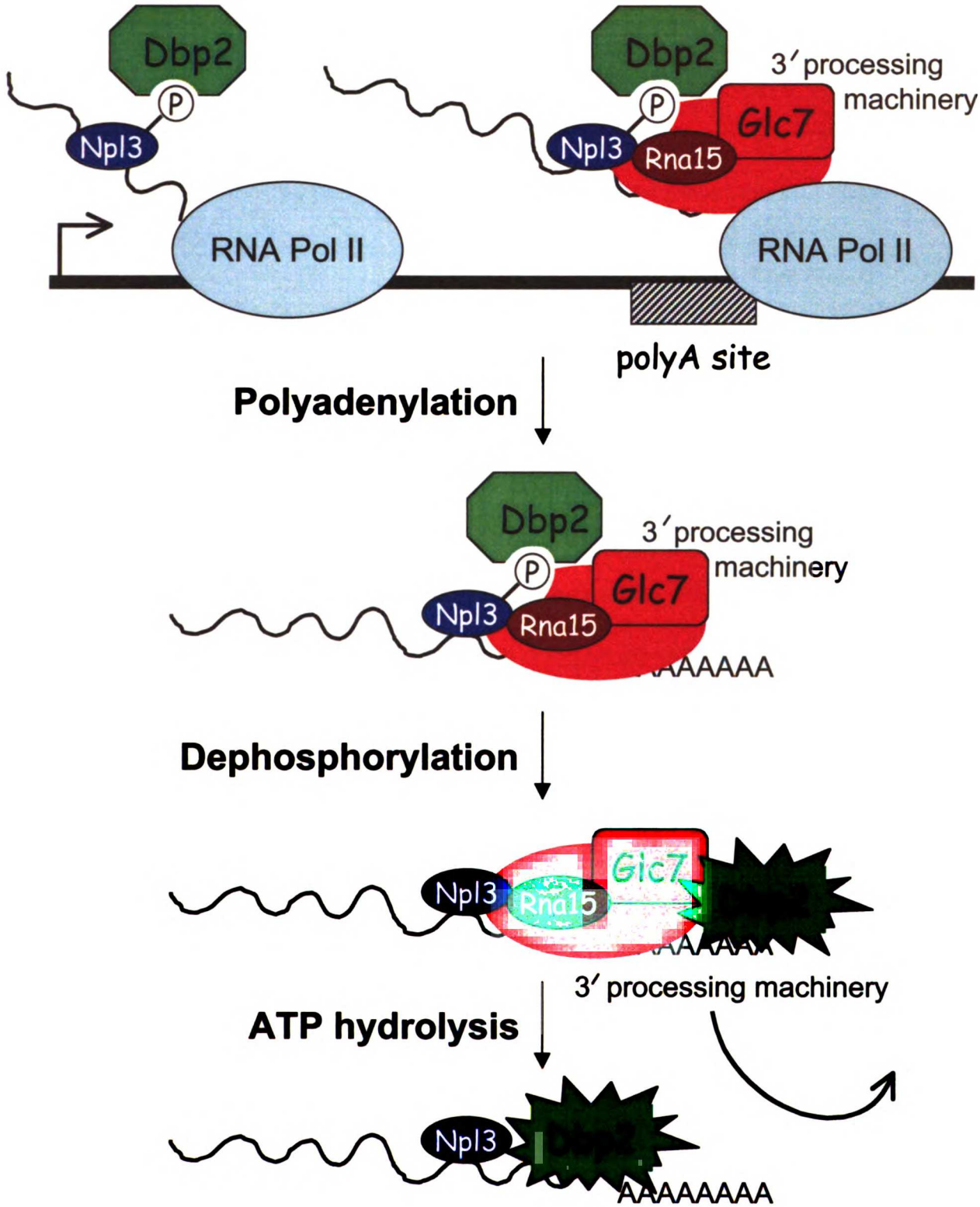

Total
TEV Eluate
WT
S411A
WT
S411A
α Sub2
90
70
60
40
Npl3zzp
Sub2p
α Npl3
90
70
60
40
Npl3zzp
Npl3p
1
2
3
4

Figure 7. Model for Regulation of Nuclear mRNP Remodeling by Dbp2p

Dbp2p is recruited co-transcriptionally to unprocessed pre-mRNA by binding to the phosphorylated C-terminus of Npl3p. When bound to Npl3p, Dbp2p is inactive as an RNA-dependent ATPase. Transcription through the poly(A) site results in association of the 3' processing machinery and Glc7p with the nascent RNP. Dephosphorylation of Npl3p by Glc7p then releases Dbp2p from its inhibitory tether, allowing Dbp2p to bind to the newly-formed 3' poly(A) tail and translocate 3' to 5', using the energy of ATP hydrolysis to dislodge Rna15p from the mRNA, thereby releasing the polyadenylated transcript from the 3' processing machinery.


Dbp2
P
Npl3
RNA Pol II
Dbp2
3′ processing machinery
P
Glc7
Npl3
Rna15
RNA Pol II
polyA site
Polyadenylation
Dbp2
3′ processing machinery
P
Glc7
Npl3
Rna15
AAAAAA
Dephosphorylation
Glc7
Npl3
Rna15
3′ processing machinery
ATP hydrolysis
Npl3
AAAAAAAA

Appendix 1

Additional Phenotypes of *Δsky1* and *npl3S411A*: Implications for Environmentally Responsive Regulation of mRNA Metabolism

Preface

Much of what purports to be a written record of the scientific process is no such thing. In our papers we often show little regard for the history of an inquiry, preferring the deception of manufacturing hypotheses *post facto* in order to present a logical, ***linear*** description of how and why we might have done a particular set of experiments had we known their outcome when we began. This misrepresentation is probably a necessary evil – it would certainly be time-consuming, and likely tedious as well, to relive all of our colleagues' idiosyncratic wanderings in order to learn their conclusions[1]. But the literature produced by revisionist history lacks a certain charm and vigor. The true "Ah-ha!" moments sometimes vanish under the weight of inevitability of the narrative, and our lame attempts at manufactured drama are heralded by the flaccid "Surprisingly, ..."

I had every intention that the following narrative would be historically accurate. I would offer the hypotheses that actually motivated each experiment (in the order in which they occurred to me!), even if hindsight revealed some of them to have been not only wrong, but wrong-headed as well. I had hoped that reading such a document might bolster the confidence of as-yet-unpublished younger students, who may see little resemblance between the published literature and their own experience of the scientific process so far. But in the process of writing, some previously missed connections became apparent to me, and I did not scruple to omit them. This proved to be the proverbial slippery slope. Ultimately, I submitted fully to the exigencies of storytelling.

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[1] If you, dear reader, have found me tedious already, pray tarry no longer and proceed immediately to the figure legends.

The unifying hypothesis behind this body of work is that nature would not have conserved SR protein kinases throughout eukarya if they were irrelevant to the fitness of the cell. Thus, since both *Δsky1* and *npl3S411A* mutants grow well under normal laboratory conditions (log phase growth in YPD at temperatures between 16°C and 37°C), and since protein phosphorylation is *the* classical, reversible modification used to modulate cellular activities in response to changing conditions, I tested *Δsky1* and *npl3S411A* strains' responses to a variety of environmental insults. Below, I present evidence that Npl3p phosphorylation affects the cellular response to one environmental stress – exposure to toxic cations – and discuss possible molecular mechanisms for adaptive regulation of gene expression via modulation of Npl3p activity.

RESULTS AND DISCUSSION

I began to think about 'adaptive' functions for Npl3 phosphorylation when following up a labmate's accidental observation: *Δsky1* cultures left in the roller drum for 10 days do not grow up readily when diluted into fresh media (C. Siebel, unpublished observations). This observation proved reproducible (W. G., unpublished observations) but I lacked an intellectual context within which to pursue a possible function for Sky1 in maintaining viability during stationary phase, since *npl3S411A* did not appear to share this phenotype[2] (W. G., unpublished observations). Nevertheless, this experimental exercise stimulated thinking about possible functions for Sky1-dependent regulation of

[2] These experiments were performed using *Δnpl3* strains covered by pRS315-NPL3/npl3S411A plasmids. Because the phenotype of *Δsky1* was sensitive to both strain background and culture conditions (W. G., unpublished observations), these assays should be repeated using the integrated, isogenic *npl3S411A/NPL3*, *Δsky1/SKY1* strains described in Chapter 2.

gene expression in response to changing environmental conditions. Thus primed, at a Cold Spring Harbor meeting in 1998 I visited a poster which might otherwise have escaped my notice. The authors found that osmotic stress in mammalian cells results in both cytoplasmic accumulation and increased steady-state phosphorylation of hnRNP A1 (now published, (van der Houven van Oordt et al., 2000). This correlation between changes in phosphorylation of a shuttling RNA-binding protein and changes in its steady-state localization, and the suggestion that these changes might be an adaptive response to cellular stress, were intriguing given that the only function of Sky1 known to us at the time was in modulating the intracellular localization of Npl3p. I naively assumed that these changes in hnRNP A1 phosphorylation and localization play a positive role in the survival of osmotically stressed human cells, and decided to explore the possibility that modification of Npl3p plays an analogous role in yeast.

Δsky1 and *npl3S411A* Mutants Are Resistant to Toxic Cations

To test the hypothesis that Sky1-dependent phosphorylation of Npl3p is required for yeast to respond to osmotic stress, I compared the growth of *Δsky1* and isogenic wild-type strains on media containing 1M NaCl. Surprisingly (really!), *Δsky1* grows much *better* than *SKY1* on YPD + 1M NaCl (Fig. 1A). *npl3S411A* similarly grows better than *NPL3* in the presence of 1M NaCl (Fig. 1B), consistent with the idea that Npl3p is a physiologically relevant substrate of Sky1p under these conditions. High concentrations of NaCl are both osmotically challenging and ion-toxic. These effects can be separated experimentally using sorbitol or KCl to produce an osmotic challenge and LiCl to test resistance to toxic cations. As shown in Figure 1, both *Δsky1* and *npl3S411A* are resistant

to 0.3M LiCl (Fig. 1C) but not 1M KCl or sorbitol (Fig. 1D) compared to wild-type cells. Thus we conclude that loss of Sky1-dependent phosphorylation of Npl3p increases resistance to toxic cations but not osmotic stress.

NaCl-treatment Causes Transient Cytoplasmic Accumulation and Increased Sky1-Dependent Phosphorylation of Npl3p

I was surprised initially that loss of Sky1 caused resistance rather than sensitivity to environmental stress. Nevertheless, this was a clear and striking growth phenotype (the first I found for *Δsky1*) attributable to changes in Npl3p phosphorylation, so I decided to pursue it. At the time, we knew that loss of Sky1-dependent phosphorylation perturbed the steady-state localization of Npl3p (Chapter 1). Thus, in an effort to relate the ion-resistant phenotype of *Δsky1* to its molecular phenotypes, I examined the effects of NaCl treatment on 1) Npl3p localization, and 2) Npl3p phosphorylation.

Treatment of wild-type cells with 0.4M NaCl for 10 minutes causes a dramatic intracellular redistribution of Npl3p from nuclear-concentrated to nuclear-excluded (Fig. 2A, upper panels). NaCl-dependent relocation is clearly visible in *Δsky1* cells as well (Fig. 2A, lower right), on top of the milder constitutive increase in cytoplasmic Npl3p caused by deletion of *SKY1* (Fig. 2A, lower left, and Chapter 1). Thus, Sky1 cannot be the primary regulator of the dramatic cytoplasmic relocalization of Npl3p induced by NaCl. Relocation of Npl3p in wild-type cells is correlated with a modest increase in steady-state phosphorylation (Fig. 2B, compare the relative intensities of the anti-Npl3 and anti-phosphoNpl3 signals in lanes 1 and 2). This increase requires *SKY1* (Fig. 2B, compare lanes 2 and 4). In fact, *Δsky1* cells, which show a 50% reduction in steady-state

Npl3p phosphorylation under non-stressed conditions (Chapter 1), show a further *reduction* in Npl3p phosphorylation after NaCl treatment (Fig. 2B, compare lanes 3 and 4).

The effects of NaCl on Npl3p are transient, peaking 2-5 minutes after addition of NaCl, and returning to pre-stress conditions within 30 minutes. Careful analysis of the relative kinetics of re-import of Npl3p in wild-type and *Δsky1* cells during adaptation to NaCl stress shows a clear delay in *Δsky1* cells (G. Whitworth, unpublished observations). The kinetics of Npl3 relocalization in *Δsky1* following NaCl-treatment are closely correlated with the changes in Npl3p phosphorylation: after 2 minutes, when Npl3p is most dramatically cytoplasmic, *Δsky1* cells show the greatest reduction in steady-state Npl3p phosphorylation. After 10-15 minutes, when Npl3p begins to re-accumulate in the nucleus, steady-state phosphorylation is approaching pre-stressed levels, and after 30 minutes, both Npl3p localization and steady-state phosphorylation are indistinguishable from the pre-stressed condition (W.G. and Gregg Whitworth, unpublished observations).

Npl3p is likely to be phosphorylated by nuclear kinases as well as by cytoplasmic Sky1p (see Appendix 2). Thus, the simplest interpretation of the changes in Npl3p phosphorylation following NaCl-treatment is that they are indirect effects of changes in localization: in wild-type cells, relocalization of Npl3p to the cytoplasm results in increased phosphorylation via increased contact with Sky1p, whereas in *Δsky1* cells, relocalization results in decreased phosphorylation of Npl3p due to loss of accessibility to nuclear kinases. It will be interesting to determine whether the transient decrease in Npl3p phosphorylation in *Δsky1* requires the activity of the nuclear Npl3p phosphatase Glc7p (Chapter 2). If so, this would suggest that even in wild-type cells NaCl-treatment

may result in transient nuclear dephosphorylation of Npl3p which is invisible in these experiments due to rapid rephosphorylation in the cytoplasm.[3]

Mutations That Increase Cytoplasmic Npl3p Confer Resistance to Toxic Cations

How might loss of Sky1-dependent phosphorylation of Npl3p increase cells' resistance to toxic cations? Wild-type yeast relocate Npl3p to the cytoplasm in response to Na/LiCl. If this relocalization plays a positive role in the stress response, other mutations (in addition to *Δsky1* and *npl3S411A*) that shift Npl3p's steady-state localization towards the cytoplasm would be expected to increase Na/Li tolerance. As shown in Figure 3A, deletion of Npl3p's import receptor Mtr10 makes cells resistant to NaCl and LiCl. Two *npl3* alleles that increase cytoplasmic Npl3p by an unknown mechanism (Lee et al., 1996), *npl3-328* and *npl3-1*, also confer resistance to NaCl and LiCl (Fig. 3B and data not shown). Finally, two mutations that are known (*mex67-5* (Liu et al., 1999) or likely (*glc7-109*)[4] to impair export of Npl3p (thus *decreasing* the cytoplasmic pool relative to the nuclear) cause increased sensitivity to LiCl (Fig. 3C and (Williams-Hart et al., 2002). Taken together, these mutational data suggest that shifting the steady-state localization of Npl3p from the nucleus to the cytoplasm confers

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[3] This could be tested by examining Npl3p phosphorylation in NaCl-treated cells when Npl3p export is inhibited. *mex67-5* should be useful in this regard, as Tartakoff and colleagues showed previously that Npl3 *protein* export requires *MEX67* even when Npl3p export is uncoupled from mRNA export by inhibiting pol II transcription Liu, Y., Guo, W., Tartakoff, P. Y., and Tartakoff, A. M. (1999). A Crm1p-independent nuclear export path for the mRNA-associated protein, Npl3p/Mtr13p. Proc Natl Acad Sci U S A *96*, 6739-6744..

[4] *glc7-109* modestly increases (nuclear) phosphorylation and poly(A) RNA crosslinking of Npl3p (W. G., unpublished observations), and thus is expected to impair Npl3p export, like *glc7-5*, by decreasing association of Npl3p with its export receptor Mex67p (Chapter 2).

resistance to toxic cations. This is consistent with the notion that the transient relocalization of Npl3p that occurs after treatment with toxic cations plays a positive role in this stress response.

Possible Mechanisms for Adaptive Regulation of Gene Expression via Modulation of Npl3p Localization

Yeast changes its transcriptional program dramatically in response to toxic cations (Bro et al., 2003; Posas et al., 2000; Yale and Bohnert, 2001), thereby changing the RNA species available to be processed, exported, translated and degraded. There is also accumulating evidence of post-transcriptional regulation of gene expression during this stress response (Bro et al., 2003; Li et al., 2003). Below, I discuss four potential mechanisms by which changes in the local concentration of Npl3p could modulate gene expression in response to toxic cations:

1. Inhibition of Npl3-dependent mRNA export via nuclear depletion of Npl3p
2. Alteration of pre-mRNA splicing patterns via nuclear depletion of Npl3p
3. Stabilization of Npl3p-associated mRNAs via increased cytoplasmic association
4. Alteration of translation of Npl3p-associated mRNAs via increased cytoplasmic association

Model 1: Rapid relocalization of Npl3p to the cytoplasm enhances Npl3-independent export of stress-induced mRNAs by reducing competition for limiting components of both export pathways.

Cole and colleagues first suggested that mRNA export is regulated during stress to rationalize their observation that heat-shock causes poly(A)$^+$ RNA to accumulate in the nucleus while SSA4 and SSA1 mRNAs (encoding heat-shock proteins) continue to be exported under these conditions (Saavedra et al., 1996). Silver and colleagues subsequently proposed that the rapid relocalization of Npl3p to the cytoplasm in response to stress is the mechanism by which cells deliberately inhibit "bulk" mRNA export to enhance the export of stress-induced transcripts (Krebber et al., 1999). This model could explain the toxic cation-resistant phenotype of mutants that shift the steady-state localization of Npl3p if the following are true: 1) Na/Li-induced transcripts are exported via an Npl3-independent pathway; 2) the mutations that constitutively increase cytoplasmic Npl3p actually decrease Npl3-dependent mRNA export; and most importantly 3) decreased flux through the Npl3-dependent pathway increases the efficiency of Npl3-independent mRNA export.

Proof of this model would require the identification of an Npl3-independent pathway. What factors are likely mediators of Npl3-independent mRNA export? Nab2p is another abundant shuttling RNA-binding protein required for mRNA export in yeast (Green et al., 2002; Hector et al., 2002). Nab2p and Npl3p associate with overlapping but non-identical populations of mRNA in vivo (Karen Kim, unpublished observations), consistent with the idea that certain transcripts may differ in their reliance on these two proteins for their export. Unlike Npl3p, Nab2p does not relocate to the cytoplasm in response to NaCl (Nanduri and Tartakoff, 2001), or LiCl (W.G., unpublished observations). Moreover, a recent quantitative study of protein expression profiles in NaCl-treated yeast identified Nab2p as one of 43 upregulated proteins (Li et al., 2003).

Lastly, Kent Duncan identified a mutant, *Δtom1*, that appears to specifically inhibit Nab2-dependent export while preserving Npl3-dependent export, raising the possibility of selective mRNA export regulated by the activity of the Nab2 effector, Tom1 (Duncan et al., 2000). I therefore tested *Δtom1*, as well as *nab2-21* (Hector et al., 2002) and *kap104-16* (a mutation in the Nab2p import receptor (Aitchison et al., 1996) for growth on LiCl plates. Strikingly, all of these mutants are hypersensitive to lithium (Fig. 4). These observations make Nab2p a very attractive candidate export carrier for ion-stress induced messages. However, recent observations suggest an alternative explanation for the Na/Li sensitivity of *Δtom1*: *Δtom1* causes constitutive hyperphosphorylation of Npl3S411 (Fig. 5A)[5]. Given that overexpression of Sky1p makes cells hypersensitive to LiCl (Fig. 5B), increased Npl3p phosphorylation is a likely explanation for *Δtom1's* ion-sensitivity.

Definitive evidence for distinct, physiologically significant Npl3- and Nab2-dependent mRNA export pathways has long been sought by members of the Guthrie lab. Given the genetic and cell biological differences between Nab2 and Npl3 described above, I believe it may be fruitful to pursue this goal by studying the ion-toxicity response.

Model 2: Depletion of Npl3p from the nucleus facilitates changes in pre-mRNA splicing that promote growth in the presence of toxic cations.

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[5] This observation calls into question the conclusion that *Δtom1* does not affect Npl3p export, which was based on the fact that a GFP-Npl3S411A reporter (on top of wild-type chromosomal *NPL3*) continues to show cytoplasmic accumulation in *Δtom1*. Since this reporter protein cannot be phosphorylated, we must consider the possibility that its behavior does not accurately reflect the behavior of wild-type Npl3p.

Nuclear depletion of Npl3p could adaptively affect splicing in response to LiCl either by reducing splicing of growth-inhibiting (stress check-point) genes or by increasing splicing of growth-promoting (stress abatement) genes. What is the evidence to suggest any role for Npl3p in splicing? Most of the work described in this thesis describes roles for Npl3 outside of the traditional realm of function of SR proteins as regulators of alternative splicing. The study of Npl3 as a splicing regulator has lagged due to the lack of appropriate assays for investigating alternative splicing in *S. cerevisiae*. This is no longer the situation, due to the development of new microarrays and computational tools that can be used to assess splicing on a genomic scale. Initial studies have revealed a new paradigm for alternative splicing in an organism in which most spliced genes contain a single intron: complex, environmentally sensitive on/off regulation of splicing of individual introns (Jeff Pleiss, Megan Bergkessel, and Gregg Whitworth, unpublished observations). Thus, the moment is ripe for asking whether the SR protein system in yeast functions in alternative splicing.

I fully expect that the answer is 'yes'. Like metazoan SR proteins, Npl3p is associated with each of the RNA reactants and products of an in vitro splicing reaction (Fig. 6) and copurifies with snRNAs and splicing complexes from yeast (Gottschalk et al., 1998). Furthermore, Npl3p associates with the transcripts from intron-containing genes in vivo (K. Kim, unpublished observations), genes that exhibit complex, highly dynamic changes in their splicing after exposure to LiCl (G. Whitworth and J. Pleiss, unpublished observations). Phosphorylation of Npl3p is likely to be an important effector of pre-mRNA splicing in yeast based on the facts that 1) *Δsky1* is synthetically lethal with specific alleles of *prp8* that affect splice site usage (Dagher and Fu, 2001), and

2) *glc7-5* shows complex changes in the genomic splicing profile at the non-permissive temperature (W. G., unpublished observations). Gregg Whitworth is currently investigating the effects of *Δsky1*, *npl3S411A*, etc. on the splicing response of LiCl-treated yeast; I eagerly await the results of his experiments.

Both of the models described so far presuppose that it is *depletion* of Npl3p from the nucleus that causes changes in gene expression required for an adaptive response to toxic cations. Below I briefly consider two models in which *accumulation* of Npl3p in the cytoplasm actively affects gene expression:

Model 3: Accumulation of Npl3p in the cytoplasm stabilizes associated transcripts, thereby enhancing their translation.

Cytoplasmic mRNA turnover has only recently been appreciated as a major point of regulation in gene expression. Many regulated transcripts are governed by AU-rich elements (AREs) in their 3' untranslated regions that modulate the rate of poly(A) tail shortening and subsequent mRNA decay (for review, see (Mitchell and Tollervey, 2000; Ross, 1996; Wilusz et al., 2001). In yeast, the cytoplasmic RNA binding protein Pub1 binds to and stabilizes some ARE-containing transcripts *in a regulated manner*; the mechanism(s) for carbon source regulation of Pub1p's ARE-binding are not known, but they do not appear to involve changes in the levels of Pub1 protein (Vasudevan and Peltz, 2001). It is not known whether ARE-mediated decay is regulated in response to toxic ions, but *Δpub1* is hypersensitive to LiCl (G. Whitworth, unpublished observations). Npl3p co-purifies with Pub1p (Gavin et al., 2002), raising the possibility that Npl3p may

stabilize the binding of Pub1p to certain transcripts. It would be interesting to determine whether the cytoplasmic relocalization of Npl3p after Na/Li treatment is correlated with increased association of Npl3p and Pub1p, and whether deletion of *pub1* is epistatic to *npl3S411A* with respect to LiCl sensitivity.

Obviously, Npl3p could have effects on mRNA stability independent of Pub1p. Most of the mutants described in this appendix as increasing cytoplasmic Npl3p and Na/Li resistance are known to increase the association of Npl3p with cytoplasmic poly(A)$^+$ RNA (see Chapter 1 for increased RNA association in *Δsky1*, *npl3S411A*, and *Δmtr10*). Another mutation that similarly causes Npl3p to accumulate in the cytoplasm (*npl3L219S/mts1-1*) is known to specifically stabilize certain mRNAs, and was proposed to increase protein expression from normally short-lived messages by inhibiting their decay (Gratzer et al., 2000). It would be relatively easy, and potentially very informative to determine whether ion stress and/or ion-resistant mutants that increase cytoplasmic Npl3p affect mRNA half-lives using genomic microarrays. Although this discussion has focused on the possibility of Npl3p-dependent stabilization of mRNAs in the cytoplasm, Npl3p might just as easily act as a transcript-specific enhancer of mRNA decay, as was recently shown for ASF/SF2 (Lemaire et al., 2002). The microarray approach could reveal either form of regulation, and identify potential mRNA targets of Npl3-dependent regulation relevant to the ion stress response.

Model 4: Cytoplasmic Npl3p directly affects the translatability of associated transcripts.

Translation initiation is very sensitive to toxic cations. Lithium treatment inhibits ^{35}S incorporation in vivo and causes the polysome profile to collapse to 80S

monoribosomes, defects that can be suppressed by overexpression of the translation initiation factor eIF4A, which is induced by lithium stress (Montero-Lomeli et al., 2002). Similarly, sodium treatment severely inhibits protein synthesis both in vivo and in vitro, and this inhibition can be partially overcome by increasing the levels of the eIF1A (Rausell et al., 2003). Thus, modulating translation initiation is a potential mechanism for increasing a cell's ability to adapt to toxic ion stress. Intriguingly, the L219S mutant form of Npl3p accumulates with 80S monoribosomes (Gratzer et al., 2000), raising the possibility that Npl3 may directly affect mRNA translation.

In support of this speculation, Npl3p copurifies with proteins involved in translation initiation: Sgn1p and Ygr054w (Ho et al., 2002). Sgn1p is a cytoplasmic poly(A) RNA binding protein that interacts physically (Gavin et al., 2002; Ho et al., 2002) and functionally (Winstall et al., 2000) with eIF4G and Pab1. *SGN1* is a candidate high-copy suppressor of *Δsky1's* lithium resistant phenotype (see below), an interaction which would suggest that the observed physical interaction between Npl3p and Sgn1p modulates some function of Npl3p in gene expression.

YGR054w is the yeast homologue of mammalian eIF2A (Zoll et al., 2002), a protein that, like the canonical eIF2 initiation factor, binds aa-tRNA to 40S ribosomal subunits, although the two factors differ in their GTP requirements (Merrick, 1979). Somewhat surprisingly, eIF2 and eIF2A are unrelated at the primary amino sequence level despite the similarity of their biochemical activities. *ΔeIF2A* yeast are viable and do not show any gross alterations of the polysome profile under standard laboratory conditions, yet the protein is found associated with 40S subunits and 80S ribosomes (Zoll et al., 2002), suggesting that eIF2A may function in the translation of a subset of mRNAs

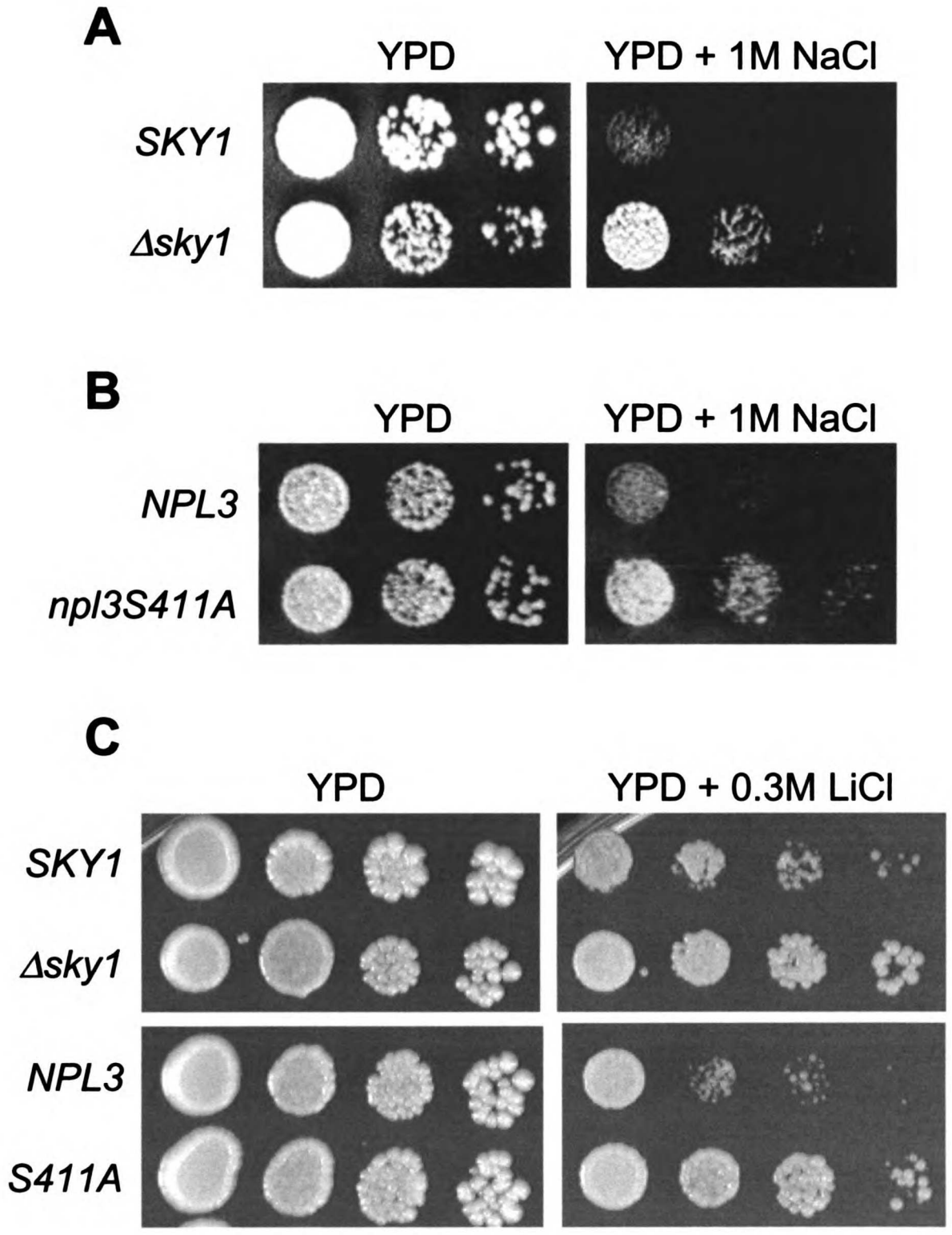
that are not required for viability. It would be fascinating to identify messages that are translated in an eIF2A-dependent manner, under a variety of stress conditions (including, of course, Li/Na treatment) by comparing polysome-associated transcripts from wild-type and *ΔeIF2A* cells via competitive hybridization on yeast ORF microarrays. This assay could be used to determine whether *npl3S411A* affects translation profiles in stressed or unstressed cells, although additional experiments would be required to distinguish primary effects on translation initiation from secondary effects of altered mRNA export.

A Final Pitch

If I have not yet persuaded you, my would-be successor, to accept the Notebook 6 torch and figure out the molecular basis for increased resistance to toxic cations in mutants that increase the cytoplasmic pool of Npl3p, I offer one last inducement: a box of plasmids. As outlined in Figure 7, I performed a screen for high-copy suppressors of *Δsky1's* lithium-resistant phenotype. I isolated 67 distinct genomic fragments (other than *SKY1*) that reproducibly restore lithium sensitivity to *Δsky1* when overexpressed. These clones include a tantalizing array of genes involved in all possible regulated steps in RNA metabolism. If mRNA export is your pleasure, how about the E3 ubiquitin ligase *RSP5*? For the splicing enthusiast, may I suggest *PRPs19*, *22*, and *45*, perhaps with *MUDs 1* and *2* on the side? Translation aficionados will enjoy *SGN1*, *TIF1* (eIF4A) and *TEF2* (EF1-alpha). COME ON DOWN, THE PRICE IS RIGHT!

Figure 1. *Δsky1* and *npl3S411A* Are Resistant to Toxic Cations

Cultures were grown overnight in YPD and then diluted and spotted by serial (6-fold) dilution onto media as indicated. Δsky1 (**A**) and npl3S411A (**B**) are resistant to toxic cations – 1M NaCl and LiCl (**C**), but not resistant to osmotic stress – 1M KCl and 1M Sorbitol (**D**).


A
YPD
YPD + 1M NaCl
SKY1
Δsky1
B
YPD
YPD + 1M NaCl
NPL3
npl3S411A
C
YPD
YPD + 0.3M LiCl
SKY1
Δsky1
NPL3
S411A

D

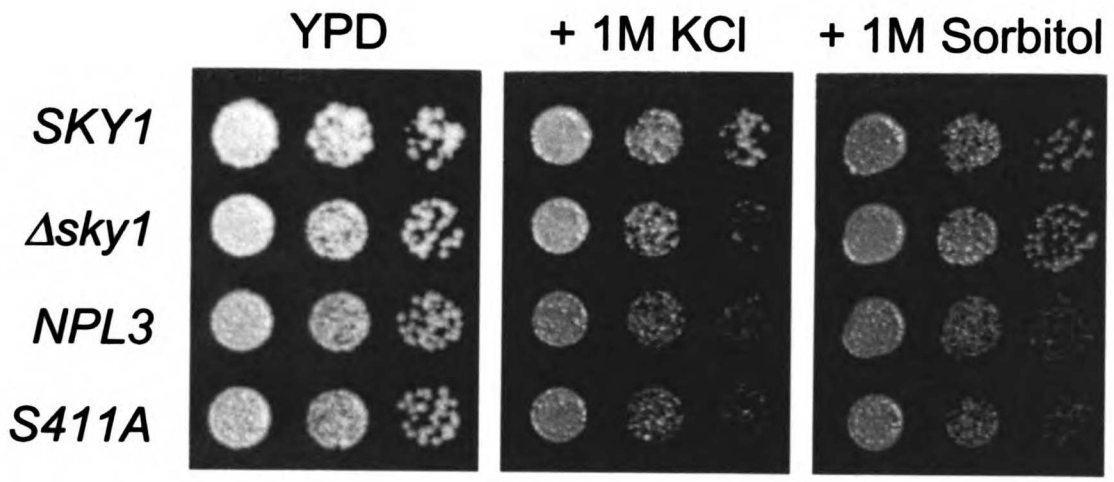


Figure 2. 0.4M NaCl Induces Transient Cytoplasmic Accumulation and Increased Sky1-Dependent Phosphorylation of Npl3p

(**A**) Log-phase (O.D. 0.15-0.3) *SKY1* and *Δsky1* cells were incubated in YPD +/- 0.4M NaCl for 10 minutes before fixation (5% formaldehyde) and spheroplasting. Npl3p was visualized in situ by immunofluorescence using rabbit polyclonal anti-Npl3p (1:400) followed by goat anti-rabbit-FITC conjugated secondary antibody (1:400).

(**B**) Total cell extracts were prepared from the same cultures by bead-beating (as described in Chapter 2). Npl3p and phosphoNpl3p were detected by Western blotting with anti-Npl3p (1:2,000) and anti-phosphoNpl3p (1:1,000) followed by goat anti-rabbit HRP conjugated secondary and ECL.

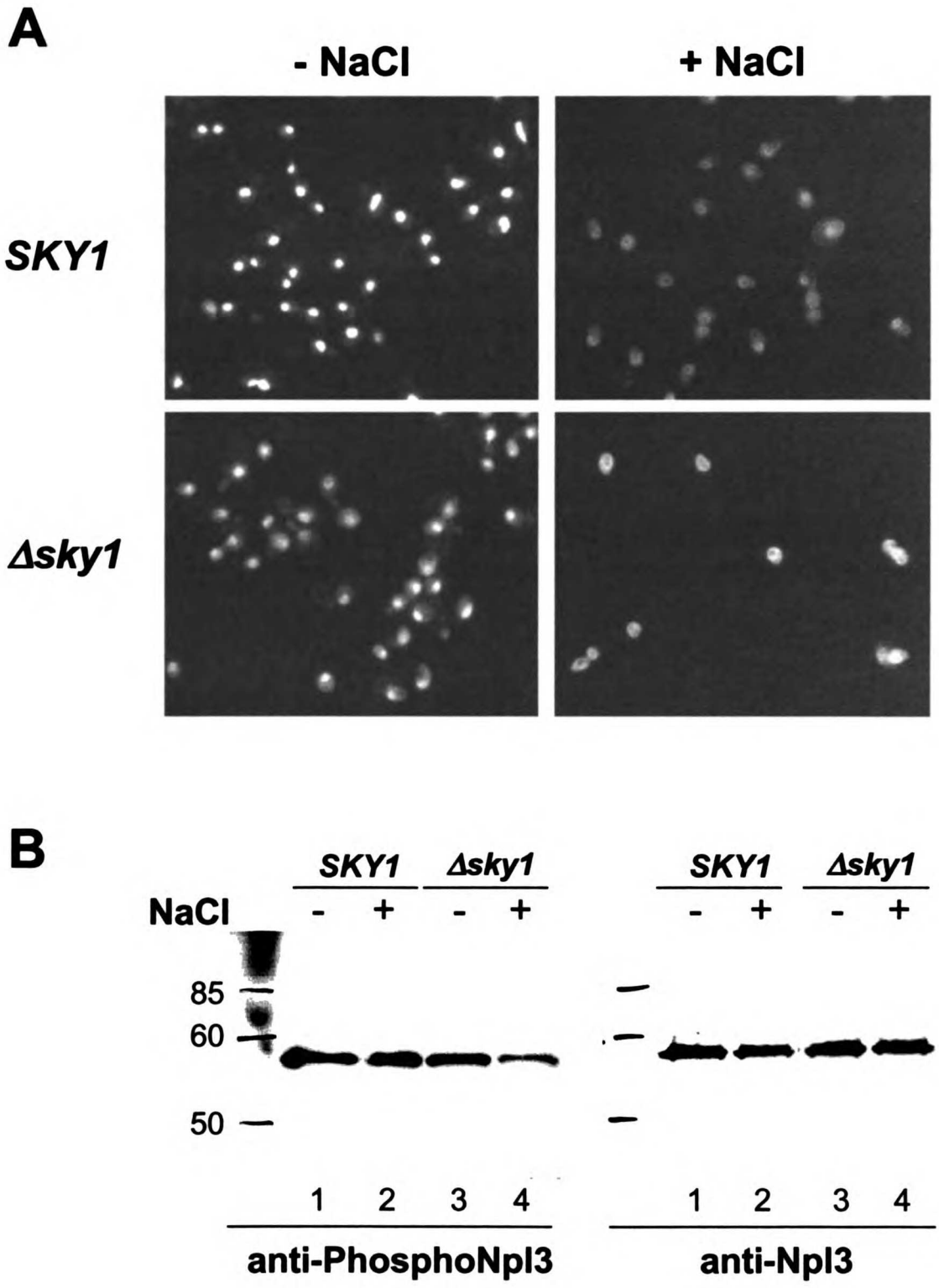

A
- NaCl
+ NaCl
SKY1
Δsky1
B
SKY1
Δsky1
SKY1
Δsky1
NaCl
- + - +
- + - +
85
60
50
1 2 3 4
1 2 3 4
anti-PhosphoNpl3
anti-Npl3

Figure 3. Increased Cytoplasmic Npl3p Is Correlated with Toxic Cation Resistance

Cultures were grown overnight in YPD and then diluted and spotted by serial (6-fold) dilution onto media as indicated. All plates were incubated at room temperature, which is permissive for growth of the temperature-sensitive mutants. *Δmtr10* (**A**) and *npl3-328* (**B**), two mutant backgrounds in which Npl3p is partially mislocalized to the cytoplasm (see text), are resistant to toxic concentrations of NaCl and LiCl, whereas *mex67-5*, which is known to be defective in Npl3p export at higher temperatures (see text), is hypersensitive to LiCl (**C**).

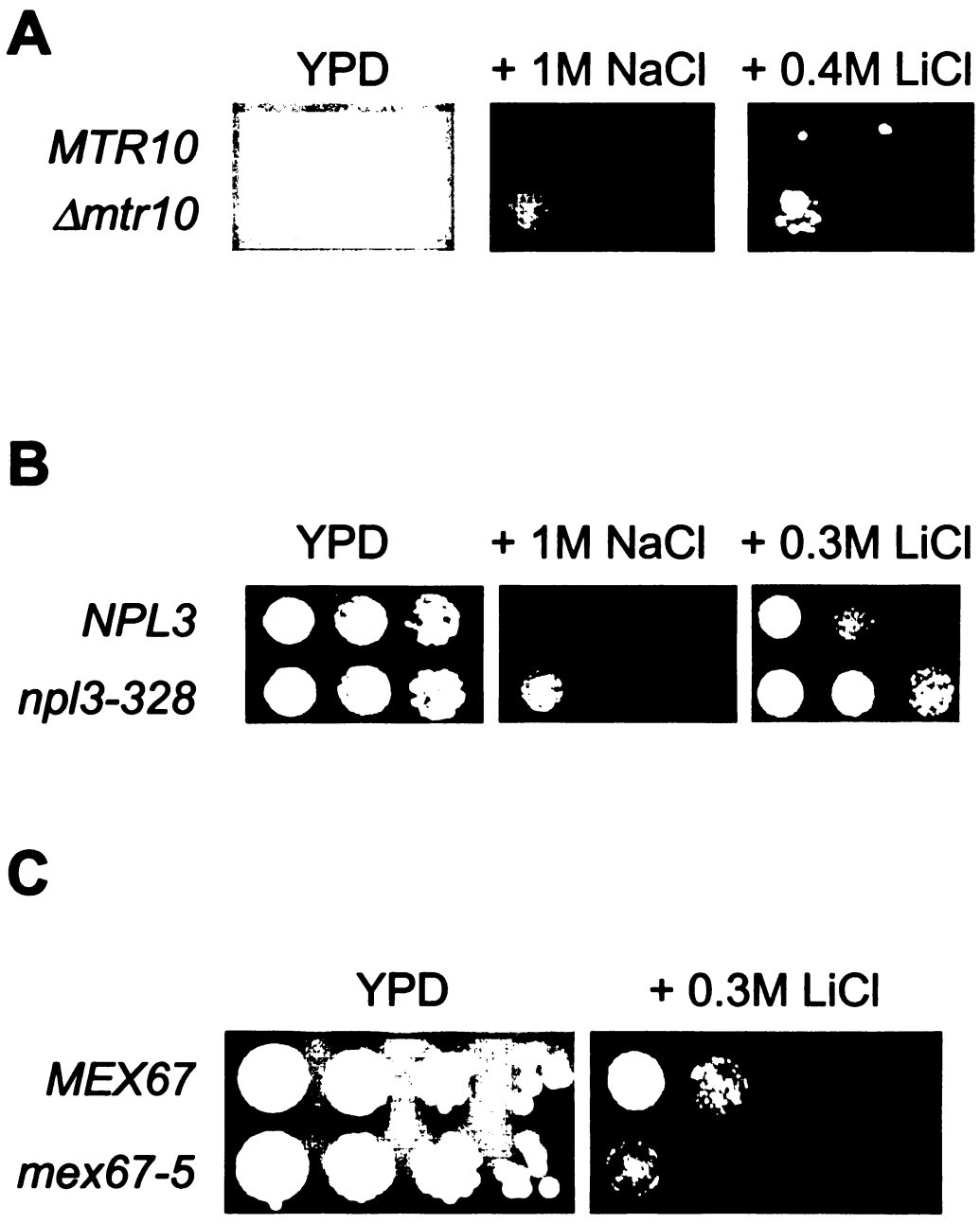

A
YPD
+ 1M NaCl
+ 0.4M LiCl
MTR10
Δmtr10
B
YPD
+ 1M NaCl
+ 0.3M LiCl
NPL3
npl3-328
C
YPD
+ 0.3M LiCl
MEX67
mex67-5

Figure 4. Mutations That Perturb Nab2 Function Cause Hypersensitivity to Toxic Cations

Cultures were grown overnight in YPD and then diluted and spotted by serial (6-fold) dilution onto media as indicated. All plates were incubated at the permissive temperature for the mutants: room temperature for temperature-sensitive *Δtom1* and *kap104-16* mutants, and 30°C for the cold-sensitive *nab2-21* strain. All three mutants are hypersensitive to LiCl compared to isogenic wild-type strains.

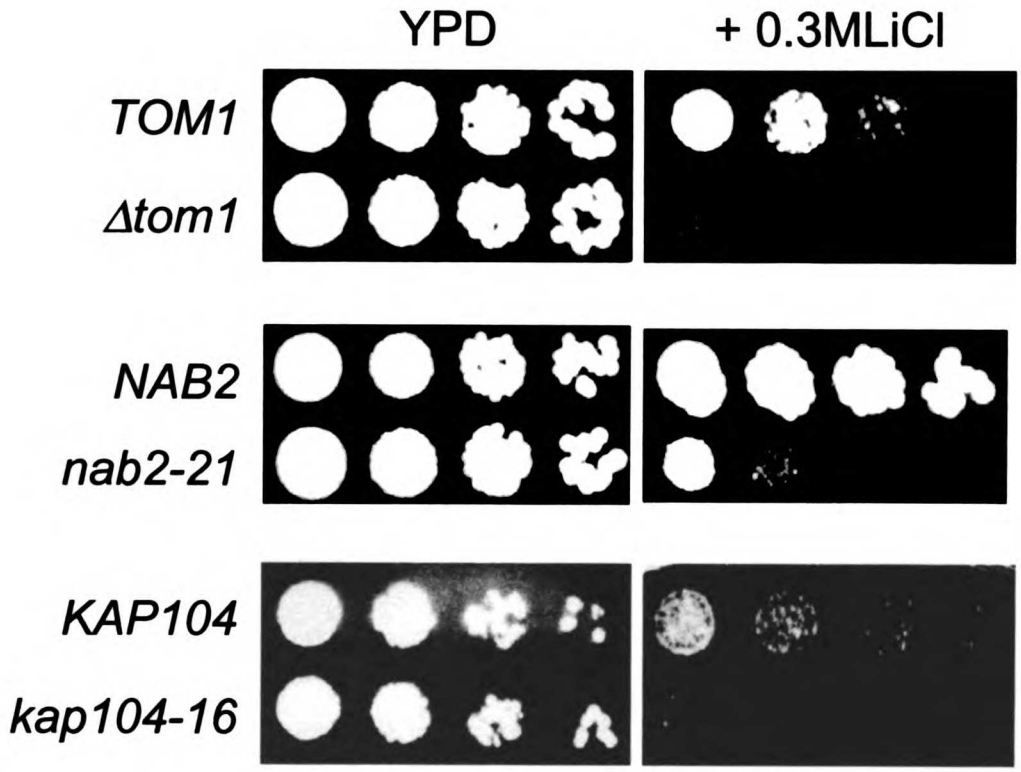
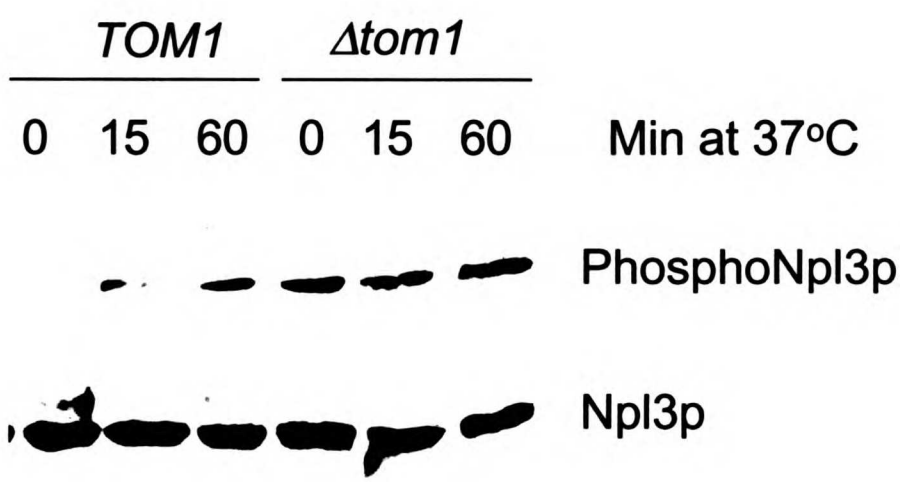

YPD
+ 0.3MLiCl
TOM1
Δtom1
NAB2
nab2-21
KAP104
kap104-16

Figure 5. Increased Phosphorylation of Npl3p Is Likely to Increase Ion-Sensitivity

(A) *Δtom1* shows constitutively increased Npl3p phosphorylation compared to *TOM1*. Note that steady-state Npl3p phosphorylation reproducibly increases in wild-type cells upon shift from room temperature to 37°C. Total cell extracts were prepared from log-phase YPD cultures, which were grown at room temperature before shifting to 37°C for the time indicated, by bead-beating (as described in Chapter 2). Npl3p and phosphoNpl3p were detected by Western blotting with anti-Npl3p (1:2,000) and anti-phosphoNpl3p (1:1,000) followed by goat anti-rabbit HRP conjugated secondary and ECL.

(B) High-copy *SKY1* (2μm plasmid) makes cells more sensitive to LiCl. Cultures were grown overnight in SD-Ura and then diluted and spotted by serial (6-fold) dilution onto SD-Ura +/- 0.3M LiCl as indicated.

A


TOM1
Δtom1
0 15 60 0 15 60
Min at 37°C
PhosphoNpl3p
Npl3p

B

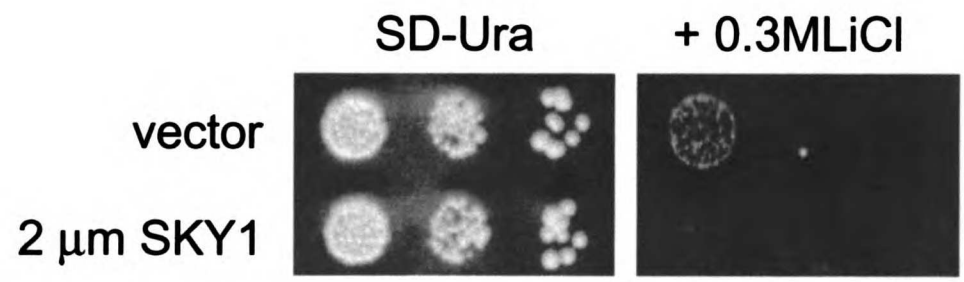

SD-Ura
+ 0.3MLiCl
vector
2 μm SKY1

Figure 6. Npl3p Associates with Splicing Intermediates

^{32}P-labeled actin precursor RNA was incubated in wild-type liquid-nitrogen splicing extract at room temperature for 15 minutes. 1/10th of the total reaction was withdrawn and the RNA isolated by phenol-chloroform extraction and ethanol precipitation. The remainder was incubated with protein A-Sepharose pre-bound to anti-Npl3p rabbit polyclonal antibody or pre-immune serum (1:2 serum:beads) for 3 hours at 4°C with gentle agitation. IP pellets were washed 3X in 50 mM Tris-HCl, pH 7.4/100 mM NaCl/0.5 mM EDTA before RNA isolation as described above. RNA species were separated by electrophoresis on 6.5% TBE-Urea gels and visualized by autoradiography.


1/10th
Total
Pre-
immune
αNpl3p
P
P

Figure 7. A Screen for High-Copy Suppressors of the *Δsky1* Li^R Phenotype

Δsky1 cells were transformed with a 2μm (YEp13, LEU2) genomic library. 13,000 transformants were selected on SD-Leu and replica-plated to 0.4M LiCl. Lithium-sensitive transformants (93 candidates) were rescued from the SD-Leu master plate, and retested by streaking on 0.4M LiCl plates. 79 out of 93 original isolates showed reproducible lithium sensitivity. To test whether the observed lithium-sensitive phenotype was plasmid-dependent, candidates were allowed to lose the plasmids passively by growth in non-selective medium. 3 Leu- isolates from each parent suppressor strain were tested for growth on 0.4M LiCl. 69 out of 79 strains required the high-copy plasmids for their lithium-sensitivity, and were pursued further. These plasmids were recovered from yeast, and assayed for the presence of *SKY1* coding sequence by PCR. 2 out of 69 plasmid inserts contained the *SKY1* ORF. The remaining 67 plasmids appeared to contain unique genomic inserts, based on restriction digest analysis with Dra I. These plasmids were transformed into the parent *Δsky1* strain to confirm their ability to confer lithium-sensitivity; all 67 plasmids retested in this assay. 22 out of 67 plasmids were also found to restore lithium-sensitive growth to the lithium-resistant *npl3-328* strain. One vector-insert junction from each plasmid was sequenced to identify the genomic region containing the suppressing activity. These sequences, together with information regarding likely candidate suppressing ORFs may be found in W. G. Notebook E8.

Screen for high-copy suppressors of *Δsky1* Li^R phenotype

Transform *Δsky1* with 2 μm genomic library
***~13,000** Transformants screened*

↓

Replica SD-Leu to 400 mM LiCl: ***93** Li^S candidates*

↓

Li^S retest (streaks): ***79** Li^S candidates*

↓

Test for plasmid-dependence
***69** plasmid-dependent Li^S candidates*

↓

Recover plasmids: ***2*** (non-identical) = *SKY1* [PCR]
(all others appear unique by DraI digest)

↓

Transform parent *Δsky1 strain*: all 67 retest as Li^S

↓

Transform *npl3-328*: *22/67* suppress Li^R
(frogging on SD-Leu + 400 mM LiCl)

↓

Sequence one end of all 67 inserts
to identify candidate genes

Appendix 2

The Search for Sky2

In Chapter 1 I showed that *Δsky1* reduces but does not abolish phosphorylation of Npl3p in vivo. As discussed in the Epilogue, this residual phosphorylation of Npl3p is functionally significant: unlike *Δsky1*, *npl3S411A* has a constitutive mRNA export defect. Here I provide evidence that phosphorylation of S411 accounts for more than 90% of Npl3p phosphorylation (Fig. 1), and present the results of my directed search for additional Npl3S411 kinases.

I focused my attention on two likely candidates, Yak1, which is the closest sequence homologue of Sky1, and Ctk1, which copurifies with Npl3p (Ho et al., 2002) and is known to recognize Ser-Pro-Thr (the sequence context of Npl3 Ser411) as a phosphorylation site (Cho et al., 2001). I found that *Δctk1* reduces Npl3p phosphorylation in vivo (Fig. 2) and in vitro (Fig. 3), consistent with the hypothesis that Npl3p is a substrate of its binding partner Ctk1p. Furthermore, I discovered that the cold-sensitive *Δctk1* strain has a constitutive mRNA export defect (Fig. 4), which indicates that Ctk1p could be the export-relevant nuclear Npl3p kinase.

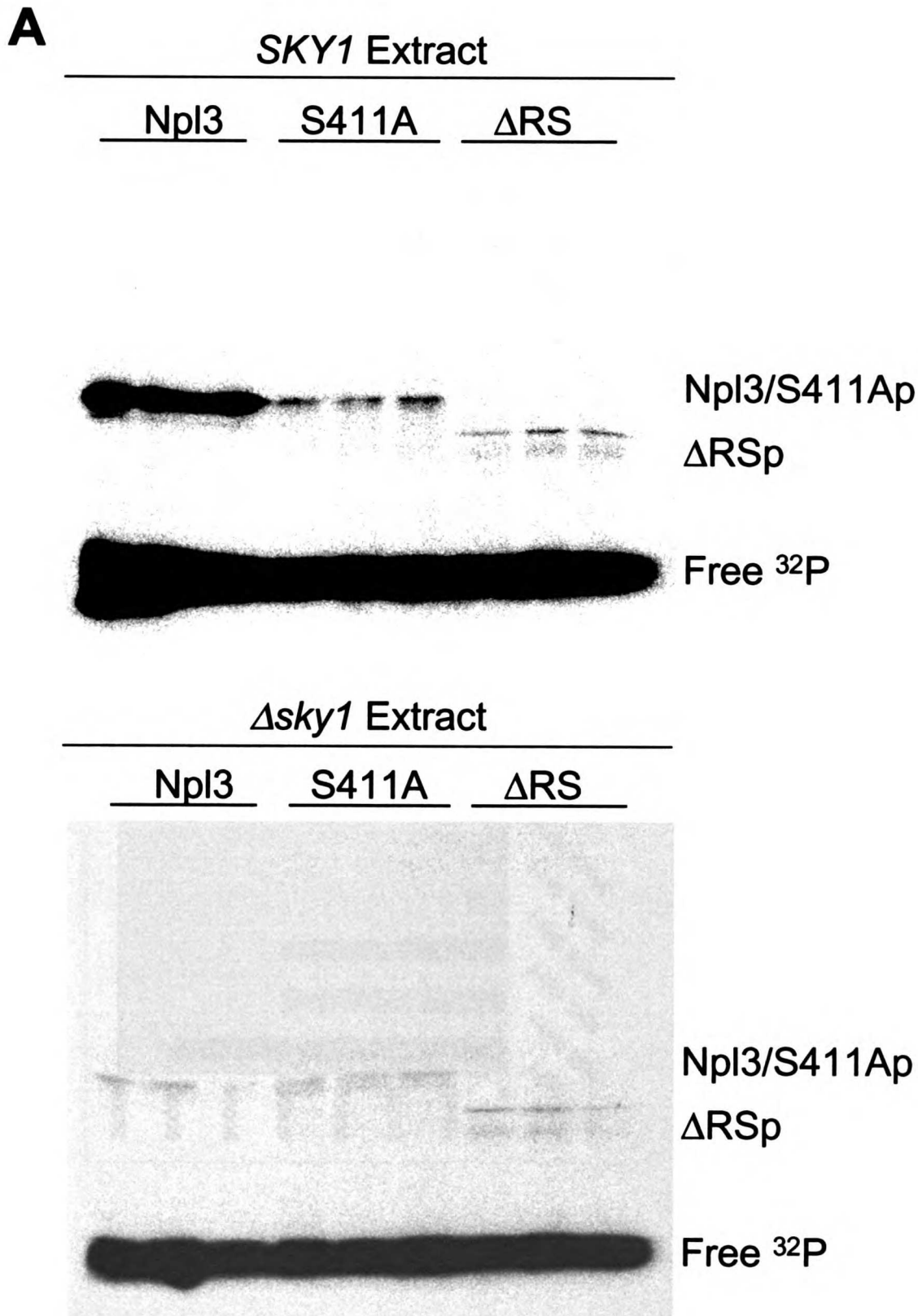
Of course, Ctk1p has another substrate that is very likely to be significant for mRNA export: Serine 2 within the heptad repeats (YSPTSPS) of the CTD of RNA pol II (Cho et al., 2001). Recent studies strongly suggest that Ctk1-dependent phosphorylation is required for coupling transcription to 3' end processing (Ahn et al., 2004; Kim et al., 2004; Ni et al., 2004). These papers assume that the CTD is the only relevant substrate of Ctk1 in this process. While there is ample evidence to suggest that the phosphorylated CTD directly affects the recruitment and/or activity of 3' processing factors (reviewed in (Ahn et al., 2004; Kim et al., 2004; Ni et al., 2004), I would humbly submit that there is also good reason to consider the possibility that the phosphorylation state of Npl3p

affects 3' processing as well. In Figure 5, I propose a model (really a Grand Canonical Ensemble *vis à vis* Npl3 phosphorylation) that incorporates functions for Ctk1-dependent phosphorylation of both Npl3p and the CTD in coordinating events in pre-mRNA processing. Finally, in Figure 6 I show that *Δsky1 Δctk1* does not eliminate in vivo phosphorylation of Npl3S411, thus opening the door for a search for 'Sky3'.

Figure 1. Serine 411 is the Major Site of Npl3p Phosphorylation

(**A**) rHisNpl3/S411A/ΔRSp (purified from E. coli as described, Chapter 1) was used in in vitro kinase reactions with ^{32}P gamma-ATP (as described, Chapter 1) using whole-cell yeast extract as the source of kinase activity. ~2μg of purified recombinant substrate protein was incubated with 10μg of total yeast protein for 45 minutes at 30°C. The substrate proteins were re-isolated via their Histidine tags by incubation with Ni-NTA, as described in Chapter 1, and detected by SDS-PAGE followed by autoradiography.

(**B**) Quantitation of phosphorylation by phosphorimager scans of the gels shown in (**A**). Mutation of serine 411 to alanine reduces phosphorylation of Npl3p in *SKY1* extracts by >90%. Deletion of the RS-domain at the C-terminus of Npl3p reduces phosphorylation to a similar extent. Finally, *Δsky1* extracts are severely defective for in vitro Npl3p phosphorylation, reducing phosphorylation of wild-type Npl3p substrate by almost 10-fold.


A
SKY1 Extract
Npl3
S411A
ΔRS
Npl3/S411Ap
ΔRSp
Free 32P
Δsky1 Extract
Npl3
S411A
ΔRS
Npl3/S411Ap
ΔRSp
Free 32P

B

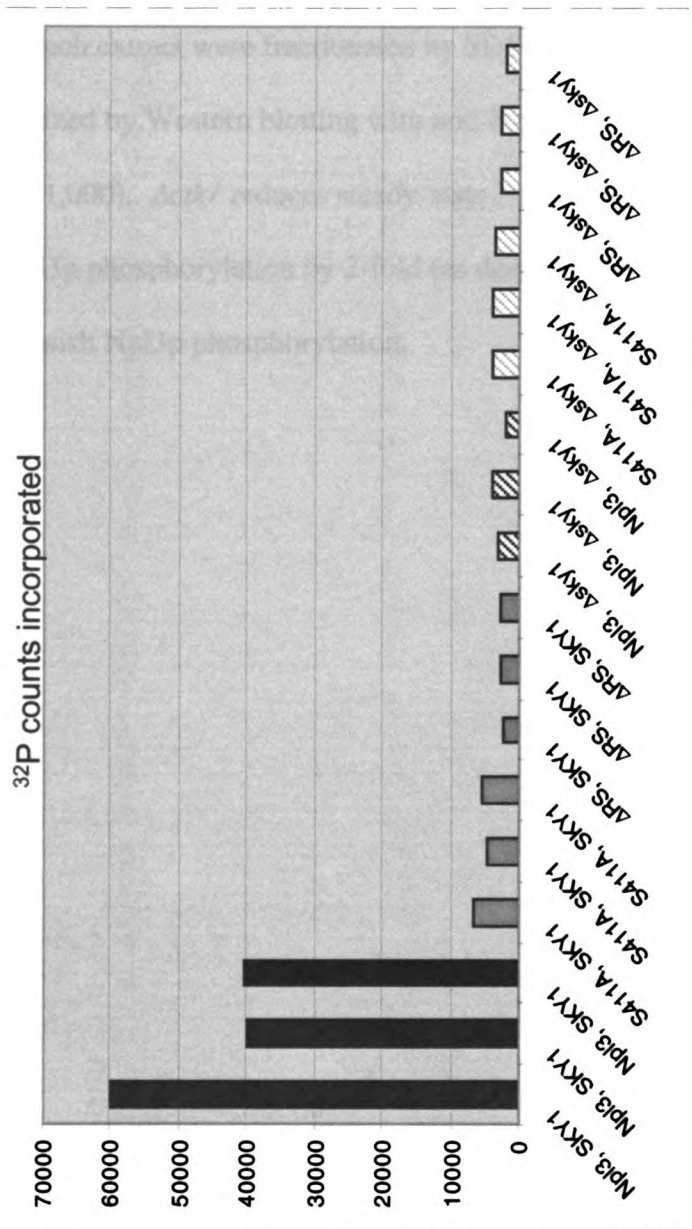

^{32}P counts incorporated
70000
60000
50000
40000
30000
20000
10000
0
Npl3, SKY1
Npl3, SKY1
Npl3, SKY1
S411A, SKY1
S411A, SKY1
S411A, SKY1
ΔRS, SKY1
ΔRS, SKY1
ΔRS, SKY1
Npl3, ASK1
Npl3, ASK1
Npl3, ASK1
S411A, ASK1
S411A, ASK1
S411A, ASK1
ΔRS, ASK1
ΔRS, ASK1
ΔRS, ASK1

Figure 2. *Δctk1* Reduces Npl3p Phosphorylation In Vivo

Steady-state in vivo phosphorylation of Npl3p was assayed in viable kinase knock-out strains essentially as described in Chapter 2. Whole-cell extracts were prepared (by bead-beating) from wild-type and mutant cultures grown to mid-log phase in YPD at 30°C. Two dilutions of each extract were fractionated by SDS-PAGE. Npl3p and phospho-Npl3p were visualized by Western blotting with anti-Npl3p (1:2,000) and anti-PhosphoNpl3p (1:1,000). *Δctk1* reduces steady-state Npl3p phosphorylation ~1.5-fold. *Δsky1* reduces Npl3p phosphorylation by 2-fold (as described in Chapter 1). *Δyak1* does not appear to diminish Npl3p phosphorylation.

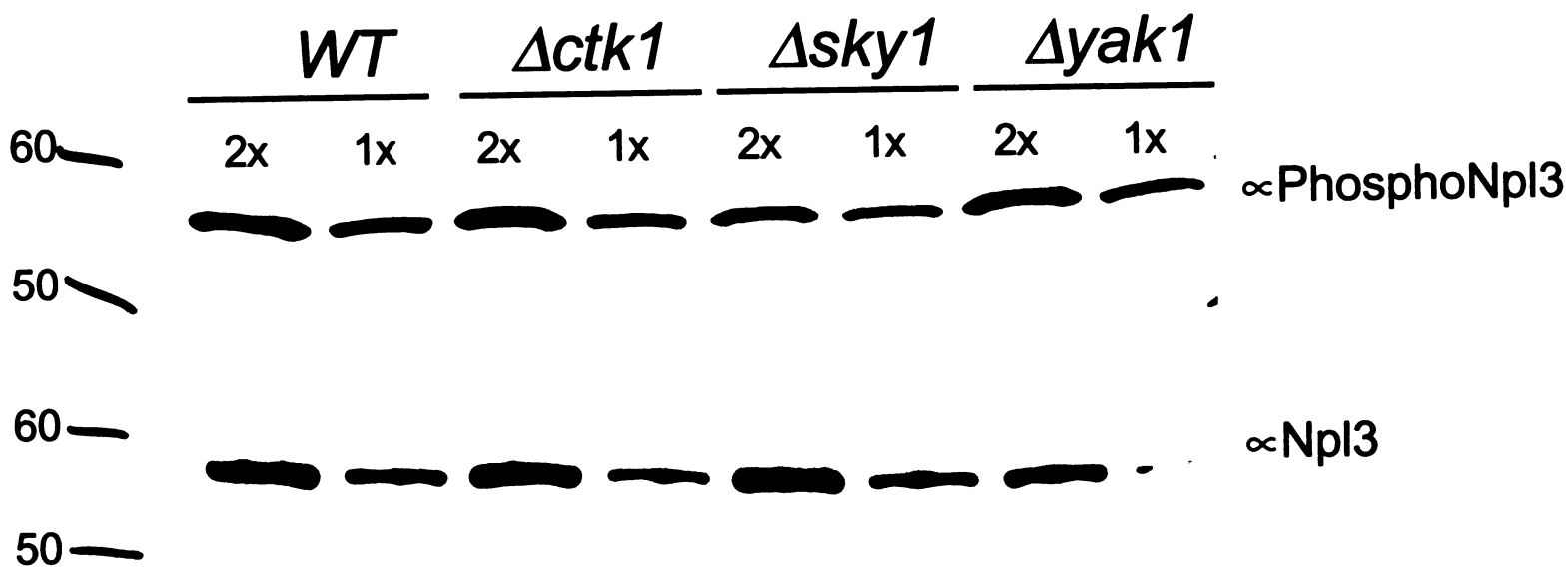
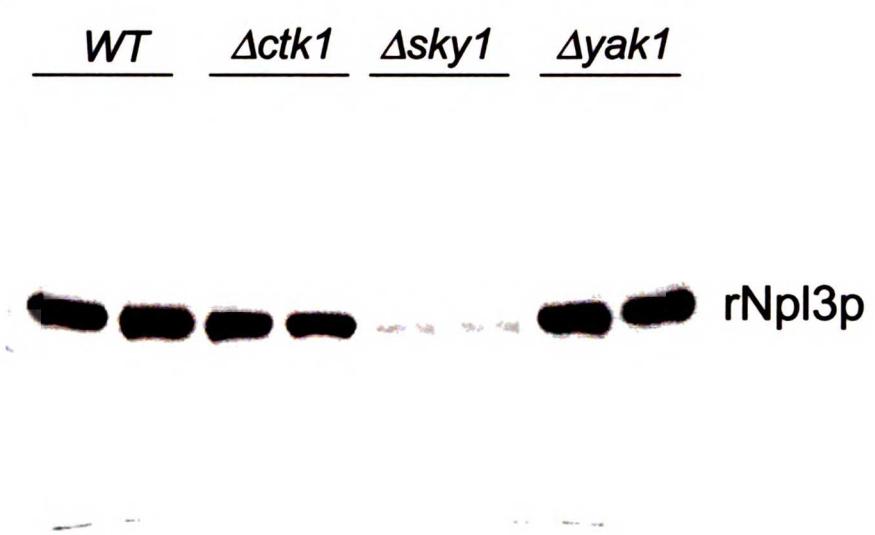

WT
Δctk1
Δsky1
Δyak1
2x
1x
2x
1x
2x
1x
2x
1x
60
50
60
50
∝PhosphoNpl3
∝Npl3

Figure 3. *Δctk1* Reduces Npl3p Phosphorylation In Vitro

In vitro kinase reactions using rHisNpl3p and whole-cell yeast extracts were performed in duplicate (**A**) and quantitated (**B**) as described in the legend to Figure 1. *Δctk1* extracts phosphorylate Npl3p to ~60% (49-73%) of the level seen in wild-type extracts. *Δsky1* extracts phosphorylate Npl3p to ~14% (12-15%) of the level seen in wild-type extracts. *Δyak1* extracts phosphorylate Npl3p to ~82% (66-101%) of the level seen in wild-type extracts.

A


WT
Δctk1
Δsky1
Δyak1
rNpl3p

B

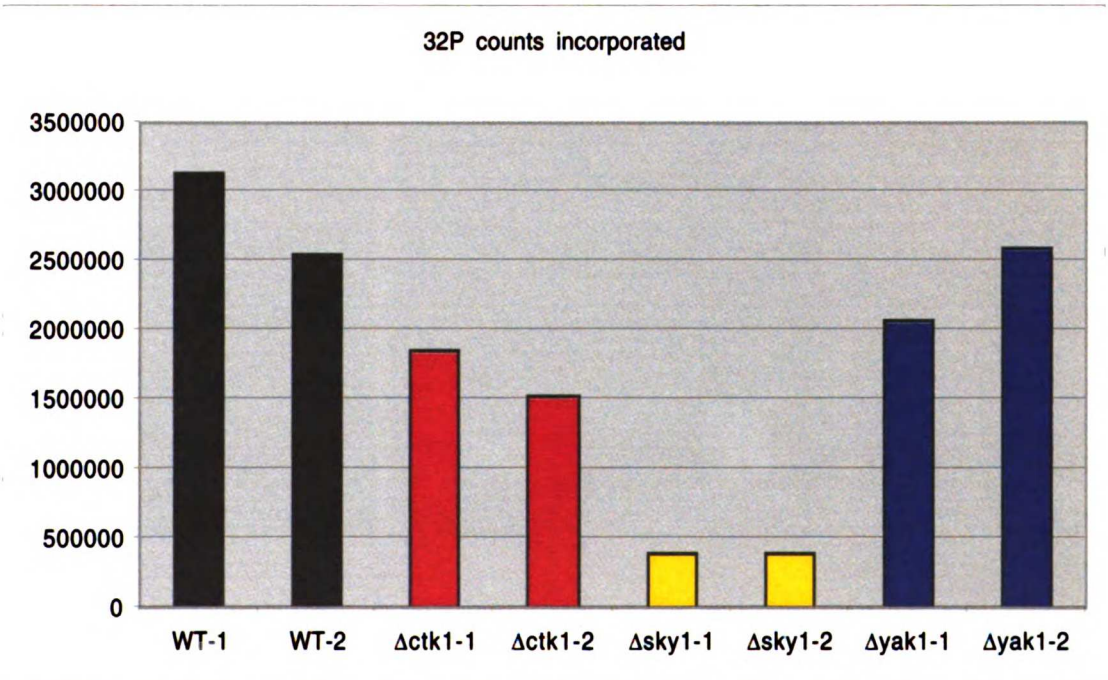

32P counts incorporated
3500000
3000000
2500000
2000000
1500000
1000000
500000
0
WT-1
WT-2
Δctk1-1
Δctk1-2
Δsky1-1
Δsky1-2
Δyak1-1
Δyak1-2

Figure 4. *Δctk1* Is Defective in mRNA Export

Δctk1 cells (left panels) show constitutive accumulation of poly(A)$^+$ RNA in the nucleus at the permissive temperature (30°C) whereas wild-type cells (right panels) show whole-cell poly(A)$^+$ RNA. Poly(A)$^+$ RNA was visualized by in situ hybridization with a FITC-conjugated oligo(dT)$_{50}$ probe as described in Chapter 2. The nuclei are visualized by staining with DAPI.

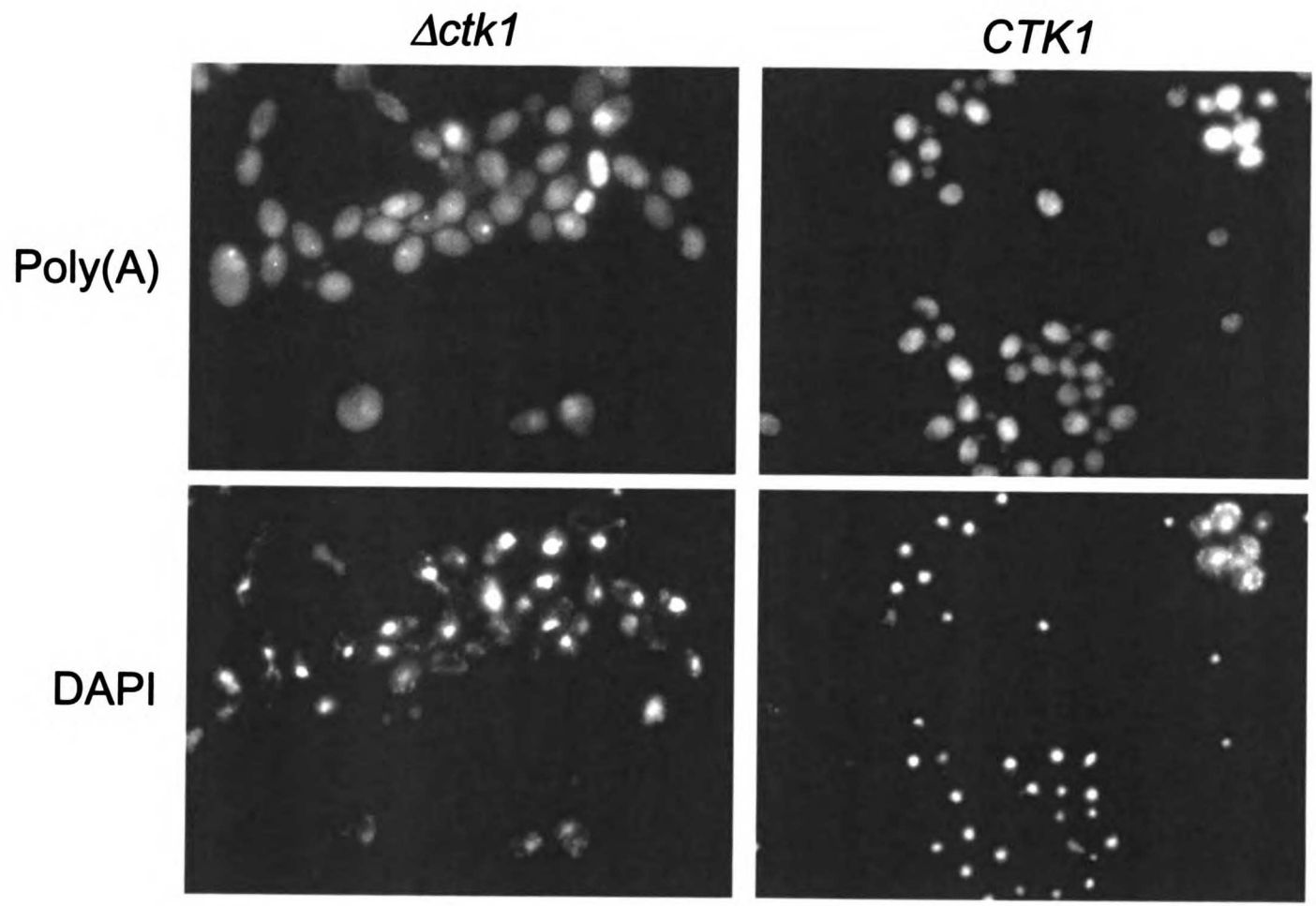

Δctk1
CTK1
Poly(A)
DAPI

Figure 6. *Δsky1 Δctk1* Double Mutants Still Phosphorylate Npl3p

Steady-state in vivo phosphorylation of Npl3p was assayed as described in Figure 2. Double mutant strains were generated by crossing haploid *Δsky1* and *Δctk1* strains, and two tetratype tetrads were analyzed to determine the relative effects of single and double kinase knock-outs on steady-state Npl3p phosphorylation in vivo. Npl3p and phospho-Npl3p were visualized by Western blotting with anti-Npl3p (1:2,000) and anti-PhosphoNpl3p (1:1,000). *Δctk1* reduces steady-state Npl3p phosphorylation ~1.5-fold and *Δsky1* reduces Npl3p phosphorylation by ~2-fold (similar to the results shown in Figure 2). *Δctk1 Δsky1* double mutants still retain ~50% of wild-type Npl3p phosphorylation, similar to the phenotype of *Δsky1* single mutants.

85
60
50
Δsky1 CTK1
SKY1 CTK1
Δsky1 Δctk1
SKY1 Δctk1
∝PhosphoNpl3
Δsky1 CTK1
Δsky1 Δctk1
SKY1 Δctk1
SKY1 CTK1
85
60
50
∝Npl3

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