Title
A role for Tom1p, a putative ubiquitin ligase, in mRNA export

Author
Duncan, Kent E.

Publication Date
2001

Peer reviewed|Thesis/dissertation

A Role for Tom1p, a Putative Ubiquitin Ligase, in mRNA Export

by

Kent E. Duncan

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

Biochemistry
in the

GRADUATE DIVISION

of the

UNIVERSITY OF CALIFORNIA, SAN FRANCISCO

DEDICATION

To the memory of Victoria Webb

A Role for Tom1p, a Putative Ubiquitin Ligase, in mRNA Export

Kent E. Duncan

ABSTRACT

In eukaryotic gene expression the synthesis of mRNAs from genes occurs in the nucleus, whereas the bulk of translation of mRNAs into protein gene products occurs in the cytoplasm. This compartmentalization necessitates transport of many macromolecules across the nuclear envelope from their site of synthesis to their site of function. Detailed understanding of the molecular mechanisms governing mRNA export remains a major challenge in cell biology. Key goals include identifying proteins that function in mRNA export pathways, determining the biochemical functions of these proteins and elucidating how these functions contribute mechanistically to mRNA export. A major additional challenge is to understand how mRNA export is coordinated with other aspects of mRNA metabolism and cellular growth processes.

In this thesis, I describe the identification and characterization of what appears to be a specific mRNA export pathway in *S. cerevisiae*. Chapter 1 presents results from genetic, cytological and biochemical approaches that I used to determine that this mRNA export pathway is differentiated by involvement of the yeast hnRNP protein Nab2p, but apparently not other hnRNP proteins, and a requirement for activity of Tom1p, a predicted hect domain ubiquitin-protein ligase, for efficient export of Nab2p and mRNA. In Chapter 2, I describe genetic and cytological evidence strongly suggesting that the Nab2p import receptor, Kap104p, plays an additional, non-import role in promoting efficient export of Nab2p and mRNA via the Tom1p-mediated Nab2p-mRNA export pathway. This chapter also describes a novel type of genetic interaction, bypass

suppression interference, between specific alleles of *NAB2*, and *PAB1*, the gene encoding the yeast homolog of the mammalian cytoplasmic polyA binding protein. Additional genetic and cytological evidence in this chapter supports a model where Kap104p and Tom1p ubiquitin ligase activity contribute to the efficiency of substitution of Pab1p for Nab2p, and that this mRNP remodeling event may be a key step in efficient mRNA export by this pathway.

The results of my thesis work provide novel molecular insights into mRNA export in *S. cerevisiae* and a mechanistic framework to guide future studies of this fundamental process in eukaryotic gene expression.

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ACKNOWLEDGMENTS

I would like to thank Maki and Jeff for not abandoning me.

PROLOGUE

The major goal of the work described in this thesis was to advance our understanding of molecular mechanisms underlying mRNA export. In particular, I wanted to determine the role that a specific protein, Ssr2/Tom1p, played in this process in the model organism, *Saccharomyces cerevisiae*. I also hoped to identify additional factors that contributed to Ssr2/Tom1p-mediated mRNA export. On the basis of sequence similarity, Tom1p was predicted to be a specificity factor in the ubiquitin protein modification pathway. Since protein modification by ubiquitin has been demonstrated to regulate a wide variety of molecular pathways, I anticipated that focusing on the role of a ubiquitin ligase might provide evidence for regulation of mRNA export and help to elucidate how this fundamental part of eukaryotic gene expression might be coordinated with other aspects of mRNA function and metabolism, as well as more global cellular growth-control mechanisms.

My thesis begins with what will undoubtedly be an increasingly common exercise in the 'post-genomic' era, so-called 'reverse genetics': the identification of a phenotype for an already identified gene, with the goal to assign a cellular and biochemical function to that particular gene product. 'Reverse genetics' contrasts with traditional 'forward genetics' where mutants with a phenotype are sought first and the gene with the relevant mutation is subsequently identified. Forward genetics has the obvious advantage of providing a phenotype of interest upfront, but both methods necessitate extensive follow-up experiments to identify how the mutation produces the phenotype, and, ideally, to verify a direct biochemical role in the process of interest.

My reverse genetics project was performed with *SSR2*, which originally arrived in the lab freezer through a 'forward genetics' project conducted by Jim Umen, when he was a doctoral student in the lab (Umen, 1995). Jim performed a UV-light mutagenesis and selected for extragenic mutations that would suppress the temperature-sensitive growth defect of a U1 snRNA mutant lacking a region called the 'fungal domain', which is conserved in fungi, but not other eukaryotes (Kretzner et al., 1990). With this approach, Jim hoped to identify proteins that would modulate the interaction of U1 snRNP with the 5' splice site of pre-mRNAs and play key roles in the pre-mRNA splicing pathway in *S. cerevisiae*. One viable suppressor strain was recovered and Jim determined that it had mutations in two genes, which he named *SSR1* and *SSR2* (Suppressor of SnRNA). His analysis, described in Appendix 2 of his Ph.D. thesis, indicated that *ssr2-1* was itself a temperature-sensitive allele and that *ssr1-1*, itself mildly temperature sensitive, appeared to be a suppressor of *ssr2-1* temperature sensitivity. Thus, a complex network of genetic interactions restored viability to yeast with a conditionally lethal allele of U1 snRNA. Jim cloned *SSR2* by identifying low-copy suppressors of the *ssr2-1* temperature sensitivity and found that the putative clone encoded a 3268 amino acid protein with a homology to E6-AP carboxy terminus (hect) domain, the signature of a family of ubiquitin-protein ligases (Huibregtse et al., 1995b; Huibregtse et al., 1994). He also showed that *ssr2-1* had a variety of positive and negative genetic interactions with U1 snRNA, U5 snRNA (allelic to *slu12-1*(Frank, 1993), and *prp3-1* (Hartwell et al., 1970) (non-allelic non-complementation). Jim also undertook a series of preliminary biochemical analyses to demonstrate a role for Ssr2p in splicing, which were also described in Appendix 2 of his Ph.D. thesis.

With this backdrop, I entered the picture, intrigued by the possibility that a putative ubiquitin ligase might be playing a role in the pre-mRNA splicing pathway. A particularly interesting possibility was that Ssr2p might contribute to regulation of splicing by catalyzing post-translational modification of a splicing factor with ubiquitin. Ubiquitin is an 8.5 kd protein that is one of the most conserved proteins in the eukaryotic kingdom. Attachment of ubiquitin to epsilon amino groups of lysine side chains of many proteins has been shown to promote degradation of these proteins by targeting them to a multicomponent protease, the 26S proteasome (Attaix et al., 2001; Bochtler et al., 1999; Ciechanover, 1994; Ciechanover et al., 2000; Hershko and Ciechanover, 1998; Hochstrasser, 1996; Hochstrasser et al., 1999).

An elaborate cascade of enzymes controls the transfer of ubiquitin to target proteins (Figure 1) (Hershko and Ciechanover, 1998). The ubiquitin activating enzyme (UBA/E1) uses the energy of ATP hydrolysis to promote formation of a thioester with its active-site cysteine residue. Subsequently, E1 can transfer ubiquitin to the active site cysteine of a ubiquitin conjugating enzyme (UBC/E2). There are 13 E2's in yeast and probably more in humans (Hochstrasser et al., 1999). The UBC's impart a level of specificity to the pathway, which is enhanced by the requirement for a ubiquitin-protein ligase (E3), operationally defined as a factor that binds to an E2 and a specific target protein and promotes specific ubiquitination of the target protein (Pickart, 2001b). Ubiquitin ligase activity of the hect proteins requires thioester formation between ubiquitin and a conserved active-site cysteine residue (Scheffner, 1998). This was originally shown with the hect domain protein E6-AP, in an elegant study that demonstrated a 'thioester cascade' from E1 to E2 to hect E3, providing compelling

biochemical evidence for the potential of hect E3's to provide specificity in ubiquitination reactions (Scheffner et al., 1995). Subsequent studies have supported the generality of this biochemical model, and have identified a wide variety of highly diverse substrates for the hect ubiquitin ligases (Beaudenon et al., 1999; Huibregtse et al., 1995b; Maki et al., 1996).

Thus, Ssr2p via its genetic interactions with splicing factors and predicted biochemical activity seemed a good candidate for a protein that might regulate splicing via ubiquitination. One intriguing prospect was that regulated proteolysis might play a role in splicing. However, at the time when I began the studies described in the rest of the thesis, a regulatory role for ubiquitin that didn't involve proteolysis (subsequently demonstrated in several cases) (Chen et al., 1996; Hicke, 1999; Hicke, 2001; Terrell et al., 1998) was unprecedented, and the prospect of identifying a novel function for ubiquitination made this project more appealing. Moreover, coordination of splicing with other cellular processes, which is widely anticipated to occur via post-translational modification, remains a fascinating but highly mysterious aspect of pre-mRNA splicing. At the time, besides the identification of the hect domain, the only other hint about the possible function of Ssr2p came from an entry in the Saccharomyces Genome Database describing it's characterization as *TOM1* (trigger of mitosis), an unpublished result from the Kikuchi lab, who had screened for temperature sensitive mutants that arrested the cell cycle in mitosis. While this phenotype now seems highly likely to be a downstream consequence of effects on gene expression in *tom1* mutants, this is the first registered name for *SSR2*, and I will therefore henceforth refer to *SSR2* as *TOM1*, and the original

ssr2-1 mutant as *tom1-ssr* in the remainder of the thesis. Thus, the function of Tom1p was essentially unknown at the time I initiated my thesis work.

Unfortunately, as I followed up Jim's original characterization of the *tom1-ssr* mutant, the effects on splicing *in vivo* in temperature shift experiments were relatively subtle and appeared substantially after the divergence of *TOM1* and *tom1-ssr* growth curves, suggesting they might be secondary defects (KD, unpublished results). Moreover, while Jim had reported that splicing extract from *tom1-ssr* was constitutively inactive for splicing *in vitro*, I used a more consistently reliable protocol to prepare extracts from *tom1-ssr* and *Δtom1* and found that these extracts spliced as well as wild type extracts, even when 'heat inactivated' under a variety of buffer conditions that had been used to heat inactivate extracts from other temperature sensitive mutants in *bona fide* splicing components. Thus, a direct role of Tom1p in splicing began to seem unlikely to me.

These negative results raised the possibility that Tom1p's primary function might be in another cellular process. I reasoned that this might be another aspect of mRNA metabolism and, using a fluorescence in situ hybridization (FISH) assay (Amberg et al., 1992; Kadowaki et al., 1992) tested *tom1-ssr* for defects in mRNA export. At that time, we surmised there must be a molecular link between pre-mRNA splicing and mRNA export, but the only evidence to suggest this was the identification of cold-sensitive *brr* mutants with defects in both processes ((Noble and Guthrie, 1996) Anne debruynKops and CG, unpublished results). Thus, it was of great interest to identify additional mutants with connections to both splicing and export. Indeed, *tom1-ssr* showed a clear defect in mRNA export using oligo dT50 FISH to analyze sub-cellular localization of the polyA

tail (Duncan et al., 2000). Since the accumulation of nuclear polyA RNA appeared within an hour of a shift to 37° C and was very strong at 90 minutes, well before growth curves diverged or splicing defects were manifest, I turned the focus of my thesis work to what seemed much more likely to be a primary defect associated with the absence of Tom1p function.

At the time when I made this transition, nucleocytoplasmic transport processes were only beginning to be dissected at the molecular level. Indeed, the identification of importin β/α heterodimers as factors that bound to basic nuclear localization signals (now referred to as the 'classical NLS') and mediated their docking to the nuclear pore complex (NPC) and subsequent translocation into the nucleus had only recently been demonstrated (Gorlich and Laskey, 1995). The role of the small GTPase Ran in transport was still unclear and one popular model was that, by analogy to G-proteins, there would be an 'activated' state that would 'license' a Ran complex to pass through the nuclear pore (Moore and Blobel, 1994). That there would be a whole family of karyopherins mediating transport (Gorlich and Kutay, 1999) was not yet known. Moreover, a relatively small set of nucleoporins had been identified and functionally characterized (Davis, 1995). Now, it is well-documented that there are multiple karyopherin family members acting as import or export receptors that promote translocation of cargo through the NPC. These proteins perform this function by binding to the cargos, often directly, and simultaneously interacting with FG repeat-containing nucleoporins. Directionality is conferred by the nucleotide state of Ran: Ran-GTP is enriched in the nucleus, stabilizing exportin-cargo interactions and destabilizing importin-cargo interactions, the opposite is true for Ran-GDP (Gorlich and Laskey, 1995; Ryan and Wente, 2000; Wente, 2000).

We knew even less about mRNA export and the mediating factors. It was clear that, like all exchange between the nucleus and cytoplasm characterized to date, mRNA export occurred through NPC's (Maquat, 1991). However, most of the factors now believed to be key mediators of this process had not yet been identified. A pioneering study using microinjection methods in *Xenopus laevis* oocytes had demonstrated that RNA export pathways were saturable and temperature-dependent, indicating they were receptor-mediated (Jarmolowski et al., 1994). Moreover, the same study supported a model in which different classes of RNA (e.g. tRNA, rRNA, UsnRNA, mRNA) did not compete with one another for nuclear export. Together, these results implied the existence of distinct pathways for export of different classes of RNA mediated by specific export receptors. The discoveries of mRNA export receptors like TAP/mex67 (Conti and Izaurralde, 2001), REF/Yra1p (Stutz et al., 2000; Zenklusen et al., 2001), and an exon-exon junction complex that promotes mRNA export (Le Hir et al., 2001; Le Hir et al., 2000) were still well in the future.

One of the major models for mRNA export that emerged in these 'early days' proposed that the so-called shuttling heterogeneous nuclear ribonucleoproteins (hnRNPs) acted as adapters for mRNA export (Izaurralde and Mattaj, 1995). The common view is that ribonucleoprotein particles (RNPs), rather than naked RNA, are the export substrates that interact with the export receptor implicated by the microinjections studies discussed above (Jarmolowski et al., 1994; Mili et al., 2001). In the nucleus pre-mRNAs and mRNAs associate with hnRNP proteins in so-called heterogeneous nuclear ribonucleoprotein particles (hnRNP's) (Dreyfuss et al., 1989; Dreyfuss et al., 1993; Weighardt et al., 1996). HnRNP proteins are nuclear-localized RNA-binding proteins

that typically associate with mRNA co-transcriptionally and 'package' the nascent transcripts into 'ribonucleosome' structures. There are more than 20 abundant hnRNP proteins in mammalian nuclei, comprising a wide variety of different classes of RNA-binding motifs in diverse modular protein domain arrangements (Dreyfuss et al., 1993).

Immunofluorescence and biochemical studies had initially demonstrated that hnRNP proteins were highly enriched in the nucleus (Choi and Dreyfuss, 1984; Dreyfuss, 1986; Leser et al., 1984; Matunis et al., 1992a; Matunis et al., 1992b; Pinol-Roma et al., 1989b) suggesting they might be confined to this compartment. However, elegant experiments using inter-species heterokaryons revealed that a subset of hnRNP proteins 'shuttle' rapidly between the nucleus and cytoplasm, presumably appearing exclusively nuclear at steady state due to rapid re-import from the cytoplasm (Pinol-Roma and Dreyfuss, 1992). Shuttling hnRNP proteins contain sequences that function as both nuclear import (NLS) and nuclear export (NES) signals, and appear to be unique to shuttling hnRNP proteins (Michael, 2000). For this reason it was proposed that these proteins might be adapters for mRNA export, actively promoting mRNA export through interaction with a specific receptor that binds to the nuclear shuttling sequence and the NPC, 'docking' the hnRNP complex at the NPC and subsequently promoting translocation of the RNP complex through the NPC.

The strongest evidence supporting the shuttling hnRNP protein-mediated model for mRNA export comes from extensive studies of the shuttling hnRNP protein, hnRNPA1. HnRNPA1 is a highly abundant hnRNP protein with a '2xRBD-Gly' domain structure featuring 2 tandem RNA binding domains of the RRM/RNP consensus type, and a c-terminal glycine rich region containing an RGG box RNA binding motif and the

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M9 transport signal (Dreyfuss et al., 1993). Import and export of hnRNPA1 is mediated by a sequence called M9 that is required for import and export of A1 and is sufficient to act as an NLS and NES in a heterologous context (Michael et al., 1995a). M9 is distinct from the A1 RNA binding domains and has no RNA-binding activity on its own, implying that M9-mediated export of A1 is unlikely to be due to A1 'piggybacking' on mRNA bound to a different export receptor. Importantly, full length hnRNPA1 has been functionally implicated in mRNA export by microinjection assays in *Xenopus laevis* oocytes (Izaurralde et al., 1997). In these studies, co-injection of high levels of A1 with mRNA export substrates specifically inhibited export of the mRNAs, presumably due to competition with endogenous hnRNPA1 export complexes for an export receptor. The M9 sequence plays a key role in this process: co-injection of A1 lacking the M9 sequence (a protein known to retain full non-specific RNA binding activity in vitro) fails to compete with endogenous factors for mRNA export (Izaurralde et al., 1997). This implies that the unidentified export receptor interacts with M9.

The shuttling hnRNP protein adapter model has become less favored of late, primarily for two reasons. First, the apparent failure thus far to identify the putative export receptors for hnRNP protein NES's has led to the belief that such factors may not exist. Second, the identification of TAP/Mex67, REF/Yra1as mRNA export factors that can associate with each other to form a complex with high affinity for both mRNA and the NPC would seem to obviate the need for hnRNP proteins to be mediators of export (Zenklusen and Stutz, 2001). Of course neither of these is a particularly compelling argument. The identification of one mRNA export receptor need not imply that there are no others, any more than the identification of one transcription factor implies there are no

others. Moreover, failure to identify an export receptor for a particular shuttling sequence simply suggests that the correct assays may not yet have been found that would enable receptor identification. Given the evidence that at least hnRNPA1 may be directly contributing to mRNA export (Izaurralde et al., 1997), it would seem premature to conclude that shuttling hnRNP proteins make no important contribution to mRNA export. Indeed, a report published within the final two weeks prior to submission of this dissertation describes a major new experimental approach that provides evidence for direct roles for specific hnRNP proteins as adapters in the export of specific subsets of mRNAs in mammalian cells, suggesting that this model may be about to enjoy a renaissance (Gallouzi, 2001).

An additional important aspect of mRNA export processes is the substitution of cytoplasmic mRNP proteins for nuclear hnRNP proteins. This process, referred to as 'RNP remodeling,' remains a poorly understood aspect of mRNA export, presumably due to the difficulty of studying what is likely to be a highly dynamic process, potentially involving multiple, short-lived biochemical interactions. Results from several different experimental systems have suggested that RNP remodeling is likely to be a dynamic, multi-stage process with some hnRNP proteins leaving the hnRNP complex prior to docking at the NPC, some leaving coincident with docking/translocation, and still others remaining associated with mRNA in polysomes (Daneholt, 1997; Daneholt, 1999; Daneholt, 2001). Moreover, a number of recent reports indicate that association of mRNA export factors in the nucleus after pre-mRNA processing represents an additional key aspect of RNP remodeling (Kataoka et al., 2000; Kim et al., 2001; Le Hir et al., 2001; Le Hir et al., 2000; Luo et al., 2001; Reed and Magni, 2001; Zhou et al., 2000).

Dbp5p is a yeast DEAD box family ATPase that is implicated in mRNA export (Snay-Hodge et al., 1998; Tseng et al., 1998). Dbp5p is primarily localized to the cytoplasmic fibrils that project from the NPC, and for this reason, and because of its predicted biochemical activity of RNA unwinding or protein displacement from RNA, it has been proposed that mRNP re-modeling would be a post-export event catalyzed by Dbp5p at the cytoplasmic fibrils. While attractive in many ways, there remains no direct evidence for this model. Even if the Dbp5p model does prove correct, it seem likely to be incomplete, given the multiple lines of evidence just described above indicating that mRNP re-modeling may not be a discrete event and may not occur primarily in the cytoplasm. In sum, 'mRNP remodeling' remains a complex, poorly-understood aspect of mRNA export.

While exact homologs of hnRNP proteins cannot be readily identified in *S. cerevisiae*, yeast have a number of shuttling hnRNP-like proteins that are implicated in mRNA export, including Npl3p/Nab1p, Nab2p, and Nab4/Hrp1p. These proteins were all found to be abundant nuclear proteins that can be UV-crosslinked to polyA tails *in vivo* (Anderson et al., 1993; Bossie et al., 1992; Henry et al., 1996; Wilson et al., 1994). It has subsequently been shown that conditional alleles of the corresponding essential genes display defective mRNA export at the non-permissive temperatures. As has been shown for mammalian hnRNP proteins, these proteins also influence diverse aspects of mRNA metabolism (Krecic and Swanson, 1999). For example, Nab4/Hrp1p is implicated in identification of the cleavage site during pre-mRNA 3'-end processing (Kessler et al., 1997; Minvielle-Sebastia et al., 1998), and in mRNA surveillance by marking mRNAs for the nonsense codon-mediated decay pathway (Gonzalez et al.,

2000). Similarly, Nab2p was recently implicated in coupling of polyA tail length regulation and mRNA export, suggesting a role analogous to mammalian PABP2 (Wahle, 1991a; Wahle, 1991b; Wahle et al., 1991), the nuclear polyA binding protein in mammals (Nykamp and Swanson, personal communication, manuscript in preparation).

The most studied of the yeast hnRNP proteins in the context of mRNA export is Npl3p. Multiple conditional alleles of *NPL3* display mRNA export inhibition, and Npl3p was the first yeast hnRNP protein shown to shuttle in both a yeast heterokaryon assay and in a different assay using a conditional allele of the nucleoporin, *NUP49*, that blocks reimport (Flach et al., 1994; Lee et al., 1996). In the *NUP49* assay, shuttling depends on ongoing mRNA synthesis (Lee et al., 1996). Consequently, Npl3p was proposed to be an 'important mediator' of mRNA export. *NPL3* mutants also display synthetic lethality with deletions of genes encoding the nuclear cap binding complex and the major yeast arginine methyltransferase, *HMT1* (Shen et al., 1998). The same study showed that deletion of *HMT1* slows Npl3-GFP export, although this had no discernible effect on mRNA export or yeast viability. Interestingly, unlike Nab2p or Nab4p, Npl3p shows significant sequence similarity to the SR family of shuttling nuclear mRNA binding proteins, which have also been shown to be essential splicing factors in metazoans (Reed and Magni, 2001; Smith and Valcarcel, 2000). Like SR proteins, Npl3p is a phosphoprotein, and an allele of Npl3p lacking the site for the cytoplasmically-localized Sky1p kinase (the yeast homolog of the SR protein kinase, SRPK1), shows increased localization in the cytoplasm and increased interaction with polyA RNA as measured in an *in vivo* UV crosslinking assay, suggesting a role for phosphorylation in promoting efficient release of Npl3p from mRNA (Gilbert et al., 2001).

An intriguing possibility, suggested by existence of multiple shuttling hnRNP proteins in both yeast and higher eukaryotes, is that there may also be multiple hnRNP-protein mediated mRNA export pathways. One exciting corollary to this idea is that specific mRNA transcripts might be biased towards or wholly committed to a particular mRNA export pathway by virtue of preferential association with specific hnRNP proteins. Clearly, if this were the case it would provide a potential additional mechanism for control of gene expression. When my thesis work was initiated there was evidence for differential association of hnRNP proteins with nascent transcripts (Matunis et al., 1993; Pinol-Roma et al., 1989b) and differential affinity for particular sequences as measured using *in vitro* selection systems (Burd and Dreyfuss, 1994; Swanson and Dreyfuss, 1988a; Swanson and Dreyfuss, 1988b). However, there was no evidence to suggest the specificity observed in these approaches had any functional consequence in mRNA export.

In Chapter 1, a reproduction of a paper originally published in Current Biology (Duncan et al., 2000), I provide evidence for distinct, differentially-controlled mRNA export pathways in yeast. The pathways are distinguished by the hnRNP proteins involved and a requirement for the nuclear ubiquitin ligase Tom1p for efficiency. In the chapter, I show that Tom1p is required for growth and efficient mRNA export at high temperatures. I also show that Tom1p localizes to the nucleus and demonstrate via site-directed mutatgenesis that Tom1p's role in promoting efficient mRNA export is intimately related to its predicted ubiquitin ligase activity. A particularly provocative finding was that the hnRNP protein Nab2p and polyA RNA accumulated at the nuclear periphery in close proximity to the NPC in *Δtom1* mutants at 37° C. None of the other

yeast hnRNP proteins I tested displayed mislocalization under these conditions. Indeed, while I found that export of Nab2p was inhibited in *Δtom1* mutants, Npl3p export was inhibited in other mRNA export mutants, but not in *Δtom1*. By *in vivo* UV crosslinking methods, I demonstrated that Nab2p association with polyA RNA increased, while that of Npl3p decreased slightly, but was still clearly present, implying not only that Npl3p was efficiently exported in *Δtom1* strains, but that it was likely to be efficiently exported in association with mRNA. These results provide evidence for the existence of distinct hnRNP export pathways for Nab2p and Npl3p, and imply these separate hnRNP protein export pathways also constitute separate mRNA export pathways. The results also raised the possibility of regulation of the Nab2p-mediated pathway by Tom1p's predicted ubiquitin ligase activity.

One of the major questions that arose from the work described in Chapter 1 surrounded the identity and mechanistic roles of additional molecular components of the Nab2p-mRNA export pathway affected by loss of Tom1p function. In Chapter 2, I describe the results I obtained using genetic and cytological approaches to address this question. One of the major implications of these studies is that Kap104p, the Nab2p import receptor (Aitchison et al., 1996), has a novel non-import function in Tom1p-dependent Nab2p-mediated mRNA export. This case is supported by our observation of an apparently novel type of genetic interaction, "bypass suppression interference," between mutations affecting the Kap104p binding domain in Nab2p and *PAB1*, the gene encoding the homolog of the cytoplasmic polyA binding protein, PABP1, in *S. cerevisiae* (Adam et al., 1986; Sachs et al., 1986). These alleles of nab2 interfere with the ability of high copy PAB1 to bypass the requirement for NAB2. Bypass suppression interference

by these particular *NAB2* alleles implicates Kap104p in mRNP remodeling from a Nab2p-associated to a Pab1p-associated mRNP during the mRNA export process. Additional studies described in Chapter 2 support this model, and also implicate Tom1p function in promoting an efficient 'Nab-to-Pab' transition during Nab2p-mediated mRNA export.

As is often the case with Ph.D. theses, many of the experiments I have performed during my thesis work have produced provocative results that are on their own incomplete contributions or the result may be clear, but the significance is not yet clear. These findings are presented in the Appendices. Appendix 1 describes experiments addressing the question of how specific the *Δtom1* effects are vis-à-vis the ubiquitination machinery and provides evidence that *ubc3/cdc34-1* mutants phenocopy *Δtom1*-suggesting that Cdc34p (Tyers et al., 1992) may be the UBC/E2 that transfers ubiquitin to Tom1p in its role in mRNA export. Appendix 2 describes a tantalizing preliminary finding that implicates the nucleoporin Nsp1p (Wimmer et al., 1992) in translocation during mRNA export, and is presented in the context of exciting future experimental avenues suggested by this result. A particularly exciting prospect is the use of this finding as the basis for establishing a new biochemical tool for probing the mRNA export pathway through the NPC. Taking an even longer term view, as described in Appendix 2, one can envisage using it as the basis for an assay to characterize the 'transportome' in any mutant background or cellular state of interest.

In sum, my thesis work has made progress toward the goals that originally motivated it, having provided insight into the molecular mechanisms underlying mRNA

export in *S. cerevisiae*, and having also provided several intriguing prospects for future analyses.

Figure 1. Overview of ubiquitin transfer reactions.

A schematic diagram of a ubiquitin thioester cascade from UBA/E1, to UBC/E2, to a hect domain ubiquitin protein ligase/E3 is shown. The thioester from the E3 is an obligate intermediate in the transfer of ubiquitin from the hect ubiquitin ligase/E3 to lysine side chains of the substrate.

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CHAPTER 1

A Putative Ubiquitin Ligase Required for Efficient mRNA Export Differentially Affects hnRNP Transport

Background: In the nucleus mRNAs are bound by hnRNP proteins. A subset of hnRNP proteins shuttle between the nucleus and cytoplasm and are believed to promote mRNA export by acting as adaptors between mRNA and the transport machinery. The existence of multiple shuttling hnRNP proteins raises the question of whether differentially-regulated, hnRNP-specific mRNA export pathways exist.

Results: We have determined that Tom1p, a conserved protein with a hect E3 ubiquitin ligase domain, is required for efficient mRNA export in *S. cerevisiae*, yet differentially affects hnRNP protein localization and export. Mutations in *tom1* predicted to abolish ubiquitin ligase activity block efficient export of Nab2p and mRNA, causing Nab2p-mRNA complexes to accumulate in a punctate pattern coincident with the Nuclear Pore Complex (NPC). Notably, the subcellular distribution of several other hnRNP proteins is not affected. In particular, Npl3p remains mRNA-associated and continues to be efficiently exported in *tom1* mutants.

Conclusion: Our results demonstrate that mutations predicted to affect the enzymatic activity of the Tom1p ubiquitin ligase differentially affect export of hnRNP proteins in association with mRNA. We propose the existence of multiple mRNA export pathways, with export of Nab2p-associated mRNAs dependent on a branch of the ubiquitin protein modification pathway.

Background

Gene expression in eukaryotic cells requires processing of heterogeneous nuclear RNA (hnRNA) to messenger RNA (mRNA), and subsequent export of mRNA through the Nuclear Pore Complex (NPC) to the cytoplasm. In metazoans, both hnRNAs and mRNAs are bound by a set of twenty or so nuclear-localized hnRNP proteins, which contain a variety of RNA recognition modules (Dreyfuss et al., 1993). Cytological and biochemical studies indicate that most hnRNP proteins dissociate from mRNA prior to or just after nuclear export, and are replaced by cytoplasmic mRNP proteins (Daneholt, 1997; Dreyfuss et al., 1993).

hnRNP proteins are classified as shuttling or non-shuttling according to whether they exit the nucleus in association with mRNA or are displaced prior to translocation through the NPC, respectively. Substantial evidence supports a role for hnRNP proteins as mediators of mRNA export (Stutz and Rosbash, 1998). Shuttling hnRNP proteins, notably hnRNP A1, have been proposed to be mRNA 'carriers' that promote mRNA export by interacting with the transport machinery. A1 contains a 38 amino acid domain, M9, that mediates A1 shuttling between the nucleus and cytoplasm, and plays a key role in A1 function in mRNA export (Izaurralde et al., 1997; Pollard et al., 1996).

Genetic evidence from yeast also supports a key role for hnRNP proteins in mRNA export. Conditional alleles of yeast hnRNP proteins Npl3p, Mex67p and Nab2p accumulate mRNA in the nucleus ((Lee et al., 1996; Segref et al., 1997; Singleton et al., 1995) and J.T. Anderson and M.S. Swanson, personal communication). Yeast hnRNP proteins, like their mammalian counterparts, are highly-expressed, nuclear-localized, RNA-binding proteins which can be crosslinked to the polyA tail of mRNA *in vivo*

(Anderson et al., 1993; Segref et al., 1997; Wilson et al., 1994). Npl3p and Nab4/Hrp1p shuttle between the nucleus and cytoplasm, suggesting they may act as mRNA export carriers in yeast (Flach et al., 1994; Kessler et al., 1997). Intriguingly, an M9-like domain in Nab2p functions in a mammalian nuclear import assay implying that hnRNP protein transport pathways may be evolutionarily conserved and that Nab2p may also be an mRNA carrier protein (Siomi et al., 1998).

The existence of multiple shuttling hnRNP proteins raises the question of whether distinct export pathways exist for individual hnRNP proteins in association with mRNA. In support of distinct hnRNP protein export pathways, hnRNP protein shuttling domains unrelated to M9 have been described (Fan and Steitz, 1998; Michael et al., 1997). However, in the absence of evidence for differential effects on export of distinct hnRNP protein-mRNA complexes *in vivo*, it has remained unclear whether specific hnRNP protein export pathways play independent, functionally significant roles in mRNA export.

In this report we present evidence for the existence of distinct, differentially-controlled hnRNP protein/mRNA export pathways in yeast. We have determined that Ssr2/Tom1p, a conserved protein with a hect E3 ubiquitin ligase domain (Huibregtse et al., 1995a), is not only required for efficient mRNA export (Utsugi et al., 1999), but also differentially affects export of shuttling hnRNP proteins. Our data suggest the existence of at least two distinct hnRNP protein/mRNA export pathways, differentiated by a requirement for ubiquitination of a nuclear Ssr2/Tom1p target protein.

Results

Temperature-sensitive *ssr2-1* mutants accumulate polyA RNA in the nucleus at 37° C

We identified *SSR2* (Suppressor of SnRNA) as a suppressor of a temperature sensitive U1 snRNA deletion mutant (Umen, 1995). *ssr2-1* mutants, which cannot grow at 33°C or higher, displayed genetic interactions with several splicing factors. However, subsequent analyses indicated that *ssr2-1* mutants do not display global effects on mRNA splicing, transcription or mRNA turnover (KD and CG, unpublished). Since *ssr2-1*'s genetic interactions suggested a role for *SSR2* in some aspect of mRNA processing, we examined *ssr2-1* mutants for mRNA export defects.

Using fluorescence in situ hybridization (FISH) to mRNA polyA tails we found that WT and *ssr2-1* strains grown at 22°C displayed polyA RNA throughout the cell, indicating efficient mRNA export in these strains (Amberg et al., 1992; Kadowaki et al., 1994; Kadowaki et al., 1992). In contrast, *ssr2-1* mutants shifted to 37°C accumulated polyA RNA in a punctate, ring-like pattern of 3-7 foci which overlapped with the border of DAPI staining of chromosomes (Figure 1A), implying the mutant cells were accumulating mRNA at the nuclear periphery. A concommitant decrease of cytoplasmic signal intensity was observed, although this decrease was less pronounced than in *prp20-1* mutants (not shown). Nuclear accumulation of polyA RNA was detectable as early as 1 hr. in 30-50% of the population and in >90% at 90 minutes (Figure 1A). WT and mutant growth curves do not diverge from one another until 3 hours of growth at 37° C (Figure

1B). The appearance of the mRNA export phenotype before the growth defect suggests that impaired mRNA export may be a primary defect of *ssr2-1* strains.

Evolutionarily-conserved Ssr2/Tom1p has a hect ubiquitin ligase domain

SSR2 was cloned by complementation and determined to be allelic to YDR457w, at the time an uncharacterized ORF. While this work was in progress, we learned that *SSR2* was allelic to *TOM1*, a gene purportedly involved in the G2/M transition (Utsugi et al., 1995). Tom1p has also been reported to affect transcriptional activation (Saleh et al., 1998) and temperature-dependent organization in mitotic nuclei (Utsugi et al., 1999). For simplicity, we will hereafter refer to *SSR2* as *TOM1,* and the *ssr2-1* allele as *tom1-ssr2.*

Tom1p contains a 350 amino acid hect domain (Saleh et al., 1998; Utsugi et al., 1999), the catalytic unit of a family of ubiquitin-protein ligases (E3 enzymes) (Huibregtse et al., 1995a). The Tom1p hect domain contains the conserved active-site cysteine and is 45% identical to E6-AP within the 350 amino acids comprising the hect domain (Figure 2), strongly predicting that Tom1p catalyzes covalent ligation of ubiquitin to specific target proteins. Tom1p has likely orthologs, including the proteins encoded by human partial cDNA KIAA0312 and *S. pombe ptr1*$^{+}$ (Figure 2). Notably, *ptr1+* was isolated in a screen for mutations causing nuclear mRNA accumulation (Azad et al., 1997), providing strong evidence for evolutionary conservation of Tom1p function in mRNA export.

Tom1p is a nuclear protein

To determine in which subcellular compartment Tom1p was likely to function we added three Hemagglutinin (HA) epitope tags to the N-terminus of Tom1p. HA-Tom1p

restored WT growth to *Δtom1* strains and was found to migrate as a single band well above HA-Prp8p (280kd) by SDS-PAGE (not shown). Using immunofluorescence we determined that HA-Tom1p signal overlapped with DAPI staining, and was concentrated in a pattern similar to HA-tagged nuclear splicing factor, Prp8p (Figure 3). We conclude that Tom1p is localized primarily in the nucleus, suggesting that it functions in this compartment to promote mRNA export.

Tom1p hect domain mutations result in temperature-sensitive growth and mRNA export

To determine whether *TOM1* was required for growth and mRNA export, we deleted the *TOM1* coding sequence. *Δtom1* strains were viable at 25°C or below, but displayed temperature sensitive growth and mRNA export (Figure 4A and B), consistent with other reports(Saleh et al., 1998; Utsugi et al., 1999). We noted that *Δtom1* strains shifted to 37° C for one hour or longer accumulated mRNA in a punctate pattern of 3-7 foci at the nuclear periphery as was observed with *tom1-ssr2* (Figure 4B). *TOM1* on a plasmid restored WT growth and mRNA export to *Δtom1* strains (Figure 4A, 4B). Because many mRNA export mutants show longer polyA tails *in vivo* (Kadowaki et al., 1994; Singleton et al., 1995), we also examined polyA tail length in *Δtom1* strains at 37° C. *Δtom1* strains show a substantial increase in average polyA tail length relative to WT strains, consistent with a decreased level of cytoplasmic tail trimming (not shown). Taken together, these results indicate that Tom1p is required for growth and efficient mRNA export at high temperatures.

To test the hypothesis that enzymatic activity of the Tom1p hect domain is required for efficient mRNA export at 37°C, we generated *tom1* alleles with hect domain mutations. *tom1-C3235A* substitutes alanine for the conserved, active-site cysteine which forms the ubiquitin thioester intermediate. *tom1-ΔC6* deletes the last six amino acids, including a conserved phenylalanine. Analogous mutations abolish ubiquitin ligase activity of other hect enzymes (Huibregtse et al., 1995a). Consistent with our hypothesis, neither *tom1* allele could rescue the temperature-sensitive growth or mRNA export phenotype of *Δtom1* strains (Figure 4A, 4B). We detected no difference in the steady-state levels or nuclear localization of the WT and hect mutant proteins at 22°C or 37°C (not shown), and therefore conclude that the *tom1* hect mutant phenotypes are not caused by destabilization or mislocalization of the mutant proteins. These results suggest that transfer of ubiquitin from Tom1p to at least one nuclear target protein is required for wild-type growth and efficient mRNA export.

tom1 mutants accumulate Nab2p with mRNA in proximity to the NPC

To test for possible effects on the localization of yeast hnRNP proteins in *tom1* mutants, we examined Green Fluorescent Protein (GFP) fusions to Nab2p and Npl3p in *Δtom1, tom1-C3235A,* and isogenic WT strains. As early as 45 minutes after a shift to 37°C, essentially all *tom1* mutant cells began to accumulate Nab2-GFP in a puncate pattern of 3-7 foci at the nuclear periphery, with a concomitant decrease in nucleoplasmic signal (Figure 5A). WT *TOM1* cells maintained Nab2-GFP in a nucleoplasmic pattern at 37° C (Figure 5A). In contrast to Nab2-GFP, Npl3-GFP remained localized in the nucleoplasm in both WT and *tom1* mutants at 37° C (Figure 5A). We detected no effect

on the localization of a number of other yeast mRNA-binding proteins and transport substrates in *tom1* mutants (Table I). Morevoer, in contrast to *tom1* strains, several other mRNA export mutants maintained Nab2p in a nucleoplasmic pattern at 37° C (Table II). The absence of punctate Nab2p accumulation in shifted *mex67-5* cells is especially interesting, since these mutants accumulate polyA RNA in discrete intranuclear foci (Segref et al., 1997). We conclude that defects in mRNA transport do not generally result in Nab2p redistribution, and that accumulation of mRNA in nuclear foci is not a sufficient condition for punctate Nab2p accumulation.

The Nab2-GFP accumulation pattern in shifted *tom1* mutants was strikingly similar to the mRNA accumulation pattern, and resembled NPC localization (Nehrbass et al., 1990), suggesting that Nab2p might be accumulating with mRNA at the NPC. To determine whether mRNA co-localized with Nab2p and the NPC protein Nsp1p (Nehrbass et al., 1990) in *tom1* mutants at 37°C, we performed combined in situ and indirect immunofluorescence assays. Unshifted *tom1-C3235A* mutants displayed WT localization patterns, with nucleoplasmic Nab2p and whole-cell mRNA (not shown). In contrast, shifted *tom1-C3235A* mutants showed marked co-localization of polyA RNA with Nab2p signal (Figure 5B). Since this strain did not contain Nab2-GFP, we conclude that endogenous Nab2p and Nab2-GFP behave similarly, and the mislocalization of Nab2-GFP in *tom1* mutants is not an artifact of the GFP fusion or its breakdown to GFP fragments. The mRNA foci also appeared to co-localize with the NPC, as judged by overlap with Nsp1p signal (Figure 5C). Detailed analysis of 122 mRNA foci indicated overlap with Nsp1p signal in 108 cases (89%). Thus *tom1* mutants shifted to 37° C

display the novel phenotype of accumulation of mRNA with a specific hnRNP protein in proximity to the NPC.

Nab2p is a shuttling hnRNP protein

To test the hypothesis that Nab2p is a nucleus-restricted hnRNP protein, in which case dissociation of Nab2p from mRNA might be a prerequisite for binding of shuttling hnRNP proteins and mRNA export we examined Nab2-GFP in a nuclear export assay (Lee et al., 1996). Nab2-GFP behaved similarly to Npl3-GFP, displaying an increase in cytoplasmic Nab2-GFP signal in *nup49-313* mutants at 37°C under conditions where new protein synthesis was blocked (Figure 6A). In contrast, Nab2-GFP appeared strictly nucleoplasmic in unshifted *nup49-313* mutants and shifted isogenic WT strains (not shown). No cytoplasmic signal was detected with non-shuttling , Histone H2B-GFP (Figure 6A). Moreover, we could not detect any evidence of cleaved products in *NUP49* or *nup49-313* cells by western with anti-GFP polyclonal sera (not shown), making it extremely unlikely that the cytoplasmic signal detected in the *nup49-313* strain results from cleavage of the GFP moiety from Nab2p. Our results demonstrate that Nab2p is a shuttling hnRNP protein, consistent with recent results obtained by Lee, et al. using a different assay (Lee and Aitchison, 1999), and suggest that it exits the nucleus with mRNA.

Nab2p export is inhibited in *tom1* strains at 37° C

To determine directly whether Nab2p export was inhibited in *tom1* strains at 37° C we constructed *nup49-313 Δtom1* double mutants containing Nab2-GFP and tested these strains in the export assay (Lee et al., 1996). In contrast to *nup49-313* strains,

which accumulated cytoplasmic Nab2GFP signal, *nup49-313Δtom1* strains displayed punctate accumulation of Nab2-GFP at the nuclear periphery and essentially no cytoplasmic signal (Figure 6B). Although overall GFP signal was somewhat lower in the double mutant, this does not explain the absence of cytoplasmic signal, since enhancement of the image produced nuclear signal similar to *nup49-313*, but minimal cytoplasmic signal (Figure 6B). Because the *nup49-313* selective import block requires extended incubation at 37° C (5 hrs. vs. 45 min. for *tom1* phenotypes), it is possible that the inhibition of Nab2p export in the *nup49-313Δtom1* strains is an indirect effect. Nevertheless, these data indicate that Nab2p cannot be efficiently exported in *tom1* mutants at 37° C.

Npl3p is efficiently exported in shifted *tom1* mutants

To test for effects on Npl3p export in *tom1* mutants we used a reporter with GFP fused to the *npl3-S411A* allele, which is partially mislocalized to the cytoplasm, but still a functional shuttling protein (CW Siebel and CG, unpublished). The use of such a construct eliminates concerns over possible indirect effects that might result from extended incubation of *nup49-313Δtom1* at 37° C . In contrast to *mex67-5* mutants, which displayed a dramatic decrease in cytoplasmic npl3-S411A-GFP signal at the non-permissive temperature, we detected no effect on npl3-S411A-GFP localization in *tom1* mutants (Figure 7), indicating that Npl3p is still efficiently exported in *tom1* mutants, despite the fact that Nab2p and mRNA accumulate in proximity to the NPC. This result can be interepreted in two ways: 1) Npl3p is no longer associated with mRNA, causing

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Nab2p-mRNA complexes to accumulate at the nuclear periphery, or 2) Npl3p-associated mRNAs exit the nucleus while Nab2p-associated mRNAs remain behind.

Npl3p remains associated with polyA RNA in *tom1* strains at 37° C

To directly assess the possibility that Npl3p dissociates from polyA RNA in *tom1* mutants we used a UV-crosslinking method (Anderson et al., 1993) to monitor Nab2p and Npl3p association with polyA RNA *in vivo*. We found increased levels of polyA-associated Nab2p and Nab2-GFP in *tom1* mutants shifted to 37° C, consistent with their co-localization with polyA RNA. In contrast, the level of Npl3p associated with polyA RNA appeared similar in WT and *Δtom1* strains, with only a slight reduction in association (Figure 8). This result contrasted with previously described behavior of Npl3p under stress conditions, where dramatic loss of Npl3p association is observed (Krebber et al., 1999). Thus, the Nab2p/mRNA accumulation phenotype does not result from dissociation of Npl3p from mRNA. Taken together with the presence of residual cytoplasmic mRNA signal (Figure 4B), the persistence of Npl3p export and Npl3-mRNA association in *tom1* mutants shifted to 37° C suggests that Npl3p-mRNA complexes are efficiently exported in *tom1* mutants.

Discussion

In this report we have demonstrated that yeast with mutant alleles of *TOM1*, a putative hect E3 ubiquitin ligase, display a novel mRNA export phenotype that supports the existence of multiple mRNA export pathways. Early after a shift of *tom1* mutants to the non-permissive temperature of 37° C the hnRNP protein Nab2p redistributes from its nucleoplasmic location to co-localize with polyA RNA in a punctate pattern coincident with the NPC. The *tom1* transport phenotype is highly specific, in that localization and export of other shuttling hnRNP proteins is unaffected in *tom1* mutants, and several other mRNA export mutants do not display punctate accumulation of Nab2p with polyA RNA. We also showed that Nab2p is a shuttling hnRNP protein, suggesting that it normally exits the nucleus with mRNA, and that Nab2p export, but not Npl3p export, is inhibited in *tom1* strains. *In vivo* crosslinking analysis of hnRNP protein association with polyA RNA in *tom1* mutants supported the localization results, showing an increase in the level of associated Nab2p. More importantly, Npl3p remains associated with polyA RNA, implying that Npl3-mRNA complexes are efficiently exported in *tom1* mutants while Nab2p-mRNA complexes accumulate in proximity to the NPC.

It is important to stress that our findings are based on analyses of events that occur within the first 1-2 hours following a temperature shift, significantly before the point at which *tom1* growth rates diverge from wild-type, and when the *tom1* growth, polyA RNA localization and Nab2p re-localization phenotypes are still reversible (not shown). The specific transport phenotypes we observed early after a temperature shift contrast with pleiotropic *tom1* phenotypes, including a cell cycle block, which were

observed 4 hours or more after a temperature shift (Utsugi et al., 1999). Because it appears early in a shift to 37° C, the Nab2-mRNA export phenotype we have described is more likely to be a primary defect due to the absence of Tom1p function.

Taken together, our results indicate the existence of distinct mRNA export pathways mediated by individual hnRNP proteins and differentiated by a requirement for Tom1p ubiquitin ligase activity (Figure 9). Important aspects of the model to address include: 1) the specific target(s) of Tom1p-dependent ubiquitination and how ubiquitin modification affects their function ; and 2) whether Nab2p-specific mRNA cargos are affected by loss of Tom1p function.

In Figure 9 we propose the simplest model, in which Tom1p acts directly to ubiquitinate a protein that associates with or is a component of Nab2p-mRNA complexes. In principle, the effect on the target could occur via its degradation by the 26S proteasome, or could occur by a non-degradative, proteasome-independent mechanism (Hicke, 1999). If protein degradation is involved, Nab2p itself is unlikely to be the substrate, since steady-state levels of Nab2p are unaltered in *tom1* mutants and Nab2p which has accumulated at the nuclear periphery can be recycled back to the nucleoplasm when *tom1* mutants are returned to the permissive temperature under conditions where new protein synthesis is inhibited (not shown). In addition, we have not thus far detected a biochemical interaction between Tom1p and Nab2p, nor have we found evidence of ubiquitinated Nab2p species (not shown). We therefore favor the idea that the effect on Nab2p-mRNA transport arises from failure to ubiquitinate some other factor.

Aside from Nab2p, the only other obvious candidate Tom1p target was Spt7p, a component of the ADA/SAGA transcriptional co-activator complex, and the only protein

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thus far shown to be ubiquitinated *in vivo* in a *TOM1*-dependent manner (Saleh et al., 1998). Two observations argue that Spt7p is not the relevant target (data not shown): 1) *Δspt7* mutants do not accumulate nuclear mRNA at 37° C, indicating that neither Spt7p nor the SAGA complex is required for efficient mRNA export; 2) *Δspt7 tom1-C3235A* strains at 37° C accumulate polyA RNA in the nucleus in the same punctate pattern observed with *tom1-C3235A* strains. Thus, the *tom1* transport phenotypes cannot result from failure to activate or negatively regulate Spt7p or the SAGA complex. In sum, the target of Tom1p that plays a role in Nab2p-mRNA export remains to be identified.

Of particular interest is the question of whether mRNAs derived from a particular subset of genes are exported primarily or exclusively by a Nab2p or Npl3p-mediated mRNA export pathway. Were this the case, it would provide the cell with an additional mechanism for regulation of gene expression. In support of this idea, there is precedent for preferential association of individual hnRNP proteins with distinct mRNA species *in vitro* and *in vivo* (Dreyfuss et al., 1993; Matunis et al., 1993; Wurtz et al., 1996). Alternatively, association of any particular mRNA molecule with Nab2p or Npl3p prior to nuclear export might be a stochastic process. In this case, specific inhibition of one export pathway, as seen in *tom1* mutants would simply reduce the fraction of total cellular mRNA reaching the cytoplasm. Determining which of these two models is correct is a challenging question for future investigation.

Ultimately, precise mechanistic understanding of Tom1p's role in Nab2p-mRNA export will require identification of the relevant protein target and affected mRNA species. Nevertheless, the *tom1* mutant transport phenotype provides *in vivo* evidence for differential effects on export of distinct hnRNP protein-mRNA complexes. Thus a major

implication of the results presented here is the existence of at least two independent mRNA export pathways, with possible pathway-specific regulation by a branch of the ubiquitin protein modification pathway.

Materials and Methods

Cloning of SSR2

SSR2 was cloned from aYCP50-based library (gift of Mark Rose (Rose et al., 1987)) by complementation of *ssr2-1* temperature sensitivity, sequencing of the insert and BLAST comparison to the Saccharomyces Genome Database. Linkage between *TOM1* and *ssr2-1* was confirmed by marking the *TOM1* locus with *HIS3*, generation of a *TOM1::HIS3/ssr2-1* diploid, sporulation, and tetrad analysis.

Yeast Strain and Plasmid Construction

Details of plasmid construction are available as supplementary information online. *Δtom1::HIS3* strains were generated in WT strain YPH399 (*MATa ade2-101 his3-Δ200 leu2-Δ1 lys2-801 trp1-Δ63 ura3-52*) by replacement of Tom1p coding sequence with *HIS3* using PCR-generated pKD12, a 'gamma deletion' construct in pRS303 (Sikorski and Hieter, 1989). Genotype was confirmed by PCR. Positives were backcrossed to YPH399. Sister spores yKD99-2B (WT) and yKD100-2D (*Δtom1::HIS3*) were used for all subsequent analyses. *TOM1* constructs used in this study contain *TOM1* with its own promoter in pRS314 (Sikorski and Hieter, 1989). *TOM1* plasmids with three copies of the hemagglutinin (HA)epitope tag at the N-terminus were generated in yeast via homologous recombination, recovered from yeast, and re-transformed into yKD100-2D. The tagged WT allele complemented the *Δssr2* growth and transport phenotypes. Hect mutant allele plasmids were constructed by introduction of annealed oligos containing the respective mutations between convenient

restriction sites, with confirmation by sequencing, followed by subcloning to appropriate plasmids. The chromosomal *tom1-C3235A* strain, yKD127, was constructed in YPH399 via two-step gene replacement (Rothstein, 1991) using integrating plasmid pKD28. Genotype was confirmed by PCR cloning of the region from yeast and restriction digest. YKD 442 (*nup49-313*) was generated in yKD99-2B via two-step gene replacement using integrating plasmid pKD63. WT *NUP49* on a plasmid rescues the ts^- phenotype of yKD442. yKD443 (*nup49-313 Δtom1::HIS3*) was a His^+ ts^- segregant from an NPD tetrad generated by sporulation of the diploid resulting from the cross yKD442 x yKD100-2A (*matα Δtom1::HIS3*).

GFP Transport Reporters

NAB2-GFP (gift from Arie Kaffman and Erin O'Shea), is under control of the *NAB2* promoter in either pRS314, pRS315 or pRS316. *pRS314-NAB2-GFP* fully complements *Δnab2*. A PCR strategy was used to replace the NAB2 promoter with the regulated *GAL1* promoter for *NAB2-GFP* plasmids pKD59 (*LEU2*-marked) and pKD62 (*URA3*-marked). *pRS316-NLS-GFP$_3$* was a gift from Arash Komeili and Erin O'Shea. *pRS315-NPL3-GFP* (pCS38) and *pRS314/315-npl3-S411A-GFP* (pCS55/61) were constructed by Chris Siebel, and will be described elsewhere. *pUN100-LEU2-MEX67-GFP* (Segref et al., 1997) and pKW466 (*XPO1-GFP*) (Stade et al., 1997) have been described previously.

FISH and Immunofluorescence Microscopy

Fluorescence in situ hybridization (FISH) for polyA RNA localization was performed according to published protocols (Amberg et al., 1992; Kadowaki et al., 1992) with some modifications (A. De Bruyn Kops, and CG, in preparation). PolyA RNA hybridized to a

digoxigenin-labeled oligo dT50 probe was detected using FITC-coupled sheep anti-digoxigenin Fab fragments (1:50, Boehringer-Mannheim). Immunofluorescence for transport reporters and the NPC was performed according to the same protocol, with addition of appropriate antibodies (see Supplementary material). Immunofluorescence to detect HA-Tom1p was performed essentially as described (Pringle et al., 1991), except fixation was performed in culture medium with 5% formaldehyde (diluted from 37% stock, Fluka) at growth temperature for 15 minutes, and blocking was performed with 1% w/v Blocking Reagent (Boehringer-Mannheim #1096176) in PBS. Detection was with mAB HA.11 (1:100, Babco) and goat anti-mouse FITC (1:400, Cappel). Images were collected and processed using an Olympus BX-60 microscope with a 100X Olympus UPlanFI NA 1.30 objective, appropriate filters, IP Lab imaging software (Scanalytics) and a Sensys CCD camera (Photometrics). Unless otherwise noted, all images to be compared within a data set were equivalently exposed and processed.

Nuclear Export Assay for Nab2p

The nuclear export assays to determine whether Nab2p is exported in WT and in *tom1* strains were performed essentially as described (Lee et al., 1996), but with the following modifications, respectively. To determine whether Nab2p shuttles, *NUP49* or *nup49-313* strains (Doye et al., 1994) expressing NAB2-GFP, NPL3-GFP, or Histone H2B-GFP in appropriate selective media with glucose were shifted to 37°C in the presence of 0.1mg/ml cycloheximide to inhibit new protein synthesis. To determine whether *tom1* inhibits Nab2p export, WT, *nup49-313*, *Δtom1* ,and *nup49-313 Δtom1* strains (yKD 99-2B, 100-2A, 442, and 443, respectively) containing Nab2-GFP under control of the Gal1

promoter (pKD62) were grown overnight in SC-Ura + 2% raffinose, induced for 2 hours with 2% galactose, spun at room temperature, washed with YEPD medium, re-spun and resuspended in 37° C YEPD and incubated at 37° C. In both cases GFP reporter localization was assayed after 5.5 hr growth at 37° C.

UV-Crosslinked RNP Purification

One liter cultures of yeast in YEPD (yKD99-2B or yKD100-2D + pRS314-Nab2-GFP) were split in half and rapidly mixed with 500 ml each of 51° C YEPD to bring the temperature to 37° C, followed by incubation in a 37° C shaker for two hours. Shifted cultures (OD=0.7-1.2) were harvested and split in half. One half was treated with UV light, as described (Anderson et al., 1993). Punctate localization of Nab2-GFP in the mutant after crosslinking was confirmed by microscopy. Lysates in RNP lysis buffer (Anderson et al., 1993) were made by shaking with 0.5mm glass beads in a multi bead-beater (Biospec) at 4° C for 2 three-minute cycles with >5 minutes on ice between cycles. Subsequent purification of proteins UV-crosslinked to polyA RNA was performed essentially as described (Anderson et al., 1993). Resuspended polyA RNA samples were normalized by A_{260}, nuclease-treated and loaded for SDS-PAGE and immunoblotting. 1.6 A_{260} units of each eluate were loaded per lane. Nab2p, Npl3p, and Nop1p were detected using primary antibodies mAB 3F2 (Anderson et al., 1993), anti-Npl3 polyclonal sera (Siebel and Guthrie, 1996), and mAB A66 (Henriquez et al., 1990) respectively, followed by HRP-conjugated secondary antibody (Bio-Rad), and detection with Super Signal West Femto substrate (Pierce).

Supplementary materials and methods

Plasmid Construction

TOM1 'gamma deletion' construct, pKD13,was generated by introducing a BamHI-BclI-digested OKD122/123 PCR product into the BamHI site of pKD12, and screening orientation. pKD12 contains a BamHI-XhoI-digested OKD 124/125 PCR product. To generate pKD11 the *TOM1* ORF +256 bp 5'/880 bp 3' was excised from the original YCP50 clone with SphI and FspI, blunted with T4 DNA Polymerase (NEB), and sub-cloned into SmaI/EcoRI (both destroyed) of pRS314. For epitope tagging of the amino terminus, PCR with Pfu polymerase (Stratagene, OKD 124/133;134/135), digestion with BamHI/EcoRI or EcoRI/XhoI, and 3-way ligation was used to construct pBluescript KS+ derivative, pKD18, which contains the first 1200 bp of *TOM1* sequence with unique EcoRI and BglII sites at the Tom1p start codon. A PCR fragment containing 3 copies of the Hemagglutinin (HA) epitope tag (OKD 126/127) was sub-cloned into the EcoRI and BglII sites of pKD18, to generate pKD22. The tagged full-length *TOM1* plasmid, pKD31, was generated in yeast using a homologous recombination strategy. The tagged fragment was excised from pKD22 and co-transformed into yeast with pKD11, which had been partially digested with NheI, thereby enriching for homologous recombination events that would generate an HA-tagged allele on the plasmid.

tom1-C3235A was constructed in pKD9 (pBluescript KS+ containing the pKD11-derived C-terminus of Tom1p from KpnI to XhoI), by introduction of annealed OKD 120/121 with the mutation between XcmI and NdeI. *tom1-ΔC6* was constructed by inserting annealed OKD 156/157 between NdeI/BglII in pKD16 *(TOM1* C-terminus with EcoRI and BglII directly before Stop codon). pKD16 was generated by 3-way ligation of a BamHI-EcoRI-digested OKD 128/129 PCR product, and an EcoRI-XhoI-digested OKD

130/131 PCR product into BamHI and XhoI of pBluescript KS+. Hect mutant fragments were sub-cloned with AgeI and XhoI into untagged and tagged full-length *TOM1* plasmids PKD11 and pKD31 to generate *tom1-C3235A* constructs pKD14 and pKD32, and *tom1-ΔC6* constructs pKD35 and pKD36. The pKD14 BamHI-XhoI insert was sub-cloned into pRS306 to generate integrating plasmid pKD28. pKD28 was linearized with AvrII to promote integration 3' of the C3235A mutation.

Integrating plsamid pKD63 was created by sub-cloning the 2.8kb SacI-BamHI fragment from pUN90-*nup49-313* (Doye et al., 1994) into pRS306 cut with SacI and BamHI. pKD63 was linearized with BsrGI to promote integration 3' of the *nup49-313* mutations.

pKD62 (URA3-marked Gal1promoter-NAB2-GFP) was constructed by sub-cloning a XhoI-BamHI fragment from pKD59 into pJL257 (Gal1 pro, URA3, gift from Joachim Li, UCSF). pKD59 was contructed in two steps. First, pKD55 was generated by sub-cloning the XhoI-BamHI fragment containing the Nab2 promoter and Nab2-GFP from pRS314-NAB2-GFP into XhoI and BamHI sites downstream from the *GAL1* promoter in pJL602 (Gal1 pro, LEU2, gift from Joachim Li). Next, a PCR fragment (OKD 160/161) containing a BamHI-site upstream of the *NAB2* ATG initiator codon and extending to the HinDIII site in *NAB2* was sub-cloned into BamHI-HindIII-cut pKD55, thereby eliminating the *NAB2* promoter and generating *GAL1* promoter-regulated *NAB2-GFP* (pKD59).

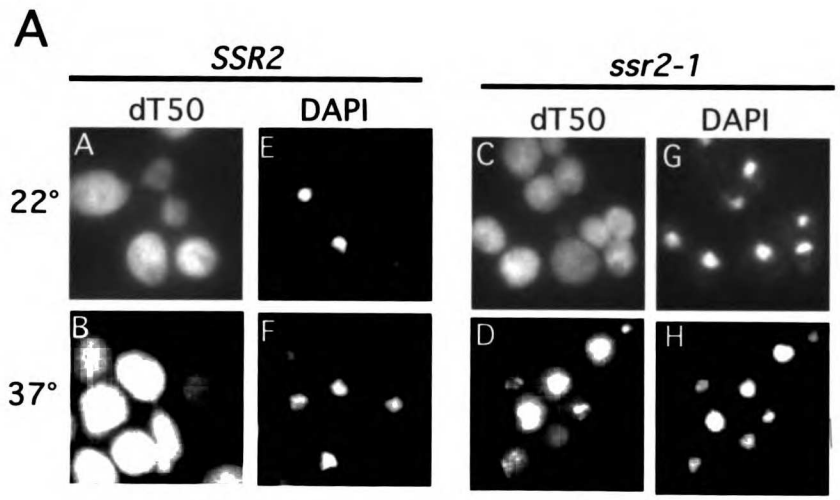
Acknowledgements

We thank Erin O'Shea, Arie Kaffman, Arash Komeili, Nicole Miller Rank, and Karsten Weis for plasmids and helpful discussions; Chris Brandl and Maurice Swanson for strains, antibodies and communicating results prior to publication; John Aris for antibodies; Ed Hurt, Joachim Li and Pam Silver for providing strains and plasmids; Anne de Bruyn Kops, Julianne Dunphy, Wendy Gilbert, Maki Inada, Amy Kistler, Erin O'Shea, Pascal Preker, and Jon Staley for critical reading of the manuscript; Henrike Scholz for much support, discussion, and assistance with the alignments; and Lucita Esperas, Carol Pudlow, and Heli Roiha for superb technical support. This work was supported by a grant from the NIH to CG and an NIH training grant. CG is an American Cancer Society Research Professor of Molecular Genetics.

Figure 1. Growth and mRNA export phenotypes of ssr2-1 mutants.

(**A**) Exponentially-growing *ssr2*-cultures in YEPD were shifted from 22°C to 37°C for 90 minutes or maintained at 22°C, as indicated. Localization of polyA RNA in formaldehyde-fixed cells was visualized by FISH (panels A, B, C, D). DNA was stained with DAPI (panels E, F, G, H).

(**B**) Equivalently dense, exponentially-growing YEPD cultures of WT (circles) or *ssr2-1* (squares) strains were shifted from 22°C to 37°C and growth was monitored by measuring OD at 600nm as a function of time at 37°C. Relative OD values are plotted on a logarithmic scale.



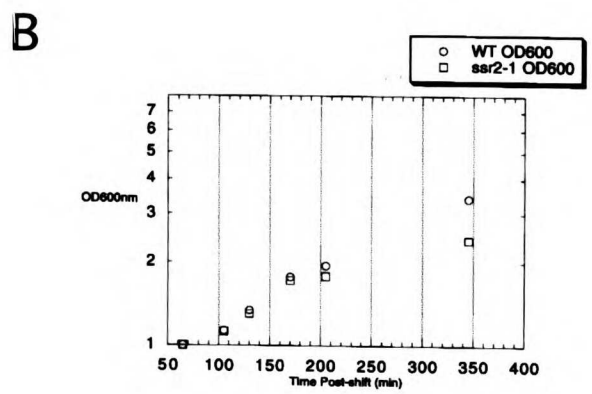


Figure 2. Ssr2/Tom1p contains a hect (homology to E6-AP carboxy terminus) domain and is evolutionarily conserved.

The c-terminal 425 amino acids of Tom1p were aligned with the c-terminal 425 amino acids of putative Tom1p orthologs from *S. pombe* (Ptr1p) and *H. sapiens* (Kiaa0312), as well as three other hect proteins from *H. sapiens* (E6-AP), *S. cerevisiae* (Rsp5p), and *S. pombe* (Pub1). Residues conserved in at least 4 family members are boxed in yellow. Residues similar in at least 4 family members are boxed in blue. Residues identical in Tom1p, Ptr1p and KIAA0312, but not conserved in the other three proteins are shown in magenta. E6-AP, Rsp5p and Pub1 have been shown to be ubiquitin ligases *in vivo* and/or *in vitro*; this activity requires formation of a ubiquitin thioester intermediate on the active-site cysteine (Huibregtse et al., 1995a; Nefsky and Beach, 1996), which is boxed in red. Alignments were performed in MacVector using ClustalW (BLOSUM 30, open gap penalty = 10.0, extend gap penalty = 0.05).

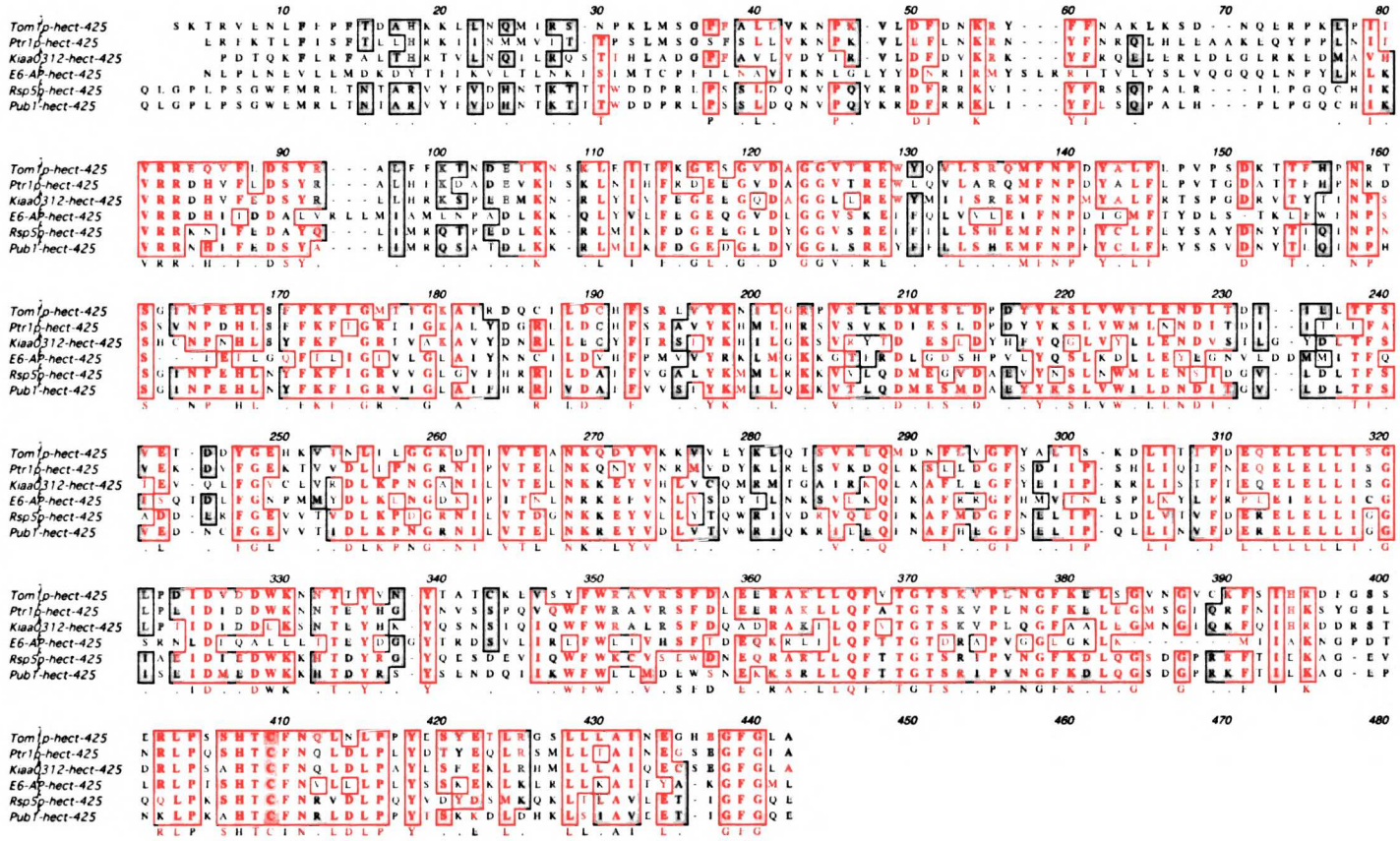

Tom1p-hect-425
Ptr1p-hect-425
Kiaa0312-hect-425
E6-AP-hect-425
Rsp5p-hect-425
Pub1-hect-425

Figure 3. Tom1p is a nuclear protein.

Exponentially-growing 22° C SD-TRP cultures of *Δtom1* strains bearing pRS314 with HA-tagged *TOM1* (panels A, B), or untagged *TOM1* (panels C, D), and a YEPD culture of cells with *HA-PRP8* (panels E, F) were processed for immunofluorescence. The HA epitope was visualized using mAb HA.11 and a FITC-conjugated secondary antibody (panels A, C, E). DNA was visualized by DAPI staining (panels B, D, F).

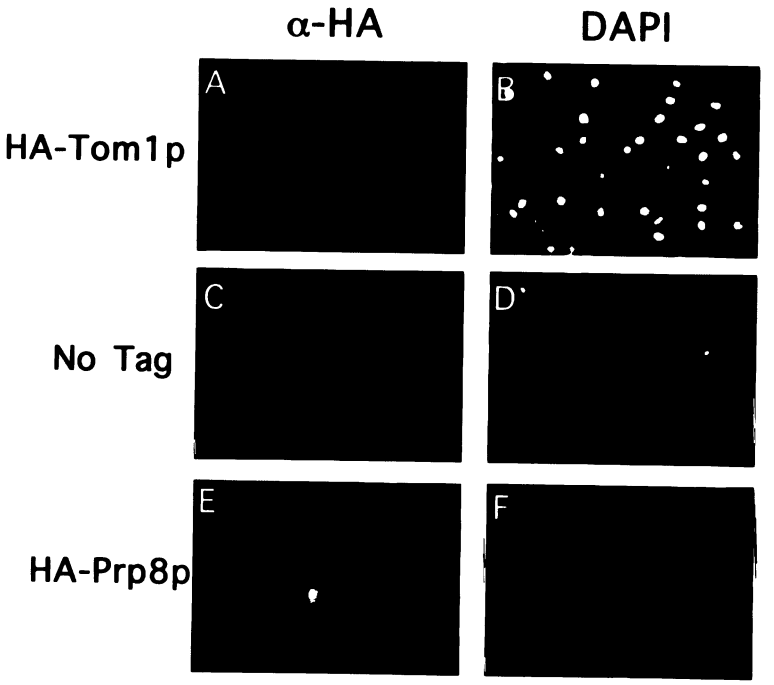
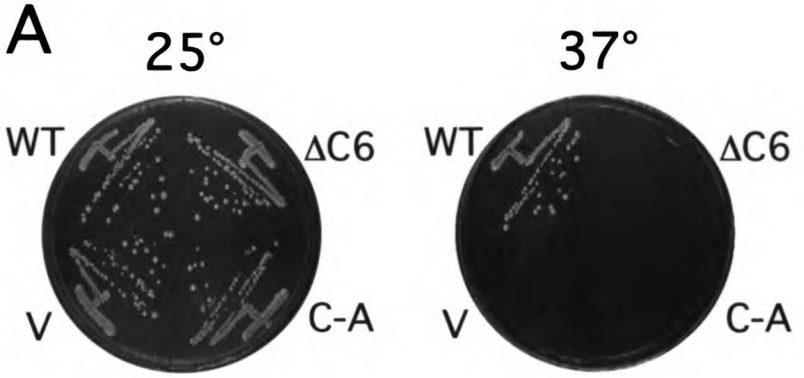

α-HA
DAPI
HA-Tom1p
No Tag
HA-Prp8p
A
B
C
D
E
F

Figure 4. *tom1* hect domain mutants affect growth and mRNA export

(**A**) *Δtom1* strains bearing pRS314 with wild-type *TOM1* (WT), *tom1-C3235A* (C-A), *tom1-ΔC6* (ΔC6), or empty pRS314 vector (V) were streaked on SD-TRP plates and incubated for five days at either 25°C or 37°C.

(**B**) Exponentially-growing 22°C SD-TRP cultures of *Δtom1* strains bearing pRS314 with wild-type *TOM1* (panels A, B), *tom1-C3235A* (panels E, F), *tom1-ΔC6* (panels G, H), or empty pRS314 vector (panels C, D) were diluted into YEPD and grown for 4 hours. YEPD cultures were shifted from 22°C to 37°C. Cells were processed for FISH with oligo dT50 (A, C, E, G). DNA was stained with DAPI (B, D, F, H).



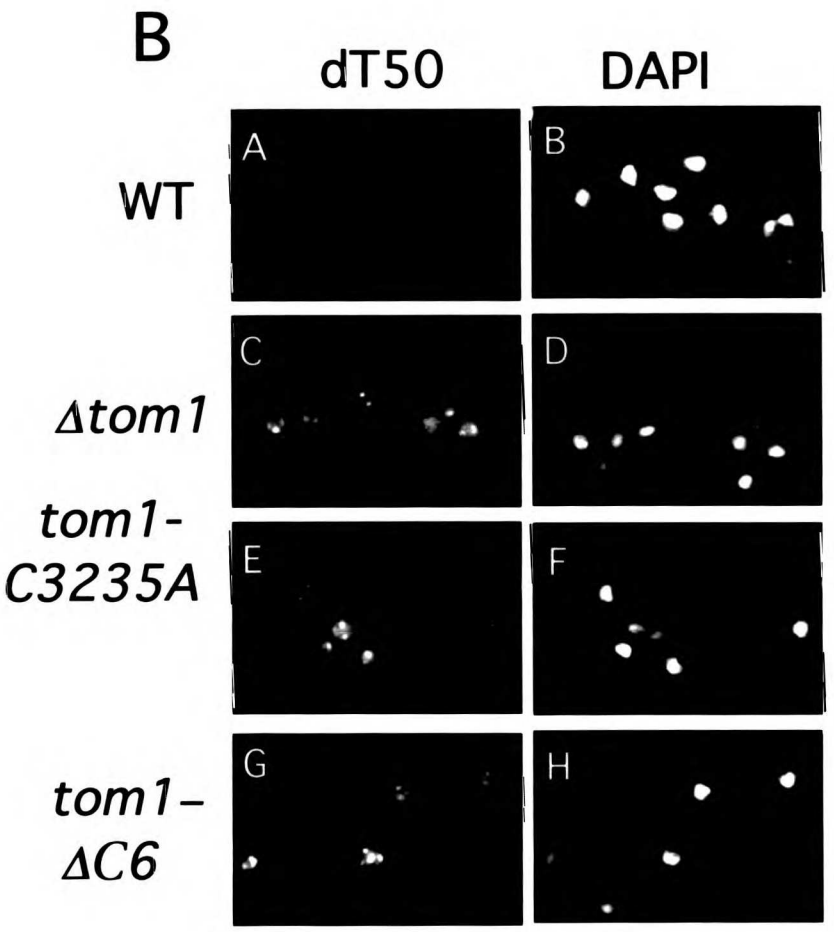


Figure 5. Co-localization of Nab2p with polyA RNA near the NPC in *tom1* mutants.

(**A**) Exponentially-growing 22°C SD-TRP cultures of WT or *Δtom1* strains bearing Nab2-GFP (panels A, B, D, E), or Npl3-GFP (panels C, F) were diluted into YEPD and grown for 2 hrs. YEPD cultures were shifted from 22°C to 37°C and GFP images were collected at the indicated times.

(**B**) Exponentially-growing *tom1-C3235A* cultures in YEPD were shifted from 22°C to 37°C for 1.5 hrs (panels D-F, G-I), or maintained at 22°C (panels A-C). Cells were processed for dT50 FISH(panels A, D, G) and indirect immunofluorescence with anti-Nab2p monoclonal and rhodamine-conjugated secondary antibodies (panels B, E, H). DNA was stained with DAPI (panels C, F). Control reactions omitting either anti-digoxigenin FITC or anti-Nab2p primary antibody showed staining for only Nab2p or polyA RNA, respectively (not shown). Pseudo-colored and merged images of the arrow-marked cell in panels B and E (panels G-I) were generated using IP Lab imaging software.

(**C**) Exponentially-growing *Δtom1* cultures in YEPD were shifted from 22°C to 37°C. Cells were processed for dT50 FISH (panel A). and indirect immunofluorescence with an anti-Nsp1p monoclonal and rhodamine-conjugated secondary antibodies (panel B).

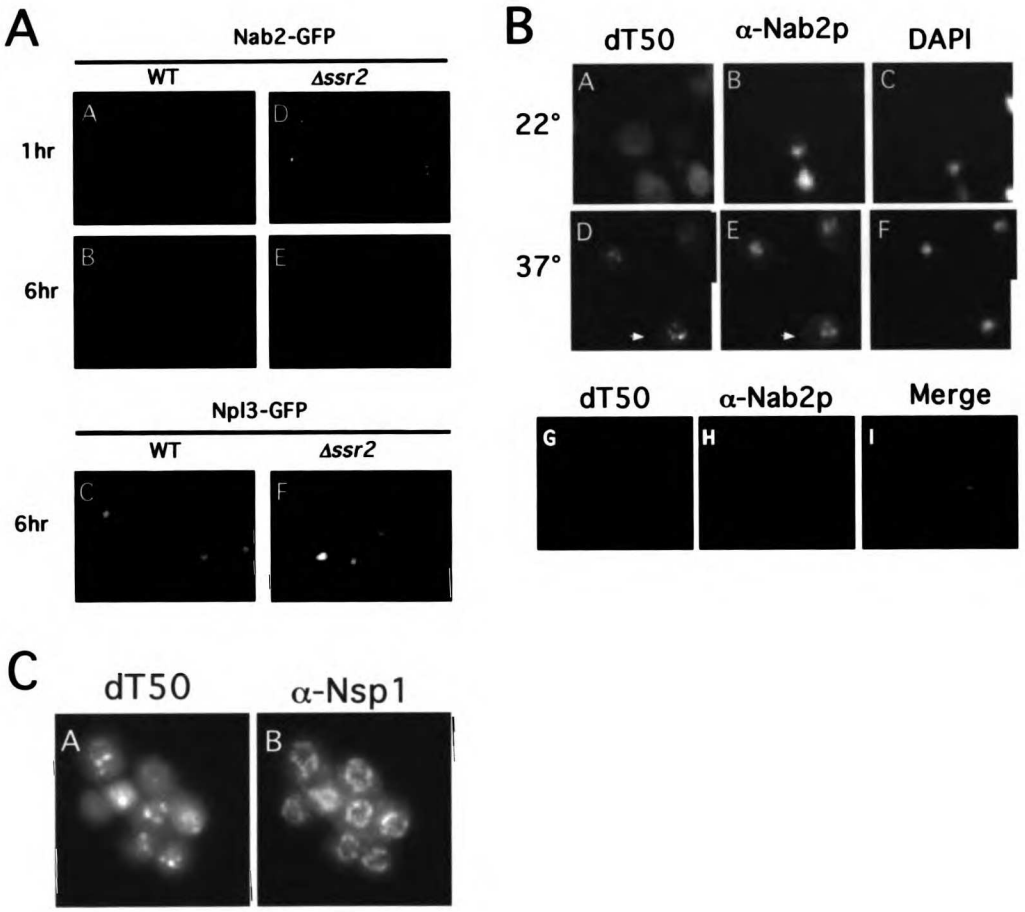

A
Nab2-GFP
WT
Δssr2
1hr
6hr
A
D
B
E
Npl3-GFP
WT
Δssr2
6hr
C
F
B
dT50
α-Nab2p
DAPI
22°
37°
A
B
C
D
E
F
dT50
α-Nab2p
Merge
G
H
I
C
dT50
α-Nsp1
A
B

Figure 6. Nab2p is exported from the nucleus in WT but not *Δtom1* strains.

(**A**) Exponentially-growing *nup49-313* cultures bearing Nab2-GFP (panels A, B), Histone H2B-GFP (panels C, D), or Npl3-GFP (panels E, F) were shifted from 22°C to 37°C in the presence of cycloheximide (100□g/ml). GFP and phase-contrast images were collected after incubation at 37°C for 5.5 hrs.

(**B**) yKD442 (*nup49-313*, panels A, B) and yKD443 (*nup49-313 Δtom1*, panels C,D,E,F) strains containing pKD62 (gal1p-Nab2-GFP) were grown overnight in SC-ura +2% Raffinose at 22° C. Nab2-GFP expression was induced with galactose for 2 hrs. Cells were transferred to YEPD medium to repress new synthesis of Nab2-GFP and shifted to 37° C for 5.5 hrs prior to exmaining Nab2-GFP localization.

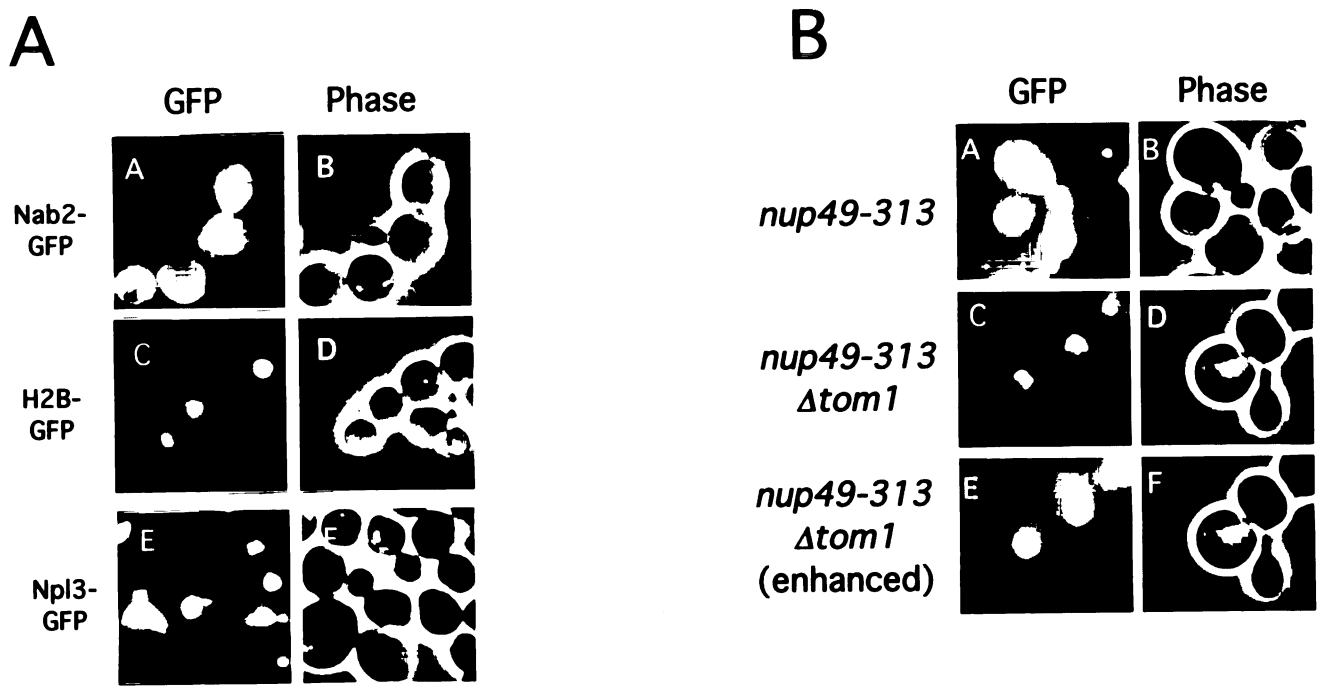

A
GFP
Phase
Nab2-GFP
H2B-GFP
Npl3-GFP
A
B
C
D
E
E
B
GFP
Phase
nup49-313
nup49-313 Δtom1
nup49-313 Δtom1 (enhanced)
A
B
C
D
E
F

Figure 7. Npl3p is exported in shifted *tom1-C3235A* mutants.

Exponentially-growing cultures of *TOM1* (panels A, C), *tom1-3235A* (panels B, D), *MEX67* (panels E, G), or *mex67-5* (panels F, H) strains expressing npl3-S411A-GFP were maintained at 22° C (top panels) or shifted to 37° C for the indicated time (bottom panels) and GFP images were collected.

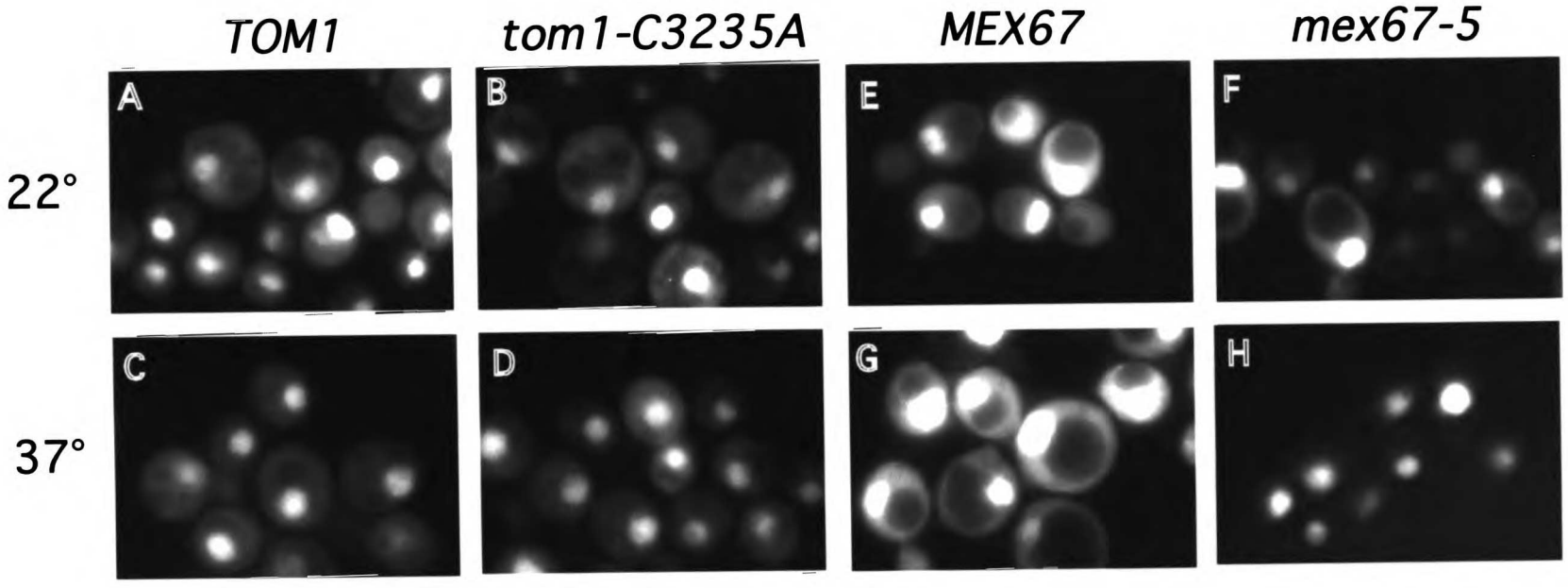

TOM1
tom1-C3235A
MEX67
mex67-5
22°
37°
A
B
E
F
C
D
G
H

Figure 8. Levels of Npl3p and Nab2p associated with polyA RNA in *tom1* strains. *TOM1* WT and *Δtom1* strains were shifted to 37° C for 2 hrs. and either treated with UV(even lanes) or not treated (odd lanes) prior to lysis and two rounds of purification of polyA RNA. Eluates from the second oligo-dT column were normalized for equivalent RNA yield and treated with nuclease prior to SDS-PAGE and immunoblotting. Npl3p was detected first. Nab2p, Nab2-GFP, and Nop1p were detected after stripping and reprobing the same blot. 'Total' lanes represent 10^{-4} of starting material. Only relevant portions of the blot are shown.

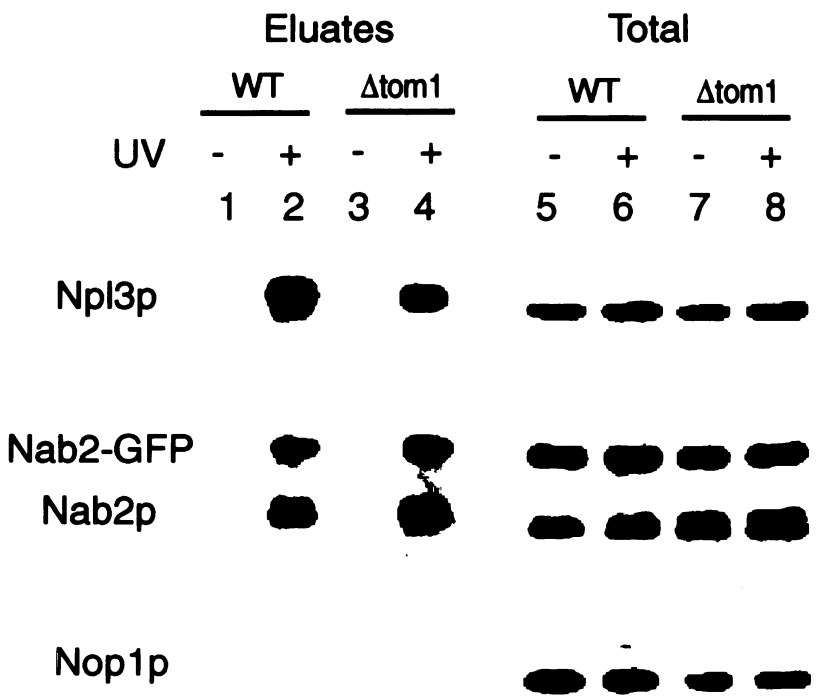

Eluates
Total
WT
Δtom1
WT
Δtom1
UV
- + - +
- + - +
1 2 3 4
5 6 7 8
Npl3p
Nab2-GFP
Nab2p
Nop1p

Figure 9. Model for Tom1p's role in mRNA export.

Modification of an unidentified nuclear factor by Tom1p is required for efficient export of Nab2p and associated polyA mRNA through the NPC. Npl3p is shown associated with a separate pool of polyA mRNA that is not associated with Nab2p and does not require Tom1p for export.

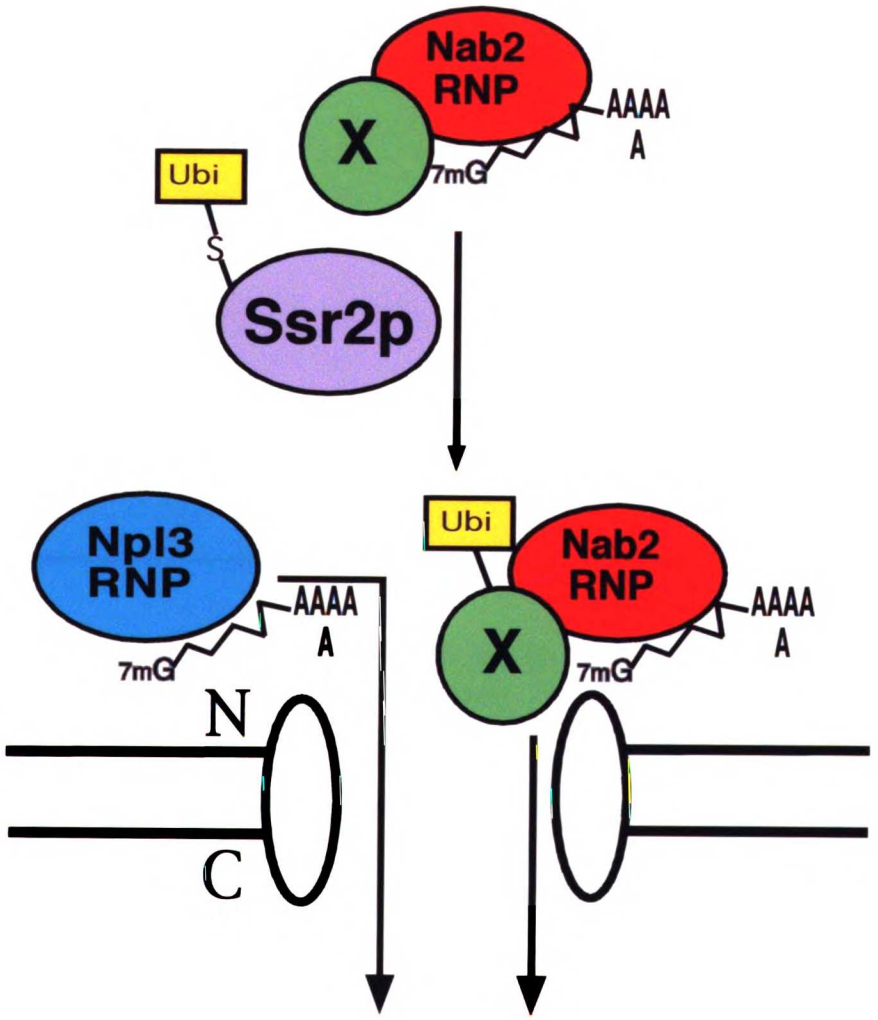

Nab2
RNP
AAAA
A
X
7mG
Ubi
S
Ssr2p
Npl3
RNP
AAAA
A
7mG
N
C
Ubi
Nab2
RNP
AAAA
A
X
7mG

Table I: Specificity of transport phenotypes in *tom1* mutants

Transport Reporter	Localization in *tom1** (at 37° C)	Class
Nab2-GFP/Nab2p	3-7 foci at nuclear periphery	Nuclear, mRNA- binding
Npl3-GFP/Npl3p	WT	"
Nab4p	WT	"
Mex67-GFP	WT	"
Nop1p	WT	Nucleolar, pre-rRNA-binding (Henriquez et al., 1990)
SV40NLS-GFP3	WT	Classical NLS cargo (Gorlich, 1997)
Xpo1-GFP	WT	Exportin for Leucine Rich NES pathway, affects mRNA export (Stade et al., 1997)

* All reporters were examined after 3 hours at 37° C, except Mex67-GFP and Xpo1-GFP which were examined after 1.5 hr. and 2 hr., respectively. SV40NLS-GFP3, Npl3-GFP and Nab2-GFP were also examined after 5hr at 37° C. Nab2p and Npl3p were also examined at 1, 1.5 and 2 hr. Nab2-GFP was also examined after 30, 45, 60, 90, and 120 minutes. Endogenous Nab2p and Npl3p, Nab2-GFP and Npl3-GFP were assayed in both *Δtom1* (yKD100-2D) and *tom1-C3235A* (yKD127) strains. Nab4p and Nop1p were assayed in *tom1-C3235A* (yKD127) strains. Mex67-GFP, SV40NLS-GFP3 , and Xpo1-GFP were assayed in *Δtom1* (yKD100-2D).

Table II: Specificity of Nab2/mRNA co-localization to *tom1* mutants

Strain	mRNA localization * (at 37° C)	Nab2p localization* (at 37°C)
WT	Whole Cell	nucleoplasmic
Δtom1, tom1-C3235A	3-7 foci at NPC	3-7 foci with mRNA
mex67-5 (Segref et al., 1997)	3-5 nuclear foci	nucleoplasmic
prp20-1(Vijayraghavan et al., 1989)	foci/whole nuclear	nucleoplasmic/ cytoplasmic
xpo1-1 (Stade et al., 1997)	whole nuclear	nucleoplasmic

* Assayed after 90 or 120 minutes. *mex67-5* and *xpo1-1* were also examined after 30 minutes.

Table III: Oligos used in this study

OKD	Length	Sequence (5'-3')
120	74	ATT TTG GTT CAT CAG AAA GAC TAC CAT CAT CAC ATA CCG CAT TCA ATC AAC TGA ATT TAC CTC CTT ATG AAT CA
121	77	TAT GAT TCA TAA GGA GGT AAA TTC AGT TGA TTG AAT GCG GTA TGT GAT GAT GGT AGT CTT TCT GAT GAA CCA AAA TC
122	33	GAC CTA AGA TCT GGG TTT GGT CTT GCC TGA TCA
123	37	GAG TCG TAG GAT CCC TGA CAA CAG TTC AAC TAA GAG C
124	21	CGC TCT AGA ACT AGT GGA TCC
125	36	GAG TCG ATC TCG AGT TCA CAC CGA GTA AAA AGC ACC
126	31	GCA TGA ATT CGG CCG CAT CTT TTA CCC ATA C
127	31	ATG AAG ATC TGC ACT GAG CAG CGT AAT CTG G
128	33	GAG TCG GGA TCC GGG CAG TTA GAT CCT TCG ACG
129	31	GCA TGA ATT CGG CAA GAC CAA ACC CTT CAT G
130	49	GCA TGA ATT CGC CAG ATC TTG ATC ATT TTT CGT GTA AAT TTA TAG CGC C
131	23	TTA CGC CAA GCT CGG AAT TAA CC
133	43	GCA TGA ATT CCA TGA ATA TAT ATT TTC AAT CAA AGG CAA AAA G
134	43	GCA TGA ATT CGC TAG ATC TGT GCT TTT TAC TCG GTG TGA AAA G
135	34	AGT CGA TCT CGA GGT AGG TAT TAA ACC CGC GGC G
156	43	TAT GAG ACA TTG CGT GGT TCA CTA TTA TTG GCA ATC AAT GAA A
157	45	GAT CTT TCA TTG ATT GCC AAT AAT AGT GAA CCA CGC AAT GTC TCA
160	33	CGT CCC CTC GAG CAT GCA TCA TCA TCA TCA TCA
161	20	TGT TGT GCG ATA TCC GAT TG

Chapter 2

Evidence for a novel, non-import role of Kap104p in Tom1p-mediated Nab2p-mRNA export

Abstract

Nab2p is a shuttling hnRNP protein essential for mRNA export in *Saccharomyces cerevisiae*. Efficient export of Nab2p-mRNA complexes requires Tom1p, a putative ubiquitin ligase. *Δtom1* strains are temperature sensitive, displaying highly-specific redistribution of Nab2p and polyA RNA to the nuclear periphery at 37° C. We hypothesized that this redistribution of Nab2p and polyA RNA requires a Nab2p interacting protein. Accordingly mutations of this factor should be epistatic to Δ*tom1*, abrogating redistribution of Nab2p and polyA RNA to the nuclear periphery. Kap104p, the Nab2p import receptor, is already known to target Nab2p to the nuclear pore complex, making it an excellent candidate for a targeting factor in Nab2p-mRNA export. To test this hypothesis we examined Nab2p-mRNA localization in temperature-sensitive *kap104-161 Δtom1* double mutants. Indeed, we find that *kap104-161* is epistatic to *Δtom1*. In contrast, nucleoporin mutants that reduce Nab2p import to the same extent as *kap104-161* do not affect Nab2p-mRNA accumulation at the nuclear periphery when combined with *Δtom1*, implying that decreased import of Nab2p is not sufficient for epistasis. In further support of our hypothesis, we have found a novel genetic interaction between *NAB2* alleles lacking the Kap104p-binding domain and *PAB1*, which encodes the yeast homolog of the cytoplasmic polyA binding protein. These *NAB2* alleles cannot complement *Δnab2*, but interfere with bypass suppression of *Δnab2* by high-copy *PAB1*. Taken together, our results imply a novel, non-import role for Kap104p in promoting export of Nab2p-mRNA complexes, and raise the possibility that this role relates to mRNP remodeling involving Nab2p and Pab1p.

Introduction

Export of mRNA from the nucleus to the cytoplasm is a fundamental process of eukaryotic cells. Early studies in *Xenopus* oocytes revealed that mRNA export is mediated by at least one export receptor that is specific for mRNA vs. other classes of RNAs (Jarmolowski et al., 1994). Presumably this receptor mediates interaction between mRNA export cargo and the nuclear pore complex (NPC), since mRNA export, like most, if not all macromolecular exchange between the nucleus and cytoplasm occurs through the NPC. Although the precise molecular composition of mRNA export cargos is not known, it is believed that mRNAs complexed with proteins in ribonucleoprotein particles (RNPs) are the export substrates that interact with the export receptor. The protein component of these RNPs is primarily composed of hnRNP proteins, nuclear-localized RNA-binding proteins that typically associate with mRNA co-transcriptionally and 'package' the nascent transcripts into 'ribonucleosome' structures (Dreyfuss et al., 1993). hnRNP proteins are themselves heterogeneous, comprising a wide variety of different classes of RNA-binding motifs in diverse modular protein domain arrangements. This diversity suggested that hnRNP proteins might have differential affinities for particular hnRNA/mRNA sequences or structures. Available evidence supports a model in which the relative concentrations of specific hnRNP proteins in association with a particular transcript not only vary, but can profoundly influence subsequent steps of mRNA metabolism, including 3'-end processing, pre-mRNA splicing, mRNA localization and mRNA stability(Krecic and Swanson, 1999). A very

recent paper provides indirect support for a similar model in mRNA export in mammalian cells (Gallouzi, 2001).

A subset of hnRNP proteins appear to 'shuttle' rapidly between the nucleus and cytoplasm, appearing exclusively in the nucleus at steady state presumably due to rapid re-import from the cytoplasm (Pinol-Roma and Dreyfuss, 1992). There is evidence that export of the shuttling hnRNP proteins occurs in association with mRNA (Visa et al., 1996). Several shuttling hnRNP proteins contain special nuclear export sequences (NES's) that are distinct from the leucine-rich class of NES recognized by CRM1, and appear unique to shuttling hnRNP proteins (Michael, 2000). For this reason it has been proposed that these proteins actively promote mRNA export through interaction with an NES-recognizing export receptor that docks the RNP complex at the NPC and promotes translocation of the RNP complex through the NPC (Michael, 2000). During the export process, cytoplasmic mRNP proteins are substituted for hnRNP proteins in a poorly understood process called 'RNP remodeling' (Dreyfuss et al., 1989; Nakielny and Dreyfuss, 1997). The shuttling hnRNP proteins are subsequently re-imported into the nucleus for another round of export. Results from several different experimental systems suggest that RNP remodeling may be a dynamic, multi-stage process with some proteins leaving prior to docking at the NPC, some leaving concomitant with NPC docking/translocation, and still others remaining associated with mRNA in polysomes (Daneholt, 1997; Daneholt, 1999; Kataoka et al., 2000; Kim et al., 2001; Le Hir et al., 2001; Le Hir et al., 2000; Luo et al., 2001; Mili et al., 2001; Reed and Magni, 2001; Zhou et al., 2000).

The strongest evidence supporting the shuttling hnRNP protein-mediated model for mRNA export comes from extensive studies of hnRNPA1. A1 is a highly abundant hnRNP protein with the "2xRBD-Gly" domain structure found in several hnRNP proteins (Dreyfuss et al., 1993). The A1 homolog in *Chironomus tentans* has been shown by immunoelectron microscopy analysis to accompany the Balbiani Ring RNP particle from the nucleus through the NPC and into polysomes (Visa et al., 1996). A 38 amino acid sequence called 'M9' is required for import and export of A1 and is sufficient to act as an NLS and NES in a heterologous context (Michael et al., 1995a; Michael et al., 1995b). M9 is distinct from the A1 RNA binding domains and has no RNA-binding activity on its own. Thus, M9-mediated export of A1 is unlikely to be due to A1 'piggybacking' on mRNA bound to a different export receptor. Importantly, full length hnRNPA1 has been functionally implicated in mRNA export by microinjection assays in *Xenopus laevis* oocytes (Izaurralde et al., 1997). In these studies, co-injection of high levels of A1 with mRNA export substrates specifically inhibited export of the mRNAs, presumably due to competition with endogenous hnRNPA1 export complexes for an export receptor. The M9 sequence plays a key role in this process: co-injection of A1 lacking the M9 sequence (a protein known to retain full non-specific RNA binding activity *in vitro*) fails to compete with endogenous factors for mRNA export. This implies that the unidentified export receptor interacts with M9 (Izaurralde et al., 1997).

To date the only specific M9-interacting factor identified is transportin1/karyopherin β2 (trn1), a karyopherinβ1/importinβ homolog, (Bonifaci et al., 1997; Fridell et al., 1997; Pollard et al., 1996). Trn1 plays a direct role in the nuclear import of hnRNPA1 via interaction with the M9 sequence (Nakielny et al., 1996; Pollard

et al., 1996). Conversely, three lines of evidence have been used to argue that transportin does not play a role in mRNA export. First, co-immunoprecipitates of Trn1 with hnRNPA1 from HeLa cell nuclei are not RNAse-sensitive and do not contain the non-shuttling hnRNP C1 protein, a major component of hnRNP complexes (Siomi et al., 1997). These findings suggest that Trn1 is not a stable component of hnRNP complexes. Second, although a ternary complex between transportin, hnRNPA1 and a short RNA with high affinity for A1 can be formed *in vitro* and detected in an electrophoretic mobility shift assay, the complex is unstable in the presence of Ran-GTP (Nakielny et al., 1996; Siomi et al., 1997). This suggests that ternary complexes between Trn1, A1 and mRNA would not be stable in the nucleus, where the concentration of Ran-GTP is presumed to be high. Finally, a monoclonal antibody against the A1 M9 sequence cannot immunoprecipitate A1 from nuclear or cytoplasmic extracts, leading the authors to conclude that the M9 sequence is inaccessible to Trn1 in hnRNP complexes (Siomi et al., 1997). On the basis of these results, and in the absence of any direct functional evidence to suggest otherwise, the idea that Trn1 might play a role in mRNA export has now been all but dismissed. However, exhaustive 2-hybrid and protein affinity based screens have failed to find any protein other than transportin that specifically associates with hnRNP A1 M9, and saturation mutagenesis of the M9 region of A1 has failed to separate the NLS and NES activities, implying that they act via an extremely similar molecular mechanism (Michael, 2000). Thus, we are left with a paradox: A1 and M9 are important for mRNA export, but Trn1, the only protein that can be found to specifically interact with the M9 sequence, is assumed not to play a role in export.

S. cerevisiae has a clear transportin homolog, Kap104p which has also been shown to be required for import of two yeast hnRNP proteins, Nab2p and Nab4/Hrp1p (Aitchison et al., 1996). Regions of these proteins that are necessary and sufficient for Kap104p binding have been identified (Lee and Aitchison, 1999; Siomi et al., 1998; Truant et al., 1998). Intriguingly, the Kap104p-binding region of Nab2p has a stretch that can be aligned with M9 sequences and shares several of the residues that are conserved among M9 sequences across phyla, some of which are required for NLS/NES activity (Siomi et al., 1998). Thus, *S. cerevisiae* appears to have the molecular components that correspond to the transportin-M9 system and therefore provides a potential opportunity to use genetic approaches to determine the contribution of a Nab2p M9-like sequence and Kap104p to mRNA export in yeast. Moreover, evidence suggests that the interaction of Kap104p with the NPC is functionally conserved in evolution, since Kap104p can successfully import Nab2p into mammalian nuclei. This underscores the notion that mechanisms of crossing the nuclear envelope deciphered in yeast will prove to be conserved.

Nab2p is a shuttling hnRNP protein that is essential for mRNA export in budding yeast ((Anderson et al., 1993) and Anderson, Nykamp and Swanson, manuscript in preparation). Recent results from the Swanson lab suggest that Nab2p may also play a key role in regulation of polyA tail length in the nucleus. Their results suggest that Nab2p in yeast may function analogously to mammalian PABP2, regulating polyA tail length in the nucleus, and coupling tail length control/proper 3'-end formation with mRNA export ((Anderson et al., 1993; Calado et al., 2000; Segref et al., 1997; Wahle, 1991a; Wahle, 1992) and Anderson, Nykamp and Swanson, manuscript in prep). In

addition, their results raise the possibility that in wild type cells a 'handoff' from a Nab2p-RNP to a Pab1p-RNP may be an important 'mRNP re-modeling' step in Nab2p-mediated mRNA export.

Our previous work (Duncan et al., 2000) (described in Chapter 1) indicated a requirement for the nuclear protein, Tom1p, a putative ubiquitin ligase, for efficient export of Nab2p-mRNP complexes. In *Δtom1* strains at the non-permissive temperature of 37° C, Nab2p and polyA RNA re-distribute from their respective nucleoplasmic and cytoplasmic localization patterns and appear to accumulate together in complexes at the nuclear periphery. This effect is selective for Nab2, insofar as localization of other yeast hnRNP proteins is not affected. Of particular note, we found that Npl3p remains associated with polyA RNA and continues to be efficiently exported in Δ*tom1* strains. Thus, the Tom1-dependent Nab2-mRNA export pathway appears to be distinct from other mRNA export pathways involving Npl3p or other yeast hnRNP proteins. However, the regions of Nab2p that mediate this effect and the additional trans-acting factors aside from Nab2p or Tom1p that participate in this apparently specific mRNA export pathway have yet to be determined.

Here we present genetic evidence that is consistent with a non-import role for Kap104p in promoting efficient mRNA export via Nab2p. We find that a temperature sensitive allele of *KAP104*, *kap104-161*, derived from the previously characterized temperature sensitive null allele, *kap104-16* (Aitchison et al., 1996) is epistatic to *Δtom1*. This implies that Kap104p is required for significant accumulation of Nab2-mRNA complexes at the nuclear periphery in *Δtom1* mutants. This might not seem surprising, given that Kap104p is required for Nab2p import. However, two lines of evidence

suggest that defective Nab2p import is not the reason for epistasis. First, we see substantial nucleoplasmic signal maintained in *kap104-161* strains at 37° C. Second, two nucleoporin mutants, *Δnup100* and *Δnup145N* (Fabre et al., 1994; Iovine et al., 1995; Wente and Blobel, 1994), display similar effects on Nab2p import to *kap104-161*, but apparently do not affect the accumulation of Nab2-mRNA complexes at the nuclear periphery in *Δtom1*. Rather, *Δtom1* appears epistatic to these two mutants for localization of Nab2-GFP. We also present genetic evidence indicating a requirement for the Kap104p binding domain for efficient Nab2-mRNA export and implicating Pab1p, the yeast homolog of the cytoplasmic polyA binding protein (Adam et al., 1986; Sachs et al., 1986) in this process as well. Taken together, our results imply the existence of a novel non-import role for Kap104p in promoting efficient export of Nab2p-mRNA complexes. We hypothesize that this non-import function could include a direct role in promoting Nab2p-mRNA export and may simultaneously, or independently contribute to export-related 'Nab-to-Pab' transition from a Nab2p-bound mRNP to a Pab1p-bound mRNP.

Results

Kap104p is required for the mislocalization of Nab2p to the nuclear periphery in *Δtom1* mutants

Temperature sensitive *Δtom1* strains shifted to 37° C display a highly-specific redistribution of Nab2p and polyA RNA to colocalize at the nuclear periphery (Duncan et al., 2000) (Chapter 1). We hypothesized that this redistribution of Nab2p and polyA RNA requires a Nab2p interacting protein. Loss-of-function mutations of this factor should be epistatic to *Δtom1*, abrogating redistribution of Nab2p and polyA RNA to the nuclear periphery (Figure 1). Thus, epistatic analysis with *Δtom1* and mutants of interest may be a general assay for identifying additional components of the Tom1p-mediated Nab2p-mRNA export pathway that are required for targeting Nab2p and mRNA to the nuclear periphery. We were particularly interested in testing Kap104p in this assay. Since Kap104p is the Nab2p import receptor (Aitchison et al., 1996), and is therefore already known to target Nab2p to the nuclear pore complex, it would seem an excellent candidate for a targeting factor in Nab2p-mRNA export. We therefore generated double mutants with *Δtom1* and a temperature sensitive allele of *KAP104*, and examined Nab2p-mRNA localization in these strains.

To generate an isogenic temperature sensitive *kap104* mutant to cross to the previously-characterized *Δtom1* strain (Duncan et al., 2000) we rescued the temperature-sensitive allele of *KAP104*, *kap104-16* (Aitchison et al., 1996, from yeast and sub-cloned

it into an integrating vector, pRS306. This allele was then integrated into the wild type yeast strain YPH399 using the two-step gene-replacement method {Rothstein, 1991 #3855). Temperature sensitive *kap104-16* integrants were isolated and an isolate that could be completely rescued by plasmid-borne wild type *KAP104* was crossed to the isogenic *Δtom1* strain. This strain was sporulated to obtain independent isolates of integrated *kap104-16* and *Δtom1 kap104-16* double mutants. The original *kap104-16* allele was generated by passage of a *KAP104* plasmid through a mutagenizing *E. coli* strain, and was demonstrated to be a temperature-sensitive null allele (Aitchison et al., 1996). However, the number of mutations comprising this allele and their locations in Kap104p were never determined (Aitchison, pers. comm.). Thus, the original *kap104-16* allele might contain multiple mutations, not all of which will necessarily be retained during two-step gene replacement. Consequently, we will refer to our temperature sensitive allele as "*kap104-161*" to reflect the fact that it is derived from *kap104-16*, but may not be identical to it. Analysis of 79 progeny from the cross of *kap104-161* to *Δtom1* revealed no evidence of suppression or synthetic growth defects between *kap104-161* and *Δtom1*. Thus, this allele of *KAP104* does not interact genetically with *Δtom1* according to growth assays on rich medium.

Since the *Δtom1 kap104-161* double mutant strains were viable at 25° C, we next examined the localization of Nab2p by immunofluorescence in *Δtom1 kap104* double mutants and isogenic single mutants and wild type control strains during a shift from 22° C to 37° C. In contrast to wild type cells, which maintained nucleoplasmic signal throughout the shift, there was clearly an accumulation of Nab2p signal in the cytoplasm of *kap104-161* strains after a 90 minute shift to 37° C (Figure 2A), consistent with the

previously reported requirement for Kap104p for efficient Nab2p import (Aitchison et al., 1996). However, in contrast to *kap104-16* mutants (Aitchison et al., 1996), Nab2p mislocalization in *kap104-161* strains was far from complete, with substantial nucleoplasmic signal representing the bulk of Nab2p signal in most cells (Figure 2A). *Δtom1* strains showed the expected punctate nuclear peripheral localization as previously described. However, *Δtom1 kap104-161* double mutants at 37° C in addition to showing increased cytoplasmic Nab2p, displayed a striking reduction of punctate Nab2p signal at the nuclear periphery and a concomitant increase in nucleoplasmic signal relative to *Δtom1* in most cells, with the nuclear staining pattern for >60% of cells indistinguishable from wild type (Figure 2A). The effect was also observed with Nab2-GFP. In this case the phenotype was even more striking, with an essentially 100% penetrant redistribution of Nab2-GFP from the nuclear periphery to the nucleoplasm and cytoplasm in double mutants relative to *Δtom1* (data not shown). This discrepancy could reflect a difference in sensitivity of the assays, or could reflect a subtle reduction in Kap104p interaction with the Nab2-GFP fusion. These data indicate that wild-type Kap104p is required for the redistribution of Nab2p seen in *Δtom1* strains, and suggest that the effect does not result from major depletion of Nab2p from the nucleus.

Kap104p is required for the mislocalization of mRNA to the nuclear periphery in *Δtom1* mutants

In order to determine whether the effect of *kap104-161* mutation on the *Δtom1* phenotype extended to mRNA, we performed the dT50 FISH assay on *kap104-161 Δtom1* double mutants and compared the results to single mutants and the isogenic wild type strain. When *Δtom1* mutants were shifted from 22° C to 37° C for 1.5 or 3 hours,

they displayed strong accumulation of polyA RNA in a punctate pattern at the nuclear periphery, as we have reported previously (Duncan et al., 2000) (Figure 2B and data not shown). In striking contrast, *kap104-161 Δtom1* double mutants displayed a major reduction in the number of cells displaying punctuate localization to the nuclear periphery. In addition, the average intensity of the phenotype in those cells that continued to display the phenotype was reduced substantially relative to *Δtom1* cells (Figure 2B). This result implies that full Kap104p activity is required not only for strong Nab2p mislocalization in *Δtom1*, but also for the mRNA mislocalization phenotype. This, in turn, supports the idea that Nab2-RNP's are the entities that are mislocalized at the nuclear periphery in *Δtom1* mutants. Finally, this result also raises the possibility that direct interaction of Kap104p with Nab2p is responsible for directing Nab2-mRNP complexes to the nuclear periphery in *Δtom1* mutants. This is a particularly intriguing possibility, since it would seem to suggest a role for Kap104p in Nab2p-mediated mRNA export (see discussion).

We also noted four other interesting observations during these experiments. First, in contrast to published reports, we observed a clear, reproducible accumulation of mRNA in the nucleus of a substantial fraction (35-50%, depending on iteration of assay) of *kap104-161* mutants (Figure 2B). This phenotype appears relatively early after a shift to 37° C (<1 hr.) and is also observed in our hands with the original *kap104-16* mutant strain (data not shown). Second, the polyA RNA accumulation pattern in *kap104-161* and *kap104-16* cells is much more condensed than is typically observed with most other mRNA export mutants. The dT50 signal is condensed into a single dot in most cases that occupies only a fraction of the nuclear volume (Figure 2B). While the significance of

accumulation in this previously unprecedented pattern is not clear, the accumulation of substantial polyA FISH signal in the nucleus at fairly early time points after a shift to the non-permissive temperature supports the possibility that Kap104p may influence mRNA export more directly than was previously envisaged. Third, we noted that *kap104-161* is not simply epistatic to *Δtom1*. The number of double mutant *kap104-161 Δtom1* cells showing a *kap104-161* pattern of mRNA localization is substantially lower than is observed with *kap104-161* alone (14% vs. 38%, based on quantification of phenotype in multiple wells in two independent assays). Finally, while mRNA no longer accumulates in a punctate pattern at the nuclear periphery in the double mutant, it also does not accumulate in the nucleoplasm. In principle, this could represent increased nuclear turnover of polyA RNA in *Δtom1 kap104-161* vs. *Δtom1*. However, we consistently observed a modest, but reproducible, increase in cytoplasmic mRNA in the double mutant relative to Δ*tom1* (Figure 2B). This suggests that there may actually be *increased* export of polyA RNA in the double mutant vs. Δ*tom1*. One possible implication of this result is that the nuclear pool of mRNA that accumulates in the absence of Tom1p function is able to access a different export pathway when Kap104p function is simultaneously impaired.

***Δtom1* is epistatic to nucleoporin deletion mutants that reduce the efficiency of Nab2p import**

As reported above, on its own the *kap104-161* effect on Nab2p localization is relatively subtle, with substantial nucleoplasmic signal clearly visible in the vast majority of cells. This indicates that *kap104-161* epistasis over *Δtom1* does not result from major depletion of Nab2p from the nucleus. Nevertheless, assuming a constant level of Nab2p in the

different strains, increased cytoplasmic Nab2p in *kap104-161* strains implies there must be a concomitant decrease in Nab2p nuclear concentration, albeit a modest one. Furthermore, the Δ*tom1* phenotype might be sensitive to the Nab2p import rate rather than the nuclear concentration of Nab2p at steady-state. A very strong effect on the import rate by *kap104-161* would increase the cytoplasmic Nab2p signal when protein synthesis is ongoing, but would not necessarily deplete the nuclear pool, since the rate of nuclear depletion is determined by the export rate. To address these concerns regarding the *kap104-161* effect on Nab2p/mRNA accumulation in *Δtom1*, we sought to generate double mutants of *Δtom1* with transport mutants that would phenocopy the increased cytoplasmic localization of Nab2p observed with *kap104-161*. The GLFG and FG repeat nucleoporins were strong candidates for such mutants since they are implicated in translocation through the NPC and several of them, including Nup100p, Nup145p, and Nup42/Rip1p have all been shown to interact directly with Kap104p by *in vitro* binding assays (Ryan and Wente, 2000).

Double mutants of the Kap104p-interacting nucleoporins *NUP100*, *NUP145* and *RIP1/NUP42* with *Δtom1* were generated and tested for effects on Nab2-GFP localization when combined with *Δtom1* and shifted to 37° C for 2 hours. In striking contrast to *kap104-161*, *Δnup100* and *Δnup145-N* showed no effect on mislocalization of Nab2-GFP in double mutants (Figure 3). Indeed, *Δtom1* appeared to be completely epistatic to these mutants with respect to Nab2-GFP localization. These results are particularly notable because, as we had hoped for when we selected them to test, these mutants by themselves phenocopy *kap104-161*, causing Nab2-GFP to accumulate in the cytoplasm, presumably due to a reduction of the Nab2p import rate (Figure 3). At least in the case of *Δnup145-*

N, the import defect, as judged by the intensity of cytoplasmic Nab2-GFP signal, appears as great or greater in strength in the single mutant to that seen with *kap104-161* (Figures 2A, 3B and data not shown). *Δtom1* was also epistatic to *Δrip1*, but the significance of this result is less clear, since *Δrip1* showed no effect on Nab2-GFP localization under the assayed conditions (data not shown). In sum, at least two other factors that shift Nab2p localization toward the cytoplasm as single mutants do not modify the Nab2p mislocalization caused by the absence of Tom1p function. Since both of these mutants are likely to directly impact nuclear translocation of Nab2p, these results suggest that the *kap104-161* effect on Nab2p in *Δtom1* is neither an effect of reduction of the amount of Nab2p in the nucleus, nor a general result of slowing of the Nab2p import rate. We conclude that Kap104p plays a special role in Tom1-mediated Nab2-mRNA export beyond simply ensuring that a sufficient Nab2p nuclear concentration/import rate is maintained.

The kap104-binding domain/RGG box is essential for Nab2p function

A Kap104 binding domain in Nab2p has been mapped by 2-hybrid and *in vitro* binding assays (Lee and Aitchison, 1999; Siomi et al., 1998; Truant et al., 1998) (Figure 4A). If binding of this site to Kap104p plays a major role in import of Nab2p one would expect the Kap104 binding domain to be essential. This is also the prediction if it plays a key role in Nab2p-mediated mRNA export. Surprisingly, to our knowledge, this was never examined previously. We therefore generated two alleles of *NAB2* that severely altered the Kap104p binding site (Figure 4B), and assayed their ability to complement *Δnab2* in *TOM1* and *Δtom1* strains using a plasmid shuffle assay (Figure 4C). Consistent with the prediction, neither allele could support significant growth at any temperature in the

absence of wild type *NAB2*, indicating that the Kap104-binding domain/RGG box is essential for Nab2p function in both *TOM1* and *Δtom1* strains (Table 1). These growth defects were completely recessive, implying a loss of function for these proteins is responsible for the associated inviability.

If the function of this region of the protein were solely to deliver Nab2p to the nucleus via interaction with Kap104p, then delivering Nab2p to the nucleus by another import pathway would be expected to rescue the phenotype. To test this hypothesis, we added a classical NLS (cNLS) to the N-terminus of *NAB2-GFP* and *nab2-Δ35-GFP* and assayed the ability of these alleles to complement *Δnab2*. *cNLS-NAB2-GFP* complemented completely, but *cNLS-Δ35-GFP* failed to support significant growth at any termperature when present as the sole copy of *NAB2*. We also examined the localization of these alleles of *NAB2* (Table 1, data not shown). *cNLS-NAB2-GFP* localization was not significantly perturbed in WT or *Δtom1* strains. In contrast, in the presence of endogenous wild type Nab2p the localization of *nab2-Δ35-GFP* was severely shifted to the cytoplasm, consistent with a requirement for interaction with Kap104p for efficient nuclear import. *cNLS-nab2-Δ35-GFP* partially rescued this phenotype, but did not completely eliminate the cytoplasmic signal (Table 1, data not shown). Thus, inviability of the *cNLS-nab2 Δ35-GFP* allele might result from incomplete rescue of the nuclear import defect of *nab2-Δ35-GFP* by the cNLS. Alternatively, it is possible that increased cytoplasmic signal reflects a shift in the rate-limiting step for import, for example to release from mRNA. Precedent for this possibility has been seen with Npl3p in the context of the interaction with its import receptor, Mtr10p (Gilbert et al., 2001).

Nab2p lacking the kap104-binding domain/RGG box displays 'bypass suppression interference'

Nab2p is essential for polyA tail length control *in vivo* and efficient mRNA export (M. Swanson, personal communication/in prep). However, inviablility of *Δnab2* strains can be suppressed by increased dosage of *PAB1*, the gene encoding the yeast homolog of PABP1, the cytoplasmic polyA-binding protein (M. Swanson, personal communication/in prep, (Adam et al., 1986; Sachs et al., 1986)). Interestingly, high copy *PAB1* seems to bypass the requirement for Nab2p for polyA RNA export without having a substantial effect on polyA tail length (M. Swanson, personal communication/in prep). This observation suggests that it is the mRNA export defect, rather than loss of polyA tail-length control, that leads to inviability of *Δnab2* strains, and that the mRNA export role is the Nab2p function that is bypassed by high-copy *PAB1*.

We were originally interested in bypass suppression of *Δnab2* by *2μPAB1* because we hoped to use it to establish a system for analyzing localization of inviable Nab2 alleles of interest (e.g. in the Kap104p-binding domain, as described above) in the absence of endogenous wild type Nab2p. However, in the process of attempting to generate such strains we observed that alleles of Nab2 that delete or heavily modify the Kap104p binding domain, *nab2-Δ35* and *nab2-KKM3* (Figure 4C) displayed marked reductions in both overall transformation efficiency and growth rate during recovery from transformation, as compared to *Δnab2 2μ PAB1* strains transformed with empty vector (Figure 5). As described in the previous section, this effect was not observed when plasmid-borne wild type *NAB2* was present rather than *2μ PAB1*, indicating that the *nab2* alleles are completely recessive to wild type *NAB2* (Figure 5B). This suggests that these

alleles are highly unlikely to be non-specifically interfering with cell viability. Thus, inviable, fully-recessive *NAB2* alleles that are predicted to lack Kap104p binding, but expected to have RNA/polyA binding activity, interfere with the ability of increased Pab1p levels to support growth by the bypass suppression mechanism. We have termed this apparently novel type of genetic interaction 'bypass suppression interference' ('BSI'). Presumably BSI results from competition between Pab1p and the mutant Nab2p alleles for at least one common binding partner, the most obvious candidate being polyA RNA itself. Binding of *NAB2* mutants is apparently 'non-productive' in the sense that the complexes fail to support a necessary additional function/interaction, and this leads to growth defects. As mentioned above, *2μPAB1* bypasses the requirement for Nab2p for efficient mRNA export. This suggests that *NAB2* alleles displaying BSI interfere with Pab1p's ability to promote mRNA export and are likely to be unable to support mRNA export themselves. Thus, BSI by alleles predicted to be unable to interact with Kap104p provides additional genetic evidence for a critical non-import role of Kap104p in promoting Nab2p-mRNA export.

Bypass suppression interference by *nab2-Δ35* is enhanced by the presence of a cNLS at its N-terminus

As reported above, *nab2Δ35-GFP* is primarily localized in the cytoplasm (data not shown). This seemed to suggest that the BSI effect may take place in the cytoplasm. However, the effect on transformation efficiency and recovery time of *nab2-Δ35* in *Δnab2 2μPAB1* strains was substantially increased by addition of a 'classical' NLS (cNLS) to the N-terminus of *nab2-Δ35*, implying that greater access to the nucleus enhanced the effect (Figure 6). Thus, BSI can be enhanced by targeting *nab2-Δ35-GFP* to

the nucleus in a Kap104p-independent manner. This result raises the possibility that BSI may actually occur in the nucleus or perhaps during export from the nucleus.

Δ*tom1* also compromises the ability of *2μPAB1* to suppress *Δnab2*

Because *TOM1* affects Nab2p localization, and interacts genetically with *NAB2* ((Duncan et al., 2000); KD and CG, unpublished results) we were interested to determine whether there was any effect of *Δtom1* on bypass suppression of *Δnab2* by *2μPAB1*. We tested growth after transformation of *Δtom1 Δnab2 2μPAB1* strains relative to *TOM1 Δnab2 2μPAB1* strains and control strains with *pNAB2* instead of *2μ PAB1* (Figure 7A). Control *Δtom1* strains containing *pNAB2* took only slighty longer to form visible colonies after transformation than did *TOM1 pNAB2* strains assayed in parallel under equivalent conditions. (Figure 7B, and data not shown). Similarly, in the context of *Δnab2 2μPAB1* strains transformed with *pRS314-NAB2-GFP*, which fully complements *Δnab2 Δtom1* strains grew only slightly slower after transformation than *TOM1* strains under these same conditions. In contrast, *Δtom1* severely slows recovery of *Δnab2 2μPAB1* strains transformed with empty pRS314 vector, implying that *Δtom1* reduces the efficiency of bypass suppression by *2μPAB1* (compare 'vector' column for *TOM1* and *Δtom1* strains in Figure 7). The BSI alleles also produce slower recovery in *Δtom1 Δnab2 2μPAB1* strains relative to their *TOM1* counterparts, but the profound effect of *Δtom1* on bypass suppression itself makes it impossible to conclude whether *Δtom1* exacerbates BSI by these alleles. We conclude that Tom1p function is required for efficient bypass suppression of *Δnab2* by high copy *PAB1*. This implies either that Tom1p directly or indirectly influences Pab1p activity or, alternatively, that *Δtom1* produces a 'cellular state' that makes bypass suppression of *Δnab2* by higher dosage of fully active Pab1p

less effective. This raises the possibility that Tom1p activity is necessary not only for efficient Nab2-mediated mRNA export, but for efficient Pab1p-mediated mRNA export as well.

Discussion

In this report we have presented genetic and cytological evidence supporting the existence of a non-import function for Kap104p in promoting efficient mRNA export via Nab2p. Our results also provide genetic evidence that this function may be to promote mRNP remodeling involving replacement of the hnRNP protein Nab2p by the yeast homolog of the cytoplasmic polyA binding protein, Pab1p (Adam et al., 1986; Sachs et al., 1986). Two major lines of evidence support these ideas. First, the temperature-sensitive *kap104-161* mutation dramatically decreased the accumulation of Nab2-mRNA at the nuclear periphery in *Δtom1* at 37° C, implying that Kap104p is required for redistribution of Nab2p-mRNA to the nuclear periphery in *Δtom1* strains. However, Kap104p does not seem to be required to maintain the nuclear concentration of Nab2p or the Nab2p import rate, as nucleoporin mutants that also reduce Nab2p import rates did not affect the accumulation of Nab2-mRNA complexes at the nuclear periphery in *Δtom1*. Second, we have identified alleles of *NAB2* that interfere with the ability of high copy *PAB1* to suppress the lethality of *Δnab2*. The alleles that show this effect are predicted to be unable to interact with Kap104p, but to retain RNA binding activity. Taken together, our genetic and cytological results imply the existence of a novel, non-import function of Kap104p in Nab2-mRNA export. They also implicate mRNP remodeling involving Pab1p as a key transition in Nab2-mediated mRNA export, and raise the possibility that Kap104p and Tom1p affect the efficiency of this step in mRNA export.

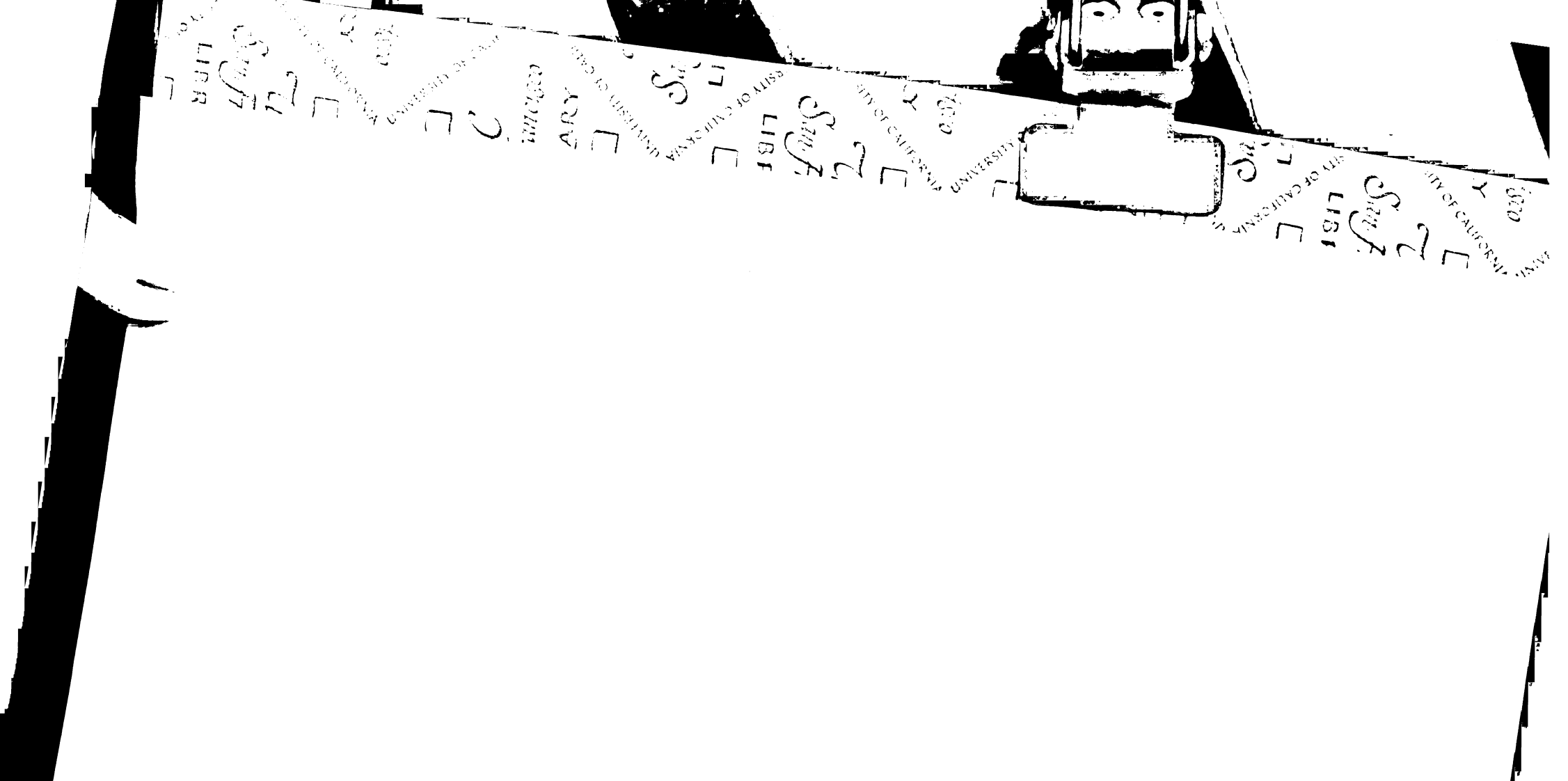
Possible non-import functions for Kap104p in Tom1p-mediated Nab2p-mRNA export

We envisage three major classes of non-import functions for Kap104p that we distinguish based on their relative timing in a 'Nab2p lifecycle' (Figure 8). Class 1/'Nab2RNP formation' functions would occur after nuclear import of Nab2p, and would promote formation of an export-competent particle. Such functions could be viewed as being 'upstream' of export (Figure 8A). Hypothetical class 1 functions might include post-import intranuclear targeting as well as more direct roles in hnRNP formation or stability. Class 2/'export complex docking' functions would be directly upstream of or coincident with export, and would involve a direct role of Kap104p in promoting docking of Nab2-mRNA complexes at the NPC (Figure 8B). Class 3/'RNP remodeling' functions would occur 'just downstream' of export- or perhaps also coincident with it- and presumably 'just upstream' of re-import and would most likely consist of displacement of Nab2p from the export RNP and subsequent inhibition of RNA binding to Nab2p (Figure 8C). This class of function for Kap104p that has been hypothesized previously based on *in vitro* biochemical results (Lee and Aitchison, 1999). We now review our results within the framework of these three classes of possible functions for Kap104p. Our goal will be to determine which function is most consistent with our results and those of others in yeast and other experimental systems.

One major line of evidence that may help to narrow the field of possible non-import Kap104p functions is the dramatic effect on Nab2p and mRNA localization in *Δtom1 kap104-161*, where accumulation of these two molecules at the nuclear periphery is greatly reduced. Importantly, this effect is unlikely to be due to different rates of onset

for the mutant phenotypes, since the rates of onset of *kap104-161* and *Δtom1* temperature sensitive phenotypes are actually quite similar (KD and CG, unpublished results). Most importantly, the phenotype is specific for *kap104-161*: nucleoporin mutants that show similar effects on Nab2p import do not reduce Nab2-GFP mislocalization in *Δtom1* (Figure 3). Taken together, we believe these results provide strong evidence for one of our major conclusions, namely that Kap104p is playing a non-import role that either ultimately or directly affects export of Nab2-mRNA complexes.

Since the putative Nab2p-mRNA complexes apparently fail to accumulate at the nuclear periphery in *kap104-161Δtom1* strains, these results seem most consistent with a Class 1 or Class 2 function for Kap104p within the nucleus. They would only be consistent with a Class 3 function in the case that this occurred in the nucleus and Kap104p binding was in some way necessary for stability of the complex. A Class 3/Nab2p displacement role would therefore predict a stable ternary complex of Kap104p, Nab2p and polyA RNA to be present in *Δtom1* mutants. This is also a prediction of the simplest class 2/direct docking model. However, to date we have been unable to detect accumulation of Kap104p with Nab2p and polyA RNA at the nuclear periphery using either anti-Kap104p antibodies or an *in vivo*-tagged Kap104-GFP strain. Although we observed pronounced nuclear signal in addition to cytoplasmic signal with Kap104-GFP, we saw no alteration in its localization in *Δtom1* strains at 37° C (KD and CG, unpublished results). In sum, our cytological results with *kap104-161* mutants are most consistent with a Class 1 role, although they are also consistent with a Class 2 role in which Kap104p functions 'catalytically' to promote docking of Nab2p-mRNA complexes at the NPC, but is not itself a stable component of these complexes.



Work by others in yeast appears more consistent with a Class 1 role than a Class 2 role (Lee and Aitchison, 1999). Even a 'catalytic'direct targeting/Class 2 function for Kap104p would require a ternary complex to form between Kap104p, Nab2p, and export-competent RNA in the nucleus or near the nuclear pore. In the one published study where this was examined the authors failed to detect any evidence of a ternary complex when recombinant Kap104p was added to recombinant Nab2p pre-bound to single stranded DNA cellulose column. Indeed, addition of a vast excess of recombinant Kap104p, but not Kap95p, was able to quantitatively elute Nab2p from the column, implying that interactions of Nab2p with nucleic acid and Kap104p are mutually exclusive. While this result is quite compelling, it is nevertheless worth noting that it may reflect the specific *in vitro* assay conditions rather than general properties of the system. For example, binding to polyA RNA might produce different results from single-stranded DNA. Alternatively, a relevant co-factor or post-translational modification might be missing. That said, Lee and Aitchison found that the most likely co-factor, Ran-GTP, actually de-stabilized the Kap104p-Nab2p complex. Moreover, this effect was greatly enhanced in the presence of yeast RNA suggesting that ternary complexes containing Kap104p, Nab2p and RNA would not be stable in the nucleus. Therefore, in light of biochemical results from Lee and Aitchison, it seems that our cytological observations appear most likely to reflect a Class 1 function for Kap104p in promoting Nab2-mRNA export.

Intriguingly, examining the literature from vertebrate studies actually makes it more difficult to draw a final conclusion. Unlike with Kap104p and Nab2p, two necessary biochemical predictions are actually satisfied with Trn1 and hnRNPA1: 1) an

abundant complex between hnRNPA1 and transportin is readily isolated from HeLa nuclear extracts (Siomi et al., 1997), consistent with the sensitivity of the Transportin-A1 complex to Ran-GTP being much lower (Bonifaci et al., 1997) than that of Importinβ-importin α- cNLS *in vitro*, and 2) a ternary complex between transportin, A1 and RNA can be formed *in vitro* (Siomi et al., 1997). The ternary complex is sensitive to Ran-GTP, but, given the clear existence of transportin-hnRNPA1 complexes in nuclear extracts, it seems plausible that ternary complexes could also exist in the nucleus *in vivo*. Obviously, even transient docking of a transportin-A1-mRNA complex to the nuclear side of the NPC would, in principle, enable transportin-mediated mRNA export. Vectorial mRNA transport from the nucleus to the cytoplasm would not necessarily require a Ran-based system; a preferred mRNA binding partner that is localized primarily in the cytoplasm and/or a cytoplasmic release mechanism may suffice.

Why then has it been concluded that transportin does not play a role in export? One major reason is that transportin is not stably associated with hnRNP complexes. However, recent biochemical reports identifying likely mRNA export complexes suggest that these complexes differ substantially from hnRNP complexes in composition and abundance (Luo et al., 2001; Mili et al., 2001; Reed and Magni, 2001; Zhou et al., 2000). Nonetheless, transportin does not seem to be a stable component of these complexes either, and, unlike for some components of these complexes, no direct functional evidence for a role for transportin in promoting mRNA export has been attained. In sum, determining whether transportin plays a direct role in export after all, and whether, despite biochemical evidence to the contrary, a similar case holds with Kap104p and

Nab2p and/or Nab4p in yeast, will clearly require substantial additional analysis and almost assuredly novel experimental approaches.

A 'Nab-to-Pab' transition may play a role in yeast mRNA export

Independent of the precise timing of Kap104p's hypothesized non-import function in Nab2-mRNA export, our results have an important additional component to them, since they also provide evidence implicating Pab1p in Nab2p-mRNA export. According to this view, one additional role for Kap104p in promoting efficient Nab2p-mediated mRNA export may be to help promote efficient mRNP remodeling, specifically Nab2p release and subsequent Pab1p binding. Surprisingly, little is known about the timing of Pab1p interaction with mRNA relative to other proteins and other mRNA processing and export. Recent work supports a model where a 'pioneer round' of translation occurs with the nuclear cap-binding complex and the nuclear polyA binding protein PABP2 associated, and that this round of translation serves a quality control function. Subsequently, mRNAs associate with the cytoplasmic cap-binding complex and polyA binding protein, PABP (Ishigaki et al., 2001). To some extent this system appears conserved, since the yeast CBC appears able to physically associate with eIF4G and promote translation initiation (Fortes et al., 2000). However, despite steady-state localizations of the components to different compartments, the actual timing of these events with respect to nuclear export remains unclear.

Recent results from the Swanson lab (Nykamp and Swanson, manuscript in preparation) support the idea that in yeast mRNP remodeling from a Nab2p-RNP to a Pab1p-RNP may not only be part of the normal progression of mRNAs, but also that Nab2p may play an important role 'upstream' of Pab1p, interacting with the polyA tail in

the nucleus, and coupling mRNA export to proper nuclear processing of the polyA tail. In this view, Nab2p, despite the lack of any apparent sequence similarity, is a functional analog of PABP2 (Nemeth et al., 1995; Wahle, 1991a; Wahle, 1991b). Intriguingly, studies of influenza virus infection implicate PABP2 as a major target for shutdown of cellular mRNA export in infected cells and there is reason to believe that loss of host mRNA export in PABP2-inhibited cells may reflect a direct role for PABP2 in this process, rather than a downstream effect from tail length defects (Neumann et al., 2000). As mentioned above, PABP2 is the protein associated with polyA tails during the 'pioneer round' of translation and PABP1 subsequently binds the polyA tail (Ishigaki et al., 2001). Based on the Swanson lab's results, Nab2p seems a particularly attractive candidate for a mediator of a hypothetical pioneer round in yeast.

The parallels between the mammalian yeast pathways are also bolstered by the finding by Swanson and colleagues that *2μPAB1* can bypass the requirement for Nab2p for mRNA export, but fails to affect polyA tail length, consistent with a key role for Pab1p in promoting mRNA export downstream of Nab2p. Pab1p is of course a major protein affecting polyA RNA stability and playing a key role in formation of a stable translation initiation complex. However, as is true for its mammalian homolog, it is found almost exclusively in the cytoplasm in wild type yeast. Since Nab2p is found almost exclusively in the nucleus, this alone suggests that Nab2p is likely to be upstream of Pab1p in the normal progression of mRNA metabolism. However, this model would be strengthened by evidence that a "Nab-to-Pab" transition is important for efficient export to occur, since bypass suppression of *Δnab2* by *2μPAB1* could simply represent an export pathway unrelated to normal mRNA export in wild type cells.

Bypass Suppression Interference provides evidence that the putative 'Nab-to-Pab' transition is critical for Nab2p-mediated mRNA export

By providing evidence that competition between Pab1p and Nab2p can occur *in vivo* our observation of bypass suppression interference (BSI) with inviable alleles of *NAB2* that lack the RGG box/Kap104p binding domain provides genetic evidence for an obligate Nab2p to Pab1p transition as an important step in the mRNA export process *in vivo*. The first example of this type of genetic interaction that we are aware of, BSI is conceptually similar to a dominant-negative effect, but differs in several key respects. First, by enabling assay of the function of non-complementing alleles in the absence of the wild type protein, BSI phenotypes should genuinely reflect an effect of loss of function. In our case, we believe the BSI effect is due to loss of function associated with the NAB35 region that contains the RGG box and is required for Kap104p binding (Lee and Aitchison, 1999; Siomi et al., 1998; Truant et al., 1998). Second, in the event that the alleles displaying BSI are completely recessive, this helps alleviate the concern over distinguishing between true dominant-negative effects and off-pathway 'neomorphic' effects. In our case this argument is bolstered by the fact that the BSI phenotypes are manifest with low-copy number vectors with the alleles under control of the endogenous *NAB2* promoter. For these reasons, we believe BSI is providing information both about requirements for Nab2p-mediated mRNA export as well as the role that Pab1p may play in this process.

It seems very likely that in our case BSI implies a competition between Pab1p and mutant Nab2p for at least one common binding partner. One obvious candidate for this binding partner is polyA RNA. Indeed, the *nab2* alleles that display BSI retain the

CCCH zinc finger RNA binding domain, which is necessary and sufficient for interaction with polyA RNA *in vitro* (Anderson et al., 1993). Thus, it is plausible that the *nab2* mutant alleles displaying BSI can compete with Pab1p for interaction with mRNA, but fail to properly perform a major downstream function that requires an additional interaction mediated by the Kap104p-binding domain/RGG box. Since addition of a cNLS to *nab2-Δ35* seems to enhance the effect of *nab2-Δ35* in this assay, increased nuclear access may enhance the ability of Nab2-Δ35p to compete with Pab1p, and raises the possibility that the BSI effect may relate to a nuclear function. Taken together with our results and those of others, we suggest that BSI with these *nab2* alleles is the result of failure of Kap104p to perform an important role in the Nab2p-mRNA export process because it cannot interact with nab2-Δ35p and nab2-KKM3p. Thus, BSI by alleles defective in interaction with Kap104p provides additional genetic evidence for a critical non-import role of Kap104p in mRNA export. In principle this could be a class 1, 2 or 3 function of Kap104p.

Since the mechanism of *2μPAB1* bypass suppression appears to be at the level of Pab1p interaction with mRNA in the nucleus to promote export in the absence of Nab2p (Nykamp and Swanson, manuscript in preparation), it is reasonable to hypothesize that efficient mRNA export from the nucleus may be a key problem for strains displaying BSI. However, it should be noted that we have not yet demonstrated this directly. Preliminary attempts to investigate this question have been equivocal: the transformants displaying BSI that do ultimately recover from transformation subsequently show only a minor decrease in growth rate relative to strains with vector alone in liquid culture or plate-based growth assays and, consistent with this, do not show any increase in number

of cells showing nuclear accumulation of polyA RNA as compared to *Δnab2 2μPAB1* strains (~20% of cells show some effect, KD and CG, unpublished results). Given that the colonies that do eventually grow up are essentially selected pseudorevertants, it is perhaps not surprising that these strains do not show a continued effect. Clearly, examination of the BSI alleles with repressible/inducible expression systems will be necessary to determine directly whether the phenomenon correlates with compromised mRNA export. For that matter, it will be important to determine whether BSI can be observed under standard cellular growth conditions, or is instead confined to particularly demanding conditions, such as recovery from transformation. We submit that BSI with *PAB1* and *NAB2* will be an intriguing topic for future mechanistic studies in either case.

Working models for the roles of Nab2p, Kap104p, Pab1p and Tom1p in mRNA export

In sum, our results provide evidence for at least one, and possibly two, novel, non-import roles for Kap104p in Nab2p-mRNA export. They also raise the possibility that Kap104p's non-import function may be related to and perhaps even mechanistically-coupled to a key mRNA remodeling step involving substitution of Pab1p for Nab2p. Tom1p is apparently also required for this transition to occur efficiently. On the basis of our results and those of others, we integrate our results with Pab1p and Nab2p, Kap104p and Tom1p and propose working models to summarize our results and to illustrate key unresolved issues to help guide future studies (Figure 9A/B). The models are distinguished primarily by whether the Nab-to-Pab transition is hypothesized to occur in the nucleus or the cytoplasm.

In the first model (Figure 9A), Kap104p performs a nuclear function important for efficient formation and/or targeting of Nab2-mRNP's to the NPC and- in a distinct, cytoplasmic role- promotes mRNP remodeling by binding to Nab2p's RGG box RNA binding domain. In this view Kap104p is effectively a 'displacer' and a 'chaperone,' in that it disrupts interactions before they go too far and prevents unwanted interactions from occurring. Alternatively, the role of Kap104p could be more directly coupled to the Nab2p-Pab1p transition (Figure 9B). In this view, Kap104p plays a nuclear or NPC-associated role as a 'matchmaker,' enabling Nab2p to get to the right place at the right time and helping to promote this key RNP transition in the nucleus prior to export. Presumably Kap104p accomplishes this by competing directly with mRNA for Nab2p binding and thereby also frees polyA tails to interact with Pab1p at or near the NPC. The model in Figure 9B hypothesizes that efficient transition from a Nab2p-RNP to a Pab1p-RNP may be a pre-export transition, occurring at the nuclear periphery. This model therefore also implies that shuttling of Nab2p is not critical to its function in mRNA export. In possible support of such a notion, significant cytoplasmic Nab2-GFP signal appears relatively slowly (~4.5 hr) in *kap104-16* mutants when protein synthesis is inhibited (Lee and Aitchison, 1999). This result may therefore suggest that Nab2p export is not directly related to Nab2p function in mRNA export. Alternatively, it may suggest a key role for Kap104p in Nab2p export. The enigmatic role of Tom1p in these processes is represented in both models by the ubiquitinated 'Factor X'. Our results suggest that Factor X ubiquitination may influence the efficiency of the Nab-to-Pab transition. Whether- as we have suggested in the figure- this effect is due to a direct effect on Factor

X's interaction with any other mRNA export pathway components, awaits identification and characterization of Factor X.

Determining which of the proposed working models is correct awaits more detailed studies including biochemical analyses. An additional challenge will be to integrate this relatively simple model for an mRNA export pathway with mRNA export mediated by other factors, such as Mex67p, Yra1p, Sub2p, and Npl3p (Zenklusen and Stutz, 2001). At this point, a role for Mex67p in Nab2-mediated mRNA export would seem likely given the central role that Mex67p and its homolog TAP play in mRNA export (Conti and Izaurralde, 2001; Katahira et al., 1999). Nevertheless, whether Mex67p indeed plays a role in Nab2-mRNA export is completely unclear at this time. Ultimately, it will also be essential to understand how Nab2p, Pab1p, Tom1p and Kap104p fit into the emerging view of mRNA export as a highly complex process that apparently involves significant coupling to other mRNA processing events (Jensen et al., 2001a; Luo et al., 2001; Strasser and Hurt, 2001)feedback inhibition mechanisms (Hilleren et al., 2001; Hilleren and Parker, 2001; Jensen et al., 2001b) and multiple layers of 'quality control' (Maquat and Carmichael, 2001) that may even include nuclear translation (Brogna, 2001; Iborra et al., 2001).

Materials and Methods

Yeast strains and manipulations

Yeast strains used in this study are all available in the Guthrie lab yeast strain database.. Unless otherwise noted, standard yeast methods were used for all manipulations. Transformations, including those used for BSI assays, were performed according to standard lithium acetate methods, except that the heat shock step was omitted.

Construction of *kap104-161* strains

To generate an isogenic temperature sensitive *kap104* mutant to cross to the previously-characterized *Δtom1* strain yKD100 (Duncan et al. 2000, Ch. 1) we recovered the plasmid containing *kap104-16* (Aitchison, et al. 1996), from *Δkap104 pRS314-kap104-16* yeast. The Sac I fragment containing kap104-16 was then sub-cloned into the Sac I site of the integrating vector, pRS306 to generate pKD69. The allele was then integrated into the wild type yeast strain YPH399 using the two-step gene-replacement method (Rothstein, 1991), with the digested with Eco 47III. Temperature sensitive *kap104-16* integrants were isolated, and an isolate that could be completely rescued by plasmid-borne wild type *KAP104* was crossed to the isogenic *Δtom1*strain. This strain was sporulated to obtain independent isolates of integrated *kap104-16* and *Δtom1 kap104-16* double mutants. The original *kap104-16* allele was never sequenced (Aitchison, per. comm.). Thus, the original *kap104-16* allele might contain multiple mutations, not all of which will necessarily be retained during two-step gene replacement. For this reason, we have chosen to refer to our temperature sensitive allele as "*kap104-161*" to reflect the fact that it is derived from *kap104-16*, but may not be identical to it.

Construction of *Δnup100* and *nup145-ΔN* strains in YPH399/*Δtom1* and *Δrip1Δtom1* strains

Δnup100::URA3 and *nup145-ΔN::URA3* (from the Guthrie lab transport strain collection) were crossed to yKD100 (*Δtom1::HIS3*) by strain mixing on YEPD media and incubation at room temperature for > 5 hrs., followed by selection of diploids on SD –His –Ura medium. Diploids were sporulated and >20 tetrads were dissected. Genotypes were determined by patching and replica plating according to standard methods. Tetratype tetrads were recovered, and transformed with *pRS314-NAB2-GFP* for use in localization assays. Essentially the same procedure was used to generate *Δrip1Δtom1* strains in the W303 background, except that the cross was between *Δrip1::trp1* (also from the Guthrie lab transport strain collection) and YKD98 (*Δtom1::HIS3* in W303), so the selection media for diploids was SD-Trp –His, and PKD68 (*pRS315-NAB2-GFP*) was used instead.

Construction of the *Δtom1 Δnab2* pRS316NAB2 strain

The Δ*tom1 Δnab2* pRS316NAB2 strain was generated essentially as described for yKD100 (Duncan et al., 2000). Briefly, pKD13 was digested with BamHI and transformed into yJA513 (from Maury Swanson). His+ isolates were recovered and an isolate that displayed temperature sensitive growth that could be rescued by plasmid-borne *TOM1* was used for all subsequent manipulations and analyses.

Construction of *TOM1 Δnab2 2μPAB1* and *Δtom1 Δnab2 2μPAB1* strains

TOM1 Δnab2 2μPAB1 and *Δtom1 Δnab2 2μPAB1* strains were constructed from the corresponding shuffle strains by transformation with *pRS314-NAB2-GFP*, followed by counterselection of pRS316NAB2 on 5-FOA medium. *Ura*$^-$ isolates of each strain were transformed with *YEP24-PAB1* (*2μURA3*, gift from Maury Swanson). Strains lacking *NAB2* were subsequently obtained by selecting for loss of *pRS314-NAB2-GFP* on 5-FAA medium. Multiple isolates obtained after verifying *trp*$^-$ phenotypes were then used for subsequent assays as indicated in the text.

Plasmid construction

Plasmids used in this study are listed in Table 4. Standard methods for plasmid construction were used throughout, except that all gel extraction and minipreps were performed with Qiagen kits. All restriction enzymes were from New England Biolabs. PCRs were performed using the Expand high-fidelity system (Roche). All kits and enzymes were used according to the manufacturer's suggestions. *pRS314-NAB2-GFP* (Duncan et al., 2000) was used as the vector for construction of all *NAB2* alleles used in this study. PCR-based sub-cloning strategies were used to generate *nab2-Δ35*, *cNLS-NAB2*, and *cNLS-nab2-Δ35* alleles. Oligos used for PCR are listed in Table 5. Regions that were amplified by PCR were sequenced to verify the presence of the desired alteration and the absence of undesired mutations. Sequencing reactions were performed by the UCSF Biomolecular Resource Center using ABI Prism Sequencing Instruments (Applied Biosystems). *Nab2-KKM3* was originally generated by Karen Kim using the Quikchange mutagenesis kit (Stratagene) according to the manufacturer's instructions,

with *pRS316-NAB2-GFP* as template, and OKK13 and OKK14 as the mutagenic oligos. Sequencing of three independent positives revealed that they all contained the sequence now known as 'KKM3.' KKM3 was subcloned to *pRS314-NAB2-GFP* using Pst I and EcoRI to generate pKD99.

Fluorescence microscopy

Localization of Nab2p using mAb3F2, polyA RNA by (dT)50 FISH, and Nab2p-GFP by live fluorescence microscopy were all performed essentially as described ((Duncan et al., 2000), Chapter. 1 this volume). Exponentially growing strains in liquid cultures were used for all microscopy experiments. Relevant information on growth conditions and other manipulations are summarized in the text and/or figure legends.

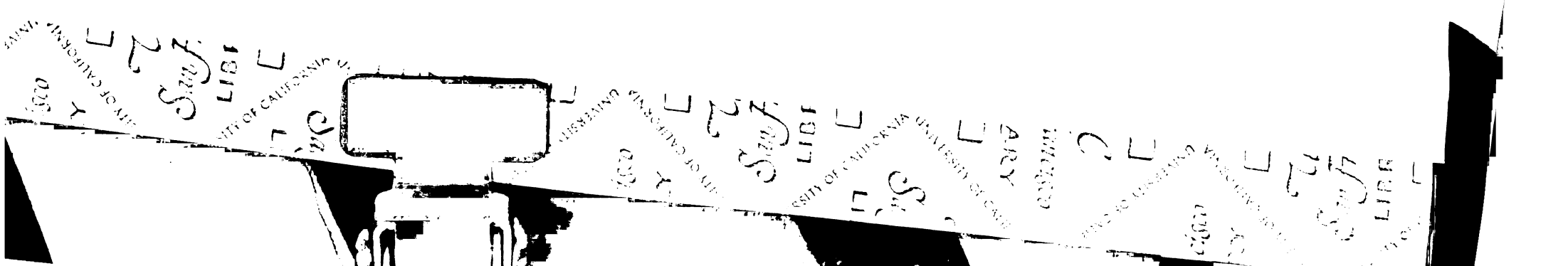
Figure 1: Epistasis to identify proteins mediating targeting of Nab2p-mRNA to the nuclear periphery.

Shematic illustration of the rationale for using genetic epistasis to identify candidate factors that may mediate targeting of Nab2p-mRNA to the nuclear periphery.

(A) In the wild type case, an upstream targeting reaction and a downstream translocation reaction both occur at wild type rates and this results in efficient export of Nab2p-mRNA complexes.

(B) Mutation of *tom1* results in a reduced rate of the translocation reaction and Nab2p-mRNA complexes accumulate at the nuclear periphery at 37° C.

(C) Mutation of an upstream targeting component, *yfg* (your favorite gene) results in loss of targeting, and Nab2p-mRNA complexes no longer accumulate at the nuclear periphery.



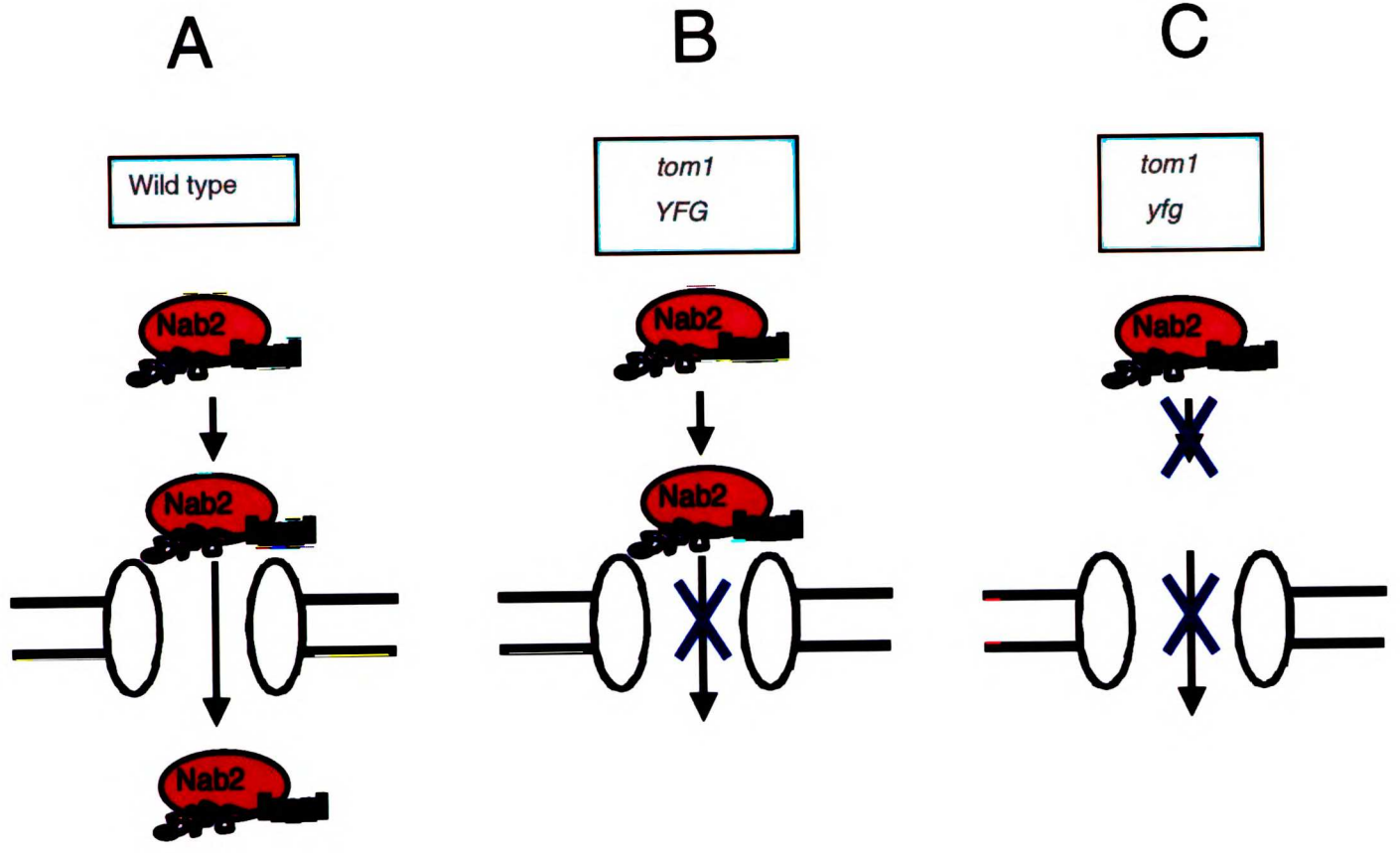

A
B
C
Wild type
tom1
YFG
tom1
yfg
Nab2
Nab2
Nab2
Nab2
Nab2
Nab2

Figure 2: Wild type Kap104p is required for mislocalization of Nab2p and mRNA in *Δtom1*.

(A) Nab2p localization. Exponentially-growing YEPD cultures of the indicated genotype were shifted from 22° C to 37° C for 90 minutes. Cells were fixed with 5% formaldehyde at 37° C for 1 hour and processed for immunofluorescence using mAB 3F2 against Nab2p (Andersen et al, 1993). Cells were examined by epifluorescence microscopy to determine localization of Nab2p. All images were collected with equivalent exposure times using a CCD camera and processed equivalently using IPLab imaging software. **(B)** PolyA RNA localization. Cells were grown and fixed as in (A), but processed for FISH with an oligo dT50 probe to detect the polyA tail. Images were collected and processed as in (A).

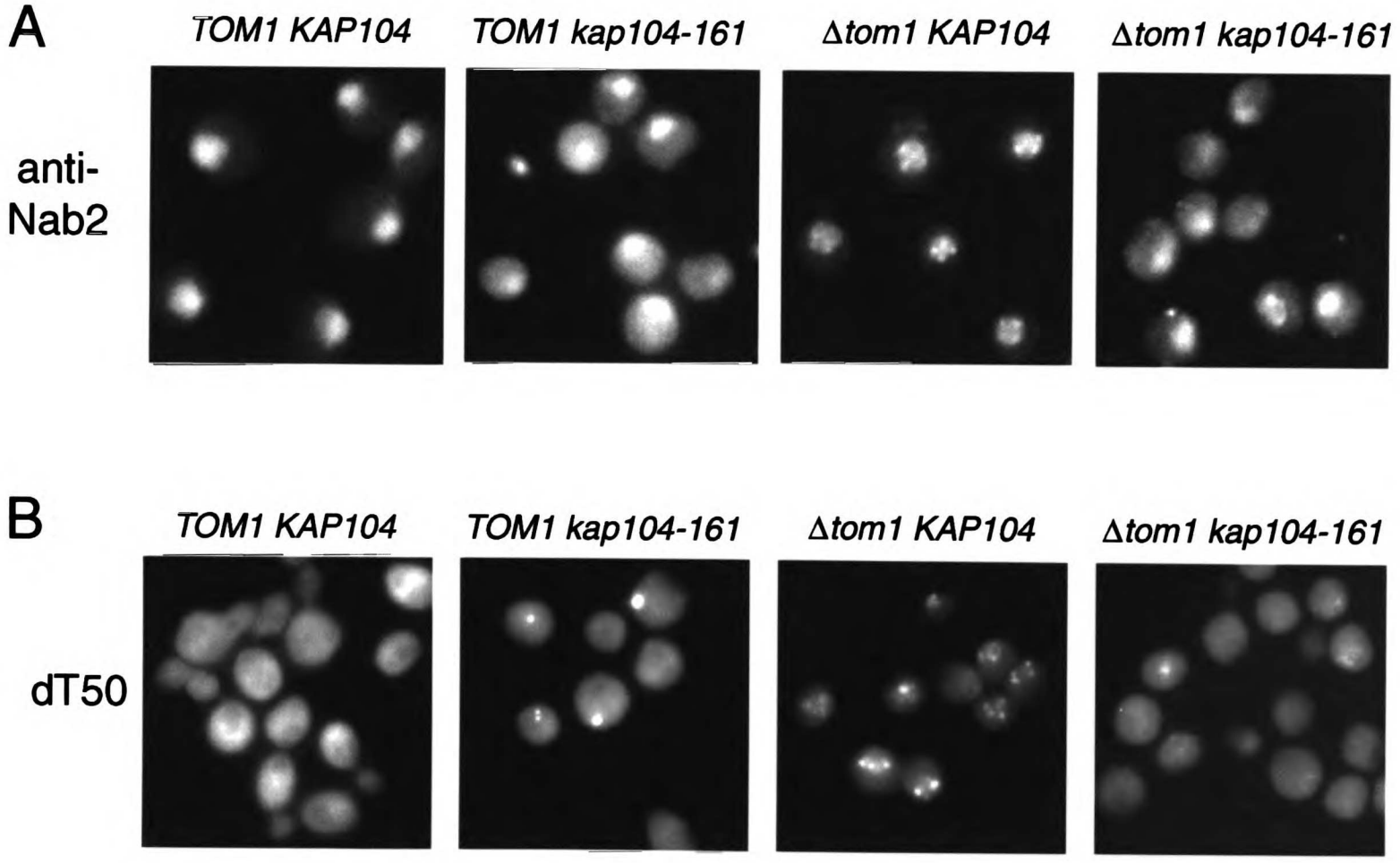

A
TOM1 KAP104
TOM1 kap104-161
Δtom1 KAP104
Δtom1 kap104-161
anti-
Nab2
B
TOM1 KAP104
TOM1 kap104-161
Δtom1 KAP104
Δtom1 kap104-161
dT50

Figure 3: *Δtom1* effect on Nab2p.

(A) Nab2p-GFP localization in *Δnup100* strains. Exponentially-growing YEPD cultures of the indicated genotype containing plasmid-borne *NAB2-GFP* were shifted from 22° C to 37° C for 120 minutes. Cells were examined by live epifluorescence microscopy to determine localization of Nab2-GFP. Images were collected as in Figs. 4 and 5.

(B) Nab2p-GFP localization in *nup145-ΔN* strains. As in (A), but with strains with the indicated alleles of *NUP145*.

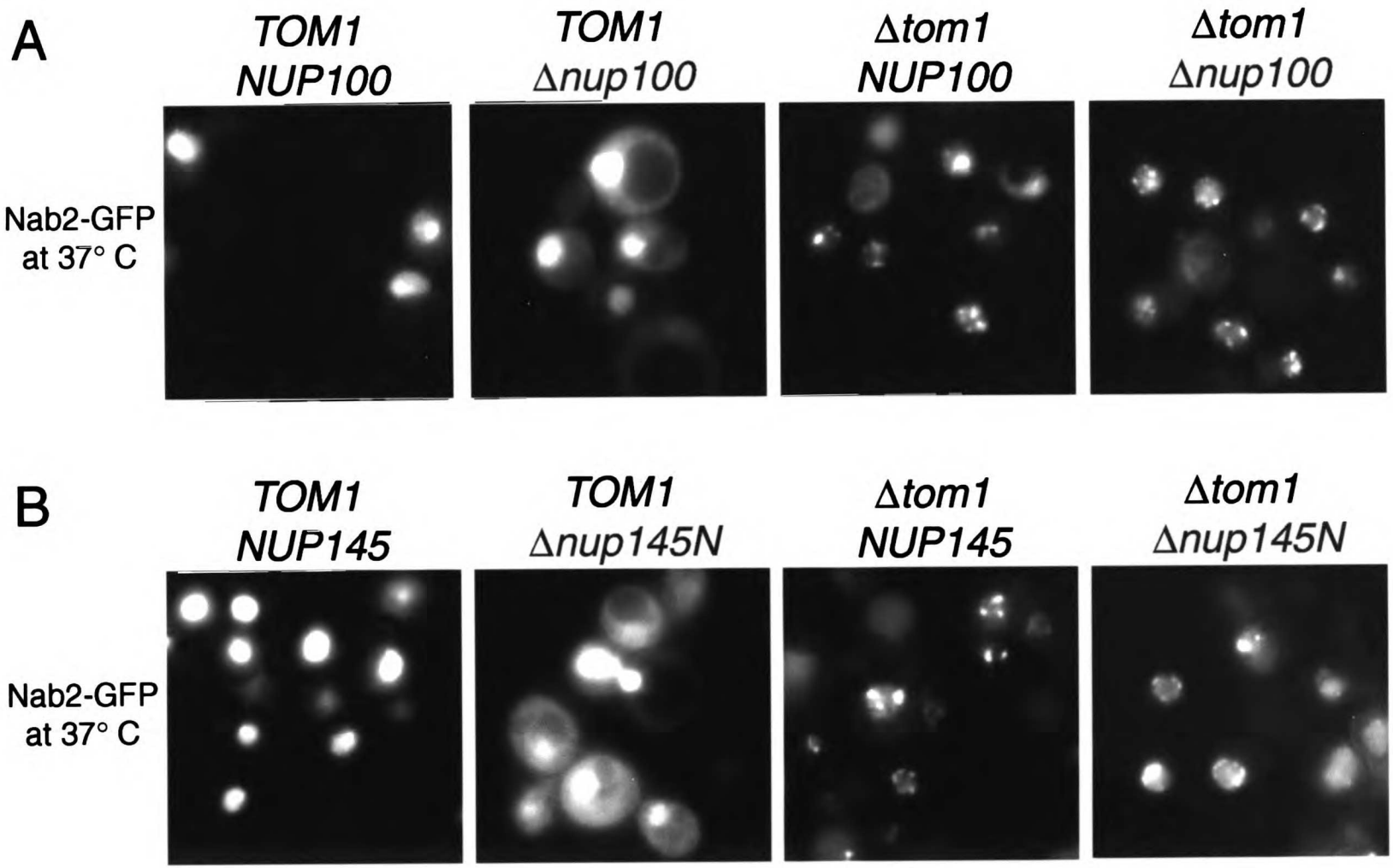
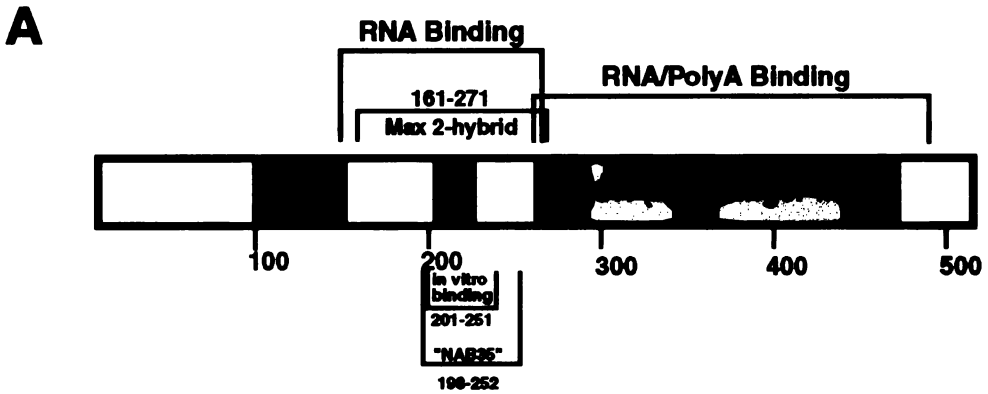

A
TOM1
NUP100
TOM1
Δnup100
Δtom1
NUP100
Δtom1
Δnup100
Nab2-GFP
at 37° C
B
TOM1
NUP145
TOM1
Δnup145N
Δtom1
NUP145
Δtom1
Δnup145N
Nab2-GFP
at 37° C

Figure 4: Generation of *NAB2* alleles affecting the Kap104p binding domain

(A) Schematic representation of the primary structure of Nab2p showing the known sequence features. "Q" is composed of an 11 residue polyglutamine tract, followed by either two or nine repeats of the tetrapeptide sequence GlnlGlnGlnPro (QQQP),depending on the yeast strain. "Max 2-hybrid" is the fragment determined to give maximal 2-hybrid interaction with Kap104p (Truant, et al.,1998). "NAB35" is the minimal fragment for 2-hybrid interaction with Kap104p mapped by Siomi and colleagues (Siomi, et al., 1998). "*In vitro* binding" corresponds to the region necessary and sufficient for detectable interaction with Kap104p in an *in vitro* binding assay using recombinant proteins (Lee and Aitchison, 1999). Regions sufficient for *in vitro* binding to many RNA homopolymers, and necessary for polyA binding are also indicated (Andersen et al., 1993) The figure is adapted from Andersen et al. and is only approximately to scale.

(B) Sequence of the NAB35 region of *NAB2* deleted in the *nab2-Δ35* allele. The sequence alterations in *nab2-KKM3* are shown aligned in the sequence below. Residues that appear conserved based on alignment of NAB35 with the M9 sequence of hnRNPA1 are boxed in magenta. Mutated amino acid residues are boxed in yellow.

(C) Schematic diagram of the plasmid shuffle assay used to test complementation of *Δnab2* by different *NAB2* alleles in *TOM1* and *Δtom1* strains.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RNA/PolyA Binding
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200
300
400
500
in vitro binding
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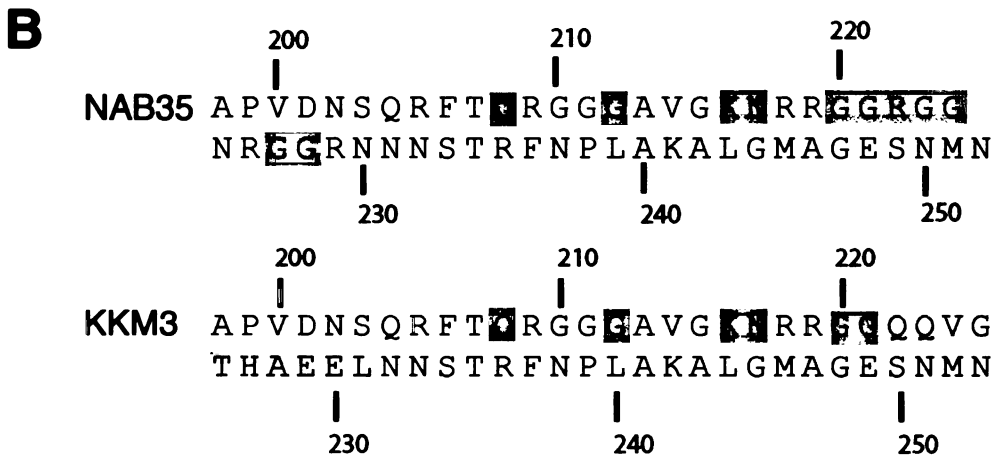
199-252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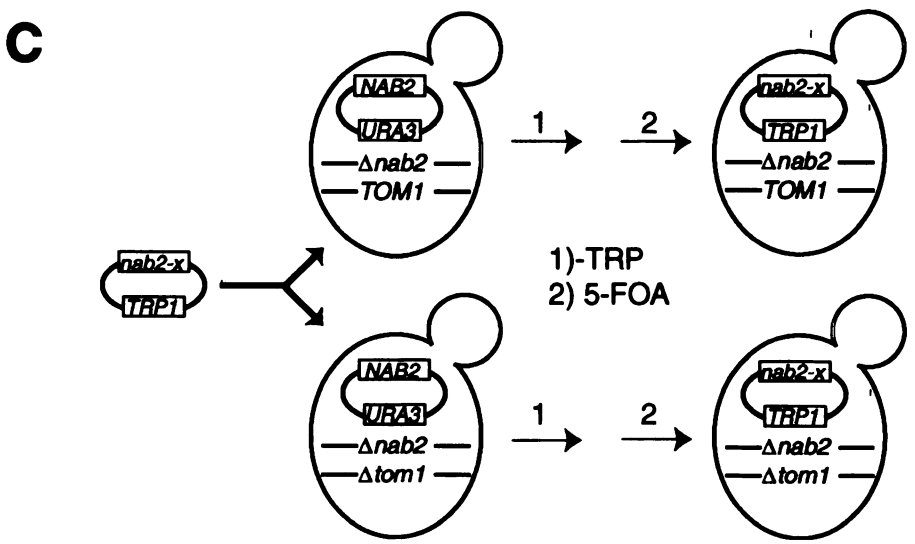
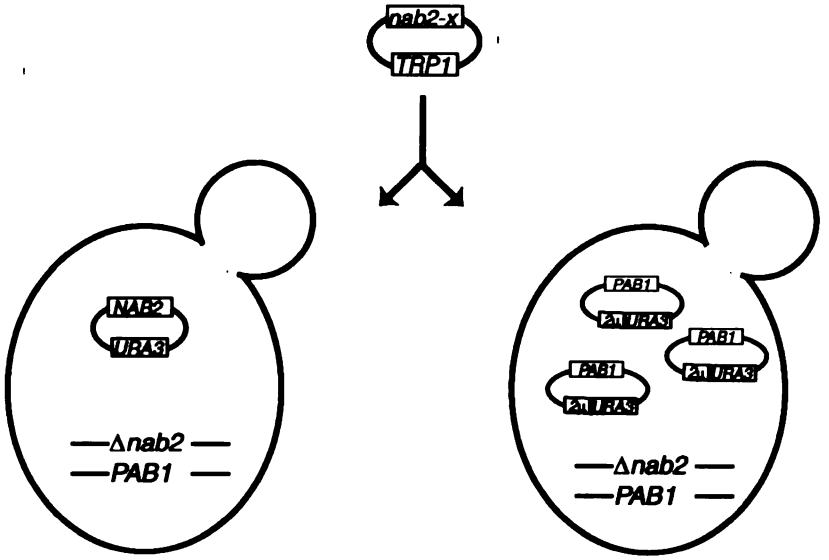

C
NAB2
URA3
Δnab2
TOM1
1
2
nab2-x
TRP1
Δnab2
TOM1
nab2-x
TRP1
1)-TRP
2) 5-FOA
NAB2
URA3
Δnab2
Δtom1
1
2
nab2-x
TRP1
Δnab2
Δtom1

Figure 5: *NAB2* alleles lacking or severely disrupting the Kap104p binding domain display bypass suppression interference in *Δnab2 2μPAB1* strains.

(A) Schematic of the transformation assay used to test for bypass suppression interference by various *nab2* mutant alleles. The strains and plasmids used are indicated. The selection of three copies of the *2μPAB1* plasmid, as shown in the figure, is arbitrary and is not intended to represent actual copy number.

(B) Strains of the indicated genotype (to left of panels) were transformed with the indicated allele of *pRS314-NAB2-GFP* (top of panels). Plates were incubated at 30° C for 70 hours (top 3 rows) or 98 hours (bottom row) prior to image collection. *Δnab2 2μPAB1*(a) and (b) are two independent isolates of this strain.

A


nab2-x
TRP1
NAB2
URA3
Δnab2
PAB1
PAB1
2μ URA3
PAB1
2μ URA3
PAB1
2μ URA3
Δnab2
PAB1

B

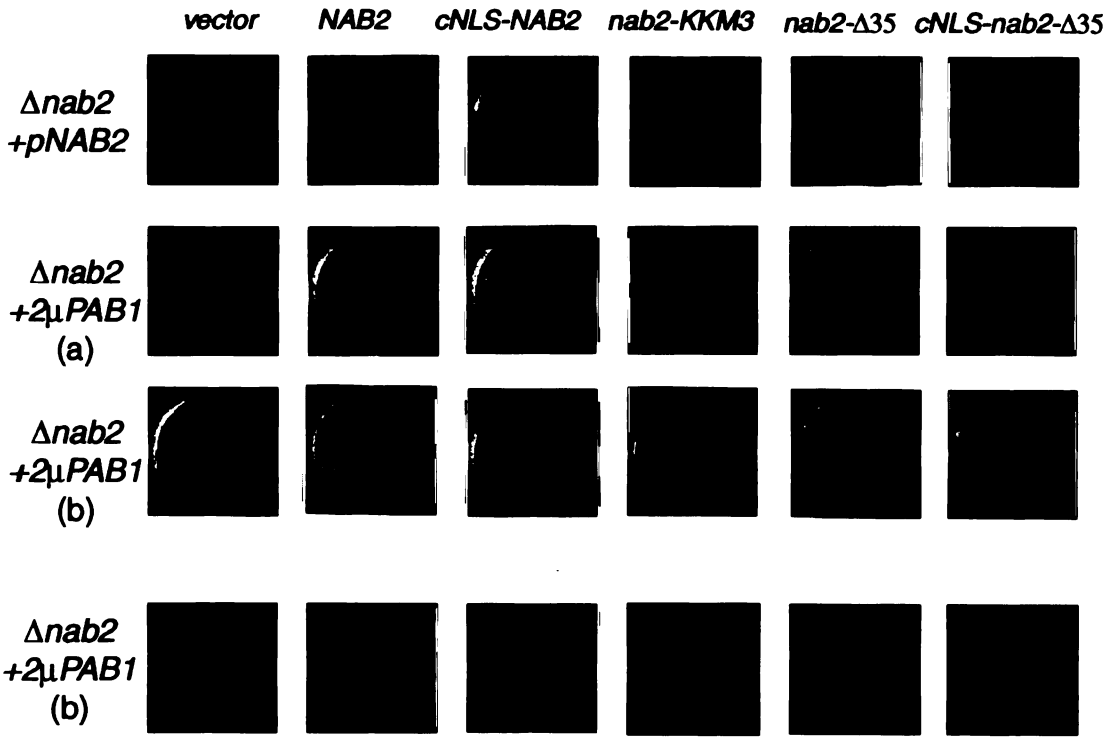

vector
NAB2
cNLS-NAB2
nab2-KKM3
nab2-Δ35
cNLS-nab2-Δ35
Δnab2 +pNAB2
Δnab2 +2μPAB1 (a)
Δnab2 +2μPAB1 (b)
Δnab2 +2μPAB1 (b)

Figure 6: Nuclear targeting of *nab2-Δ35* by addition of a cNLS enhances bypass suppression interference.

Strains of the indicated genotype (to left of panels) were transformed with the indicated allele of *pRS314-NAB2-GFP* (top of panels). Plates were incubated at 30° C for ~106 hours prior to image collection. *Δnab2 2μPAB1*(a) and (b) are two independent isolates of this strain.

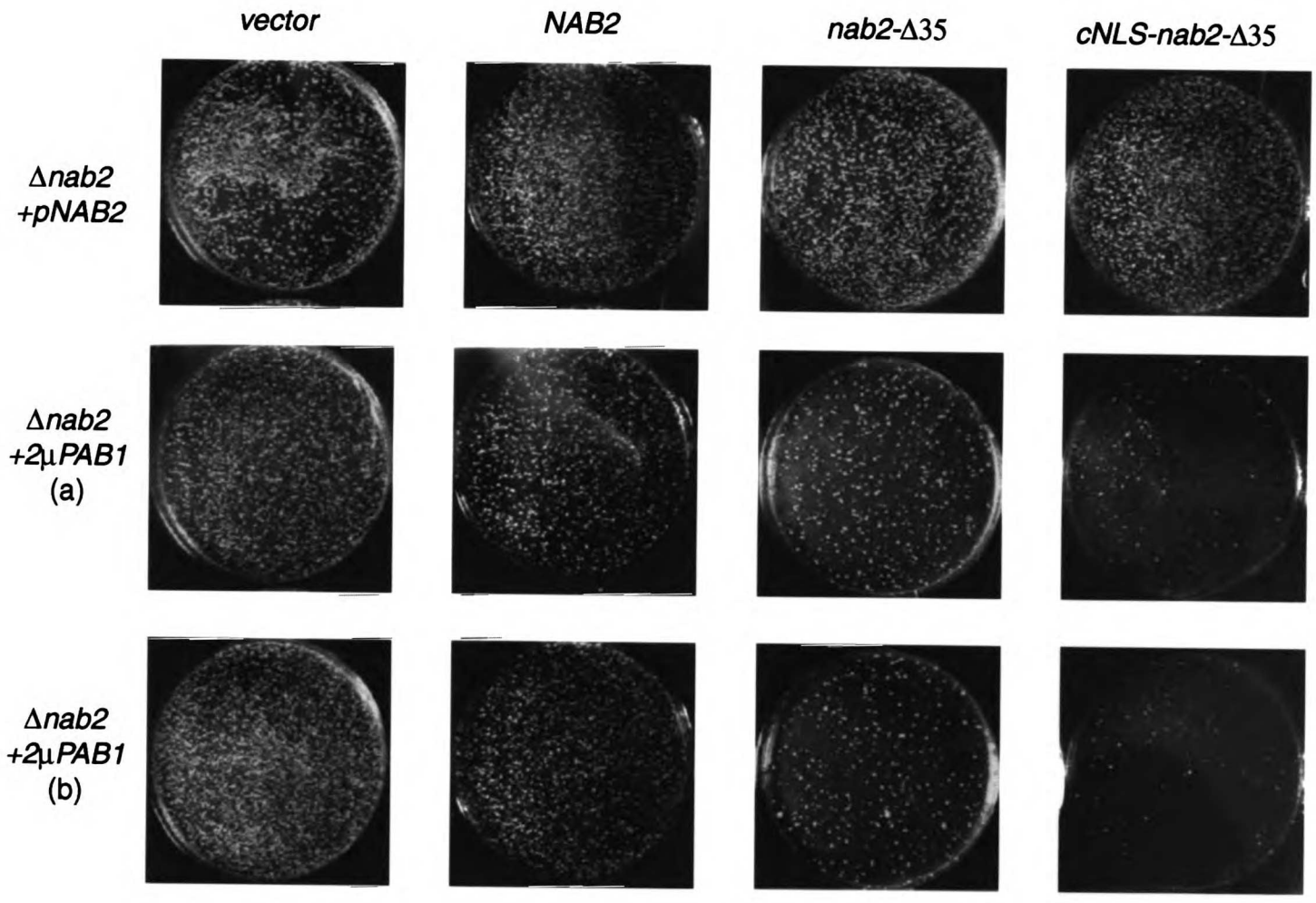

vector
NAB2
nab2-Δ35
cNLS-nab2-Δ35
Δnab2 +pNAB2
Δnab2 +2μPAB1 (a)
Δnab2 +2μPAB1 (b)

Figure 7: *Δtom1* reduces the efficiency of *2μPAB1* bypass suppression of *Δnab2*.

Strains of the indicated genotype (to left of panels) were transformed with the indicated allele of pRS314-NAB2-GFP (top of panels). Plates were incubated at 30° C for 70 hours (top row) or 98 hours (bottom two rows) prior to image collection. The bottom panels showing *Δnab2 2μPAB1* (b) strains are the same as in Figure 5.

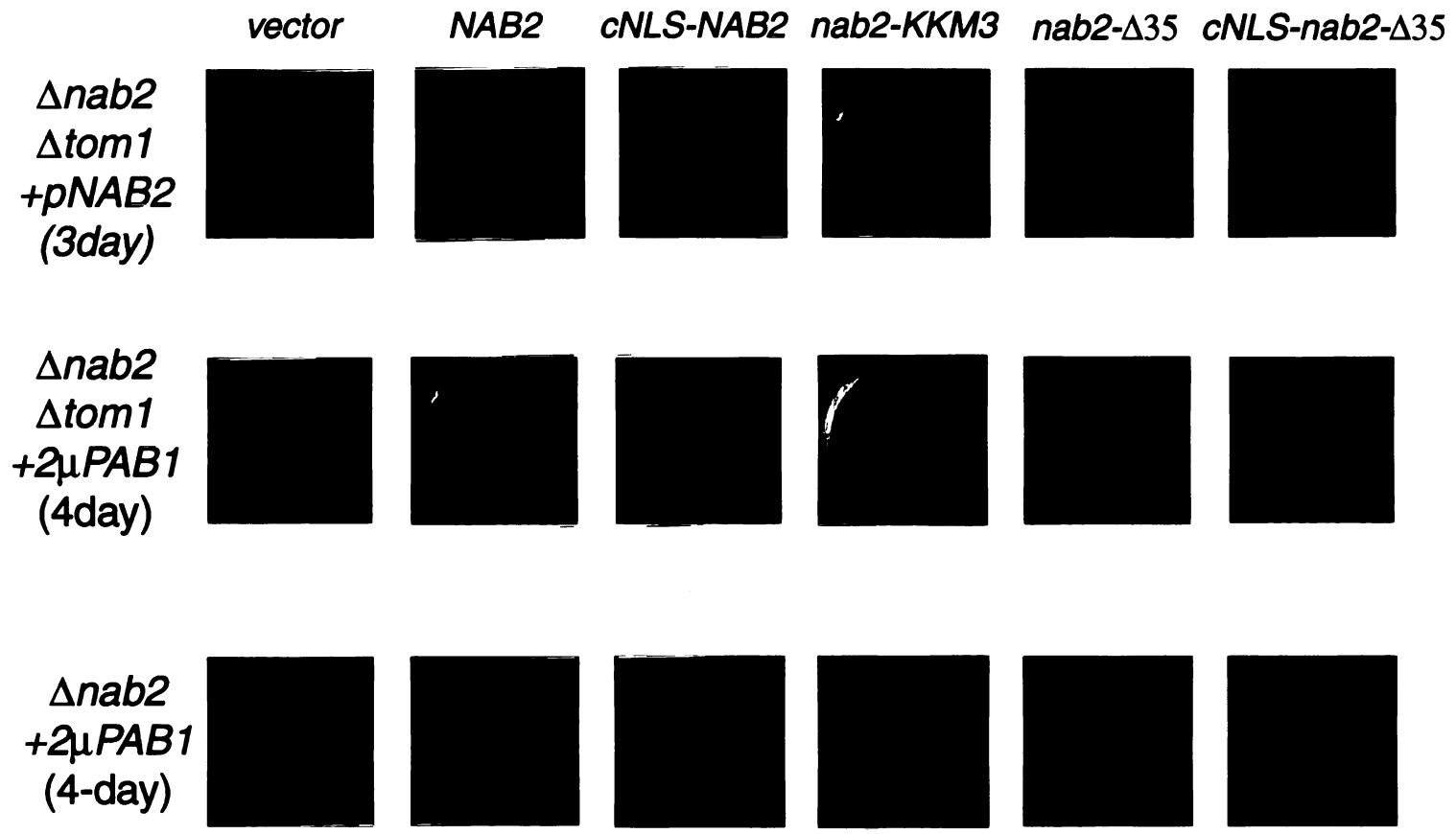

vector
NAB2
cNLS-NAB2
nab2-KKM3
nab2-Δ35
cNLS-nab2-Δ35
Δnab2
Δtom1
+pNAB2
(3day)
Δnab2
Δtom1
+2μPAB1
(4day)
Δnab2
+2μPAB1
(4-day)

Figure 8: Possible classes of non-import functions for Kap104p.

The different classes of hypothetical non-import Kap104p functions are illustrated, as described in the text.

(A) Class 1 functions are proposed intranuclear functions required for efficient formation of an export-competent Nab2p-polyA RNP complex.

(B) Class 2 functions involve nuclear Kap104p-mediated docking of Nab2p-mRNA complexes to the NPC to promote export.

(C) Class 3 functions are distinguished by competitive binding of Nab2p by Kap104p to promote mRNP remodeling required for efficient mRNA export. Such functions are presumed to occur in the cytoplasm after export, and are thus illustrated in this way. See discussion text for details.

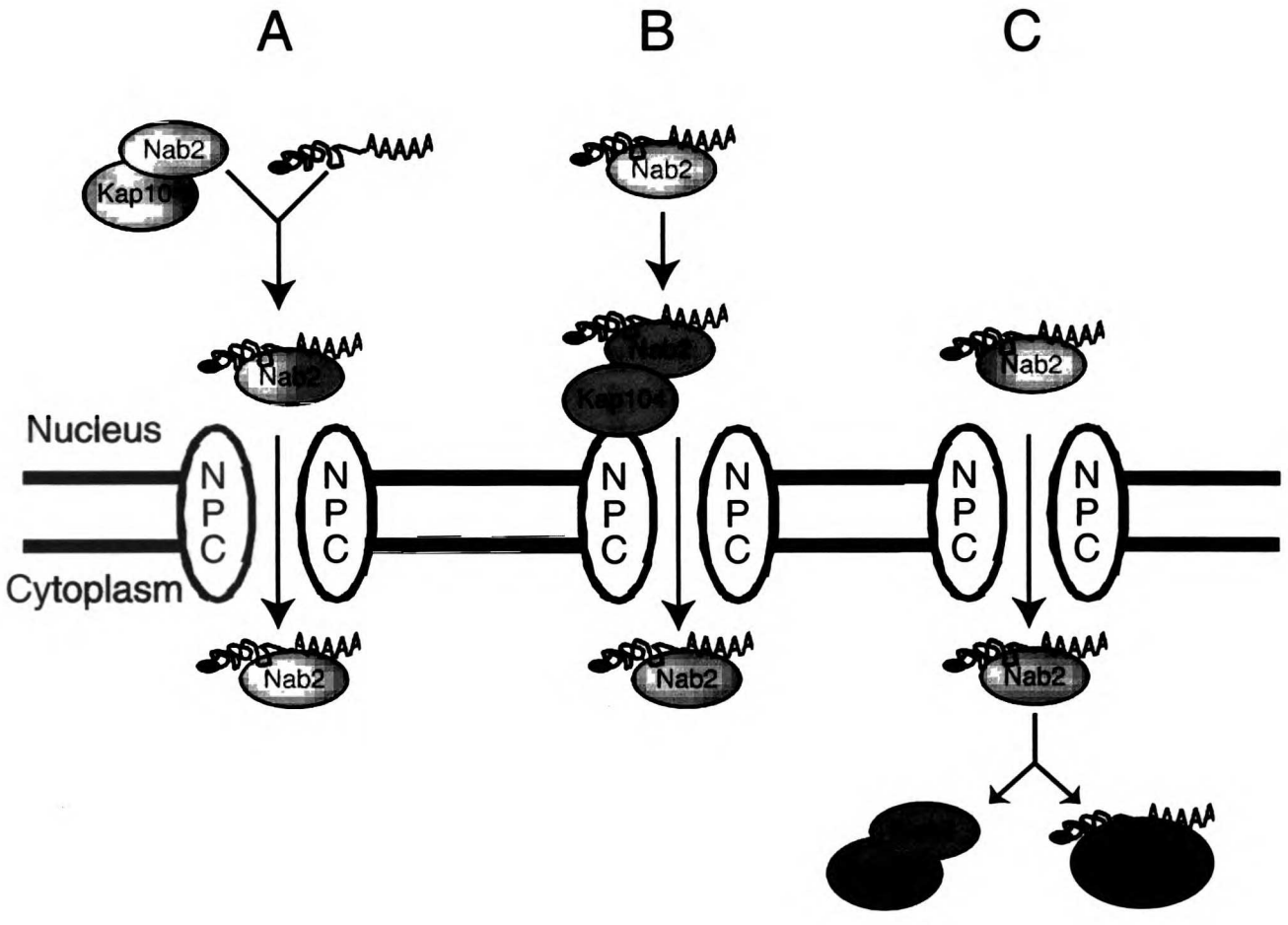

A
B
C
Nab2
Kap104
Nucleus
Cytoplasm
NPC

Figure 9: An integrated model for Nab2p-dependent mRNA export.

Working models for the Nab2p-dependent mRNA export pathway that incorporate Pab1p, Nab2p, Kap104p and Tom1p functions are depicted. In both models Tom1p function is represented by the hypothesized 'Factor X,' an unidentified target of Tom1p's putative ubiquitin ligase activity. For simplicity ubiquitinated Factor X is depicted to interact directly with Nab2p-mRNA complexes, although the effect could be indirect. The unidentified component that mediates interaction of Nab2p-mRNA complexes with the NPC is shown as a '?'.

(A) A model where Kap104p has two distinct non-import functions. One function is a Class 1 function in the nucleus. The other is a cytoplasmic Class 3 function. Pab1p's role in mRNP remodeling is depicted as primarily cytoplasmic.

(B) A model where Class 3 Kap104p functions are integrated with Pab1p-mediated mRNP remodeling and occur in the nucleus prior to mRNA export. In this view, Nab2p exchange for Pab1p requires competitive binding Kap104p to Nab2p at the NPC or nuclear periphery. The efficiency of this step is influenced by the strength of interaction between Nab2p and Kap104p, the concentration of Pab1p, and Tom1p function. Kap104p's cytoplasmic role is primarily to block non-productive interactions of newly-synthesized Nab2p with cytoplasmic RNA and to deliver the nascent Nab2p to the nucleus. It would do the same with any Nab2p that escapes the nucleus.

A
Nab2
Kap104
X
NPC
NPC
NPC
Nucleus
Cytoplasm
B
Nab2
X
Pab1
NPC
NPC
NPC

Table 1. Complementation and localization of *NAB2* alleles.

Plasmid	Viability in *TOM1*[a]	Viability in *Δtom1*[a]	Localization at room temperature[b]
Vector	-	-	N/A
NAB2GFP	+++	+++	N
Δ35	-	-	C
cNLS Δ35	-	-	N/C
KKM3	-	-	N/T

a. Tested on 5-FOA plates at 16, 25, 30, 33, and 37 ° C. All alleles behaved equivalently at all temperatures.
b. Assayed in both *TOM1* and *Δtom1* strains in the presence of endogenous wild type *NAB2*. N=nuclear, C= cytoplasmic, N/C= nuclear and cytoplasmic, N/A= not applicable, N/T= Not tested.

EPILOGUE

The major goal of the work described in this thesis was to advance our understanding of molecular mechanisms underlying mRNA export. Specifically, I aimed to determine the role that Tom1p played in this process. I also hoped to identify additional factors that contributed to Tom1p-mediated mRNA export. On the basis of sequence similarity, Tom1p was predicted to be a specificity factor in the ubiquitin protein modification pathway. Since protein modification by ubiquitin has been demonstrated to regulate a wide variety of molecular pathways, I had hoped that focusing on the role of a ubiquitin ligase might provide evidence for regulation of mRNA export and help to elucidate how this fundamental part of eukaryotic gene expression might be coordinated with other aspects of mRNA function and metabolism, as well as more global cellular growth control mechanisms. The work described in this thesis has achieved all of these objectives to some capacity.

The results in chapters 1 and 2 raise a number of intriguing topics for future study. Chief among these are : 1) the nature of export pathways for specific hnRNP protein-mRNA complexes; 2) the nature of Tom1p's effects on Nab2p-mRNA export; and 3) how mRNA export is coordinated with other processes in gene expression to enhance efficiency and enable regulation of cellular responses to changing environmental conditions. Each of these questions represents a potentially vast territory for future scientific exploration. Here, I will review them primarily in the context of the results presented in this thesis. One of the main goals will be to define future experimental directions that represent logical extensions of my thesis work.

Export Pathways for Specific hnRNP-protein –mRNA complexes

I have presented evidence for the existence of specific hnRNP protein export pathways that contribute differentially to efficient mRNA export in *S. cerevisiae*. As mentioned in the prologue and the introduction to chapter 1, this idea was always viewed as a possibility given the existence of multiple shuttling hnRNP proteins, but there was previously very little evidence to support such an idea. Given that my results are obtained *in vivo*, and result from inactivation of particular gene products, they provide particularly strong evidence not only that these pathways exist, but also that they make functionally significant contributions to yeast cell gene expression and physiology. In the following sections I will discuss some of the possible functional roles that the export pathways might play, and conclude by proposing some future experimental approaches that may help to test the different hypotheses.

What is the potential value to the cell of having distinct mRNA export pathways?

What might be the purpose of distinct hnRNP protein-mRNA export pathways? In the most general view, one can envision two very different possibilities based on whether the association of any given message with a particular hnRNP is a completely stochastic event, or alternatively, individual mRNAs derived from particular genes are biased toward or completely dependent on a particular export pathway.

The stochastic association model: utility from redundancy

In the first scenario, where there is no specificity for particular mRNAs in the mRNA export pathways, the most obvious idea is that the systems are completely redundant. According to this view, Npl3p and Nab2p simply represent two independent routes to the cytoplasm for any mRNA they happen to bind, much in the way that two

elevators in a building can represent two independent, but essentially redundant routes between different floors. Why have multiple completely redundant export systems if one will do? An obvious possible purpose of redundant cellular systems is the same as redundant systems made by humans: to provide a backup in the event that, for whatever reason, one system fails. Of course, the backup system may not be exactly redundant. Continuing with the elevator analogy, it might be that Npl3p could be viewed as the 'elevator' and Nab2p could be viewed as the 'stairs', redundant systems in terms of their overall function (moving people between floors), but mechanistically quite distinct. Pushing the analogy even further, one system may be preferentially utilized under certain conditions and the other under a different set. For example, Npl3p apparently dissociates from polyA RNA under cellular stress conditions, and this has been proposed to contribute to differential control of bulk polyA RNA export vs. 'stress message' export (Krebber et al., 1999). While the effect on Nab2p under these conditions remains to be determined, one might imagine that it is responsible for the bulk of export during stress.

A final possibility is that the pathways are 'redundant' in the sense that both proteins bind to mRNAs derived form an overlapping set of loci, but non-redundant in that they differentially recognize some other aspect of the RNA, e.g. those that have long polyA tails or those with shorter polyA tails. Technically this isn't really stochastic association, since some feature of the mRNA is presumably recognized, but the feature is not distinguished by the assay. The biological basis of this type of function might be to differentially influence cytoplasmic events by relaying 'nuclear history.' There is precedent in some systems for this type of phenomenon (Loflin et al., 1999).

The differential association model: lessons from the transcriptome

In contrast to the stochastic association model, the differential association model posits that hnRNP proteins that access different specific mRNA export pathways will interact differentially with mRNAs derived from specific loci, and that this will target these mRNAs to a specific export pathway for that hnRNP protein. Differential association is most obviously envisaged as a discrete phenomenon, wherein mRNA X would associate exclusively with Nab2p and mRNA Y would associate exclusively with Npl3p. In this 'all-or-none' model dedicated hnRNP protein export factors are absolutely required for export of the subset of transcripts they bind. An alternative possibility is that differential association might be continuous, rather than discrete. This view is consistent with the previous *in vivo* studies of hnRNP export mentioned above, that concluded there would be a 'unique constellation' of hnRNP proteins for any given mRNA (Matunis et al., 1993; Wurtz et al., 1996). In this case, dependence on a particular hnRNP protein for export of a specific mRNA will not be absolute, but will instead reflect a bias of any given mRNA toward a particular pathway that is commensurate with the bias in binding. In the continuous differential association model there would be 'differential flux' through a particular hnRNP protein-mediated mRNA export pathway for individual transcripts. Thus, mRNA X might have 70% of its export flux through a Nab2p-mediated pathway and 30% of its flux might go through an Npl3p pathway, whereas for mRNA Y the converse could be true. Under conditions where hnRNP protein interactions are limiting for export, changes in activity of Npl3p would be expected to have a larger effect on how much mRNA Y reaches the cytoplasm, as compared to mRNA X. When the differences in bias for mRNAs are of the relatively small type arbitrarily proposed here, there would

clearly be no huge effect on cytoplasmic mRNA levels by modulating the function of a particular hnRNP protein. Under these conditions, differential flux might function primarily as a mechanism for fine tuning the relative levels of particular mRNAs, rather than as an on/off switch type of mechanism

A similar concept of continuous, overlapping effects appears to hold for the transcription machinery. Transcriptome studies with transcription factor mutants and microarray methods clearly show that some mutations in transcription factors affect almost every gene's transcription to a fairly high level and are consequently essential for viability. However, many more transcription factors affect only a subset of messages to varying degrees, enabling definition for any given mRNA of a 'percent contribution' made by any particular transcription factor under specified conditions (Devaux et al., 2001; Lee et al., 2000). It seems likely that a similar situation may hold for downstream steps in gene expression involving the hnRNP proteins.

Previous evidence for differential association of hnRNP proteins with individual mRNAs

Until recently, the case for differential association of hnRNP proteins with specific mRNAs rested on only two lines of evidence. One line of evidence came from *in vitro* characterization of vertebrate hnRNP protein association with RNA. It had been shown that individual hnRNP proteins displayed preferences for different ribohomopolymers (i.e. polyA, polyG, etc.), and would select different 'winner sequences' during *in vitro* selection experiments (Abdul-Manan and Williams, 1996; Burd and Dreyfuss, 1994; Buvoli et al., 1990; Gorlach et al., 1994; Swanson and Dreyfuss, 1988a; Swanson and Dreyfuss, 1988b). These results do not seem surprising,

given the many structural differences among the hnRNP proteins (Dreyfuss et al., 1993; Gorlach et al., 1993). However, their relevance to hnRNP protein association with nascent mRNA *in vivo* is still not clear.

The second line of evidence came from *in vivo* studies that analyzed the density of staining at discrete loci in polytene chromosome puffs using monoclonal antibodies to different hnRNP proteins and immunoelectron microscopy techniques (Matunis et al., 1993; Wurtz et al., 1996). These methods enabled the conclusion that different transcripts were differentially decorated, implying variations in the relative concentrations of individual hnRNP proteins associated with specific mRNAs. Based on these data, the authors of this study proposed that any given mRNA transcript may have a unique constellation of hnRNP proteins associated with it. The generality of the conclusions from the Matunis study was supported by Wurtz et al., who reached a similar conclusion based on analyses of Balbiani Ring particle transcripts (Wurtz et al., 1996).

It is important to note however, that neither of these studies provided any evidence that differential binding plays a role in mRNA export, or indeed any functional role in the nucleus whatsoever. Conversely, as mentioned above, my results with *tom1* mutants described in the previous chapters provide no evidence for differential affects on specific mRNAs. The significance of all of the studies in aggregate would be greatly increased by direct evidence for differential association *in vivo* and direct demonstration that this differential association results in differential effects on the function of that RNA in the cell, e.g. targeting it to a specific mRNA export pathway. Ultimately, it will be important as well to know whether the cell takes advantage of any specificity to selectively regulate gene expression.

Suggested experimental approaches to the differential hnRNP protein association/specific mRNA export pathway hypothesis

Obviously, a major future direction for this project is to determine whether the pathways are completely redundant, completely independent, or somewhere on the continuum between these two extremes. The most obvious approach to this problem in the era of genomic methodologies is to develop assays to characterize the complete set of mRNAs that associate with an hnRNP protein of interest under a controlled condition, the 'bindome' for the RNA-binding protein of interest. By comparing the relative differences between two hnRNP proteins of interest, in this case Npl3p and Nab2p, it will be possible to determine the extent to which the venn diagrams describing their respective bindomes overlap. In principle, this will identify mRNAs that are the best candidates for follow-up assays to test for biological consequences associated with differential interaction. For example, the specific-transcript mRNA export pathway hypothesis predicts that individual mRNAs that are heavily enriched in the bindome of one hnRNP protein relative to another will display a greater requirement for that hnRNP protein for export. This prediction can be tested by using specific probe *in situ* hybridization methods (Hurt et al., 2000; Long et al., 1995; Saavedra et al., 1996)or other mRNA localization technologies (Brodsky and Silver, 2000) in combination with conditional alleles of the hnRNP protein of interest, e.g. *NPL3* and *NAB2*.

The most straightforward approach to bindome characterization would be to perform affinity selection methods (e.g. immunoprecipitation) to isolate the hnRNP proteins of interest from yeast lysates under physiological buffer conditions that preserve *in vivo* RNA-protein interactions to the greatest extent possible. This approach, an

obvious follow-up to the results described in Chapter 1, was initially attempted as a rotation project by Karen Kim, a UCSF graduate student in the PIBS/ tetrad program. Karen joined the lab and the hnRNP protein bindome assay has subsequently become the basis for her Ph.D. thesis project. Karen has made substantial progress in developing the assay and has found exciting evidence indicating that the binding profiles of Npl3p and Nab2p appear to overlap to a great extent, but not completely (Karen Kim and Christine Guthrie, unpublished results).

A major concern about this assay as currently performed is that it may reflect associations that occur after lysis rather than those that occur in the nucleus *in vivo*. Clearly this is a serious concern, since the goal is to determine the *in vivo* interaction profile. The problem is compounded by the fact that making the yeast cell lysates mixes the nuclear and cytoplasmic compartments causing interaction and/or competition between components that are normally sequestered from one another. Thus, Nab2p and Npl3p may be forced to compete with cytoplasmic RNA binding proteins under these assay conditions. If this is true, differences in binding observed with the assay could simply reflect differential ability of Nab2p or Np3p to compete for binding with cytoplasmic RNA binding proteins, clearly not the parameter that we intended to measure with the assay. That this concern may be valid is reflected in recent results from Swanson and colleagues suggesting a competition between Nab2p and Pab1p for the polyA tail exists in whole cell extracts, and that this competition masks a role for Nab2p in polyA tail length regulation. Such a role for Nab2p is clearly indicated by *in vivo* assays (Nykamp and Swanson, manuscript in prep.).

The original experimental approach that was proposed, and the one initially attempted by Karen, was to use *in vivo* crosslinking to trap genuine hnRNP-mRNA complexes in situ prior to lysis and then subsequently to isolate protein-RNA complexes under relatively harsh binding and washing conditions, so as to characterize only those associated by covalent linkages. Such an approach has been used routinely with formaldehyde in so-called 'chromatin IP's' (Aparicio et al., 1997; Hecht et al., 1999; Jackson, 1978; Jackson, 1999; Solomon and Varshavsky, 1985; Strahl-Bolsinger et al., 1997). The advantages of formaldehyde are that it is relatively cheap, readily-available, and reversible, although there is no reason why other cell permeable reversible cross-linking agents could not also be investigated. In principle, this approach deals handily with the caveats discussed above. In practice it appears to be no panacea. The protocol is much more complex and introduces a number of additional variables and incubation steps. Of particular concern is that the decrease in RNA yield necessitates an enzymatic amplification step prior to cDNA labeling, which will inevitably increase the noise in the data. Moreover, while confidence in the positives will be increased using this method, absence of association of a particular transcript could simply reflect a decreased crosslinking yield for a particular transcript-protein pair that brings it below a detection threshold, rather than a true absence of interaction *in vivo*. On balance, the crosslinking approach's problems remain theoretical, whereas those of the simpler, standard co-IP seem inherent to the assay. One reasonable compromise might be to do a rigorous side-by-side comparison of the two methods and thereby decide which approach to favor. In the event the data are indistinguishable, this would suggest that the non-crosslinking approach faithfully reflects the *in vivo* bindome. Use of mRNA export mutants and a

comparison of the nuclear hnRNP proteins vs. the cytoplasmic mRNP proteins such as Pab1p may also be useful in helping to determine how great a concern mixing of the nucleus and cytoplasm proves to be.

One additional important caveat that should be acknowledged here relates to follow-up experiments. It seems obvious that even if differential affinity proves to be real, this will not mean, of course, that this reflects differential control of mRNA export. A less obvious concern is that even should differential dependence of a particular mRNA on a particular hnRNP protein for efficient export be demonstrable, this too could be a red herring with respect to the step of gene expression the cell actually regulates. In other words, differential hnRNP protein requirements for mRNA export pathways might be correlated with regulation that occurs at a different upstream or downstream step in gene expression; the actual regulation of gene expression based on differential association of hnRNP proteins with particular transcripts may affect a pre or post-export step that responds to the transcript's RNP composition. Differential association might also result in targeting to a specific export pathway, but the different export pathways themselves may be entirely unregulated. On some level this may sound like a theoretical or even semantic debate, but it is most definitely not. There is substantial evidence that hnRNP proteins influence a wide variety of both nuclear and cytoplasmic RNA processing events, indeed the evidence for this is perhaps stronger than that for a direct role in mRNA transport (Krecic and Swanson, 1999). Failure to appreciate this nuance could also have serious practical consequences, since significant time might be invested to test hypotheses that ultimately prove incorrect.

One additional possible approach deserves mention here: in silico data mining to investigate possible biological meaning in the bindome data. Presumably this approach would involve characterization of the bindome for the proteins of interest under a large variety of cellular conditions, and statistical hypothesis testing or perhaps hypothesis-free data mining using the bindome data and other information of interest. This approach suffers from the absence of any expertise in computational biology in our lab, but might prove quite powerful with the right collaboration. One can imagine that such an approach might help to narrow the field of mechanistic hypotheses to a limited set that would be experimentally testable. Perhaps this would be a useful complement to testing the hypotheses that are already envisaged. A strength of the data mining approach is its potential to reveal novel biological phenomena that could not be easily found using other methods.

Connections between Ubiquitin and mRNA metabolism

My thesis work began with the goal of determining the role that Tom1p, a putative ubiquitin protein-ligase was playing in mRNA export. Results described in Chapter 1 and 2 provided compelling evidence that Tom1p is likely to be a ubiquitin ligase, and that this activity is central to its role in maintaining wild type mRNA export rates via a Nab2p-mediated pathway, thus implying some connection between ubiquitin and mRNA export. Data presented in Appendix 1 show that a large panel of strains with mutations in ubiqutin pathway components did not affect mRNA export. This clearly demonstrates that the connection between ubiquitin and mRNA export is not a general phenomenon, but appears instead to be related to a specific branch of this post-translational modification pathway. Apparently this branch involves Tom1p and possibly

also Cdc34/Ubc3p, since I show in Appendix 1 that *cdc34-1* mutants phenocopy *Δtom1*. Unfortunately, at this time we do not know what the relevant target is, and therefore have no mechanistic insight into how this activity might influence these cellular processes, nor do we know whether the effect of *tom1* mutations on Nab2p-mRNA export is direct or an indirect consequence of a more global effect on the 'cellular state.' Here I will briefly discuss at a general level the possible effects on Nab2p-mediated mRNA export pathways that ubiquitin might be expected to have. I will then review the few other connections between ubiquitin and mRNA metabolism that have been identified to date, and propose experimental approaches for determining the identity of 'Factor X,' the elusive target of Tom1p's ubiquitin ligase activity in Nab2-mRNA export.

Possible roles of ubiquitin in Nab2p-mRNA export

Formulating detailed mechanistic hypotheses about the function of ubiquitination of Factor X are impossible without knowing the identity of this protein. Nonetheles, it is useful to consider at a more general level the possible roles for ubiquitin in Nab2p-mRNA export. This topic has already been discussed extensively in Chapter 1. I will summarize that discussion here, and update it to incorporate new findings presented in Appendix 1.

The most obvious role for ubiquitin in mRNA export would be in turnover or regulated proteolysis of components of the mRNA transport machinery by targeting that factor to the proteasome (Ciechanover et al., 2000). However, ubiquitin has also been implicated in almost as many cellular processes as phosphorylation, many of them not directly related to protein turnover, including stimulation of endocytosis, promoting phosphorylation, and activating transcription factors (Chen et al., 1996; Hicke, 1999;

Hicke, 2001; Kaiser et al., 2000). Thus, it may be equally probable that ubiquitination of Factor X by Tom1p serves a non-turnover function in Nab2p-mRNA export.

The data in Appendix 1 argue that whatever the role of ubiquitination of Factor X may be, it is highly specific. I found that deletion of both *ubc4* and *ubc5*, shown to lead to loss of major turnover pathways for aberrant proteins, and a fairly severe growth defect, has no effect on mRNA localization by oligo dT50 FISH. Similarly, *Δrad6/ubc2* strains show major reductions in ubiquitination of a number of substrates and are temperature sensitive, but show no effect on polyA RNA localization at 37° C. As described in Appendix 1, mutation of only one tested *UBC*, *cdc34*, caused nuclear accumulation of polyA RNA at 37°C. Moreover, two well-characterized mutations that each result in a general decrease in the proteasome's activity and lead to cell cycle arrest also showed no effect on polyA RNA localization. These results clearly argue against a model where general loss of ubiquitin pathway activity or protein turnover results in nuclear retention of mRNA. Instead they imply a fairly specific effect due to loss of Tom1p and possibly Cdc34p function.

Previously described connections between ubiquitin and mRNA metabolism

As mentioned above, ubiquitin has been implicated in a number of cellular processes. However, aside from transcription factor effects, reported connections between ubiquitin and mRNA metabolism can be counted on the fingers of a single hand. One report describes the identification of *CDC34* as a high copy suppressor of temperature sensitive alleles of the yeast mRNA cap methyltransferase, *ABD1* (Schwer et al., 2000). Additional genetic interactions, including a synthetic growth defect between *abd1* mutants and *cdc34-2* suggest that ubiquitin transfer capabilities are necessary for

suppression. The authors propose a model where cap methylation or cap utilization is negatively regulated by a factor that is degraded when Cdc34p is overexpressed. Whether this observation relates to Tom1p function in any way is completely unclear; Cdc34p interacts with a large number of different ubiquitin ligases, primarily those of the cullin/SCF family (del Pozo and Estelle, 2000; Deshaies, 1999; Jackson et al., 2000; Kipreos and Pagano, 2000). These results are intriguing though, given that *cdc34-1* mutants were the only *ubc* mutants that phenocopied *Δtom1* for effects on mRNA export. Moreover, the cap has been shown to play a stimulatory role in mRNA export (Dargemont and Kuhn, 1992; Hamm and Mattaj, 1990).

Another hint of a connection between ubiquitin and mRNA export comes from analysis of the major mRNA export receptor, TAP, the human homolog of Mex67p. TAP has a ubiquitin-associated (Uba) domain, which is also found in several proteins involved in the ubiquitin pathway (Bertolaet et al., 2001; Braun et al., 2001; Hofmann and Bucher, 1996; Suyama et al., 2000; Wilkinson et al., 2001; Withers-Ward et al., 2000). The Uba domain is conserved in Mex67p and required in human TAP for interaction with the NPC (Braun et al., 2001). Whether this domain reflects a connection between Mex67p and ubiquitin, and whether it relates in any way to Tom1p function is currently unknown.

Claire Moore and colleagues have identified two ubiquitin pathway-related factors as specific interacting proteins in a two-hybrid screen using polyA polymerase (PAP) as bait (del Olmo et al., 1997). The functional significance of this result is supported by the finding that PAP is phosphorylated and ubiquitinated in a cell-cycle regulated manner (Mizrahi and Moore, 2000). PAP has a long half-life, and the

functional significance of PAP ubiquitination is not clear. As for Mex67p/TAP, whether there is any connection to Tom1p remains to be investigated.

Finally, two other published connections between ubiquitin and mRNA metabolism involve mRNA decay via AU-rich element (ARE)-mediated turnover. ARE's promote the rapid turnover of a variety of mRNAs, in particular those encoding cytokines and proto-oncogenes (Sachs, 1993). A large number of hnRNP proteins interact with these elements to both stabilize and destabilize the transcripts in response to different environmental cues or in cancerous cells (Chen and Shyu, 1995; Chen et al., 1995). A recent report indicates that the product of the von Hippel Lindau (VHL) tumor suppressor gene, pVHL, interacts with hnRNPA2 and regulates its expression (Pioli and Rigby, 2001). Reduced pVHL function results in increased levels of hnRNPA2, which, in turn, stabilizes the AU-element containing mRNA GLUT1 mRNA a known target for hnRNPA2 binding (Pioli and Rigby, 2001). Interestingly, pVHL has already been shown to be a ubiquitin ligase for the transcription factor HIF1, a regulator of hypoxia induced genes, via interaction with the SCF family (Carmeliet et al., 1998). Pioli and Rigby's results suggest that this activity also affects stability of hypoxia-inudced genes through hnRNPA2, although they cannot detect hnRNPA2 ubiquitin conjugates. These data may be relevant to Nab2p in yeast, since the hnRNPA2 RGG box and its methylation state have been shown to modulate nuclear localization of hnRNPA2 (Nichols et al., 2000). How this might relate to Tom1p, however, is not obvious. One purely speculative possibility is that Tom1p might function as an auxiliary factor in Cdc34p/SCF-mediated ubiquitin ligase reactions.

The second reference demonstrates a clear role for ubiquitin and the proteasome pathway in mediating the rapid decay of ARE-containing mRNAs in HeLA cells (Laroia et al., 1999). These authors showed that rapid decay involves the ARE-binding proteins AUF1/hnRNP D, which formed a complex with hsp/hsc70, eIF4G and PABP1. ARE-mediated decay was associated with displacement of eIF4G from AUF1, ubiquitination of AUF1 and degradation of AUF1 by proteasomes. Incredibly, induction of hsp70 by heat shock , down-regulation of the ubiquitin-proteasome network, or inactivation of E1 all resulted in hsp70 sequestration of AUF1 in the perinucleus-nucleus and all three blocked decay of AU-rich mRNAs and AUF1 protein. This suggests a correlation between sub-cellular localization of AUF1p and decay of AU-rich mRNAs, perhaps implying that mRNA export (or its absence) might affect the rate of turnover (i.e. the mRNA half-lives are different in the different compartments). In support of this notion, a recent report has revealed that another ARE binding hnRNP protein, HuR, previously shown to stabilize mRNAs with AREs, is also a major export adapter for ARE-containing mRNAs (Gallouzi, 2001).

In thinking about how these results might relate to Tom1p, in yeast, it should be noted that no E2 or E3 was implicated in AUF1-mediated degradation by Laroia, et al. The connections with PABP1 and eIF4G are also interesting, given the genetic connections between Δ*tom1* and *PAB1* described in Chapter 2. Moreover, the observed sequestration of specific hnRNP complexes containing AUF1 and ARE-mRNAs in the nucleus or perinucleus during heat shock bears uncanny resemblance to Δ*tom1* phenotypes, where Nab2p and polyA RNA selectively redistribute to the nuclear periphery in yeast. Finally, the recent identification of ARE-mediated decay in yeast

(Vasudevan and Peltz, 2001) makes it more likely that a similar ubiquitin-mediated decay pathway may exist in yeast. One could imagine that Tom1p might pariticipate in the rapid turnover of these mRNAs via a Nab2p-dependent export pathway. The most important predictions of this hypothesis to test would be 1) Nab2p associates with ARE mRNAs in yeast and this affects their turnover rate; 2) ubiquitination of a specific protein in the ARE-mRNP by Tom1p will be important for this rapid turnover. In principle, both are testable.

Chasing the X: suggested experimental approaches for identifying Tom1p's target

Genetic screens comprise one obvious route for trying to identify Factor X. Genetic approaches will never actually identify Factor X, but will provide candidates for use in follow-up biochemical assays. However, key advantages of this approach are its relative ease and the fact that it may provide information about Tom1p function even if it never identifies Factor X. The main disadvantage is that because of the underlying biology it may not be trivial or even possible to design the screen or selection in a way that enables identification of Factor X. For example, it may be impossible to identify Factor X by selecting suppressors of *Δtom1*. It is not clear that increased copy number of Factor X would be expected to rescue defects caused by loss of its ubiquitination by Tom1p. A similar, but reversed argument can be made for extragenic suppression. This type of screen might seem more plausible if we assume that Tom1p has a traditional role in turnover of Factor X: a compensatory decrease in Factor X levels might compensate for gain of function effects resulting from loss of *tom1* function. However, suppressor screens also assume that effects on Factor X will be sufficient to rescue *tom1* mutant growth. In the likely event that Tom1p has additional targets that need to be

ubiquitinated for viability, such approaches will never identify Factor X. A synthetic lethal screen with *Δtom1* has not yet been reported and is likely to be informative, but it is not clear whether Factor X would be predicted to display this genetic interaction with *Δtom1*.

I attempted to set up a different type of screen for Factor X, based on the idea that hect proteins are modular, and therefore Factor X is likely to interact with the N-terminus/non-hect domain of Tom1p. The concept was to overexpress an allele of *TOM1* that either had a non-fucntional hect domain, or lacked it completely. I assumed this would titrate substrates from wild-type Tom1p and thereby produce a dominant-negative phenotype. The plan was then to screen for factors that would rescue this phenotype when present in higher copy number. One can imagine that a target protein might suppress the dominant-negative phenotype by squelching the effect of the mutant N-terminus and thereby increasing the amount of ubiquitinated endogenous target proteins in the cell. In principle this method or variations of it using smaller fragments of Tom1p should be able to identify targets including Factor X even if there are multiple ones important for viability, since increased dosage of a given one could potentially rescue the loss of all targets in a dominant-negative scenario. In practice this approach didn't work. Curiously, it still might. The major problem with this method is that I constructed the hect$^-$ *tom1* alleles under control of the *GAL1* promoter and then screened for a dominant-negative effect on growth on galactose-containing media. To my surprise, not only did I not see a dominant-negative growth defect, I didn't even see a strong growth defect on GAL media with *Δtom1* strains at any temperature (KD, unpublished results). I believe this approach might be worth trying again in a modified form enabling screening on

glucose-containing media. It will also be interesting to see whether the carbon-source selectivity of *tom1* represents a specific phenotype. If so, it might help define Tom1p's cellular role. In this vein, it has been reported that yeast lacking one or both CBC proteins grow extremely slowly on glucose-containing media, but are relatively healthy when using other carbon sources (Das et al., 2000). Given that Das et al. implicate CBC in mRNA turnover, it is tempting to imagine this relates somehow to the variation in acitivity of the nuclear exosome as a function of carbon source (Bousquet-Antonelli et al., 2000).

Given the concerns and lack of success thus far with genetic approaches, I favor a 'brute force' biochemical approach to this problem. There are a number of different options within this realm. One approach would be to screen for evidence of ubiquitination of candidate proteins by simply looking for Tom1p-dependent higher mobility species or reactivity of putative conjugates with anti-ubiquitin antibodies. These assays have already been attempted with various candidates (KD and CG, unpublished results). However, identification of known ubiquitinated proteins in yeast lysates can be notoriously difficult (Kornitzer, in press). It would be worthwhile to repeat these assays using a relatively new approach involving HIS-tagged ubiquitin and lysis under denaturing conditions that is reported to provide better preservation of ubiquitin conjugates (Kaiser et al., 2000). The use of proteasome mutants or proteasome inhibitors in strains that respond to them (Lee and Goldberg, 1996; Lee and Goldberg, 1998) may also increase the chances of detecting a relevant conjugate.

An alternative, ambitious approach would be to try to establish an *in vitro* assay for Tom1p-dependent ubiquitin transfer. This would involve purified E1, the relevant E2

(Cdc34p would be the obvious choice given that it phenocopies *Δtom1*-see Appendix 1), ATP and ^{32}P-labelled ubiquitin, as well as partially purified HA-Tom1p or HA-tom1-C3235Ap (negative control), both of which are available. *Δtom1* extracts or a fraction thereof would be starting material. NEM could be used to inactivate any endogenous ubiquitin transfer enzymes in the extract, should this prove necessary. Tom1p-dependent targets can be identified using mass spectrometry, and then subsequently screened in silico and by genetic and cytological methods to determine which are most likely to be Factor X vs. Tom1p targets affecting other processes. A major caveat to this approach is that such assays have been established for other ubiquitin pathway components, but have never been used to identify the substrates, only to examine requirements for ubiquitination of an already known substrate. Nevertheless, the approach is conceptually appealing because it looks directly for exactly what is of greatest interest, namely, specific targets of the Tom1p's putative ubiquitin ligase activity. In a related approach, such a system could also be used to screen candidate substrates.

An alternative, much simpler, biochemical approach would be to assume that Factor X will stably bind to Tom1p and try to identify it by affinity chromatography. Like the synthetic lethal screen, this approach is appealing because it is likely to be informative about Tom1p function even if it fails to yield Factor X. I made preliminary attempts at this problem during my thesis work, but did not identify any interacting factors. One problem I faced was trying to resolve bands that were breakdown products of Tom1p from those that were interacting proteins. I attempted to address this issue by mixing 35Smet-labelled *Δtom1* extracts with HA-tom1p extracts prior to isolating HA-Tom1p, but the background was too high to see any specific co-purifying bands. This

approach may be worth repeating and optimizing. However, Deshaies and colleagues have described 'proteasomal proteomics' using a mass spectrometry approach that apparently can get sufficient peptide peak resolution from a crude purification, obviating gel purification (Verma et al., 2000). If this technique can perform consistently in general applications, it is likely to be a particularly promising approach with Tom1p, since it would render moot concerns about distinguishing Tom1p breakdown products from co-purifying proteins. In combination with an efficient purification scheme this might prove a straightforward and powerful approach.

Towards an integrated model of mRNA metabolism: What role might Tom1p play?

Coupling of pre-mRNA splicing and mRNA export

When the work described in this thesis was initiated one hope was that *TOM1* by virtue of its genetic connections to both splicing and mRNA export might provide evidence for and perhaps mechanistic insight into the hypothesized coupling of pre-mRNA splicing and mRNA export. While this hope has not materialized for Tom1p, exciting progress has been made on this front with other mRNA export factors (see below) and involvement of Tom1p is still possible.

An exciting recent set of reports provide genetic, biochemical, and cytological evidence for coupling of splicing (Jensen et al., 2001a; Luo et al., 2001; Strasser and Hurt, 2001). In these studies it has been shown that the DExD/H box RNA-dependent ATPase Sub2p/UAP56, well-characterized by our lab and others as a splicing factor (Kistler and Guthrie, 2001; Libri et al., 2001; Zhang and Green, 2001) appears also to play an important early role in mRNA export. Sub2p forms a strong complex with

Yra1p/Aly/REF (Stutz et al., 2000; Zenklusen et al., 2001) that is mutually exclusive with Yra1p's interaction with the mRNA export factor Mex67p (Strasser and Hurt, 2000). In the analogous case for mRNA export in metazoans, Aly is recruited to complexes during spliceosome assembly, presumably by UAP56, and the presence of Aly appears to promote the enhanced rate of export associated with post-splicing mRNP's. These data suggest a general biochemical model for coupling of completion of splicing with mRNA export via Sub2p/UAP56 and Yra1p/Aly (Luo et al., 2001).

In the case of Tom1p and the Nab2p-mediated mRNA export pathway, evidence that Tom1p acts as a coupling factor between splicing and mRNA export is limited to genetic connections to both processes. *nab2* mutants do not show effects on splicing of at least one pre-mRNA (Swanson, personal communication), suggesting this protein may not generally be required for splicing, but this question warrants analysis of more transcripts. Whether a connection exists between Tom1p and the export pathway mediated by Sub2p, Yra1p and Mex67p is not currently known. This should certainly be tested by both genetic and biochemical approaches in the future. Identification of a connection with *SUB2* might help to explain tom1 phenotypes affecting Nab2p and mRNA export, and would provide an additional genetic and biochemical handle for investigating Tom1p's role in mRNA export. Of course it is also quite possible that Tom1p's influence on Nab2p-mRNA export is through an effect that is unrelated to Sub2p/ Yra1p function. In this regard it may be relevant that Yra1-GFP localization is not perturbed in *Δtom1* mutants at the non-permissive temperature (KD and CG, unpublished results). It will be very interesting to see whether there are connections between Yra1p-Mex67p mediated mRNA export and Nab2-mediated export, as might be

expected, or whether they truly represent completely independent pathways. The 'short list' for addressing this question would be to look for synthetic lethal interactions with Δtom1 and co-immunoprecipitation of the proteins with HA-Tom1p.

Coupling of mRNA export and ribosome biogenesis

One possible alternative explanation for the genetic connections between *TOM1* and splicing factors may involve coupling as well, but between mRNA export and ribosome biogenesis, rather than pre-mRNA splicing. Genes encoding ribosomal proteins make up the lion's share of the roughly 5% of yeast genes that have introns. Consequently, many of the components of the splicing machinery were originally identified in a screen for mutants with decreased rRNA production/stability (Hartwell et al., 1970). These effects can be simply viewed as indirect downstream effects of the mutations, but an alternate, popular view is that the dependence of ribosome biogenesis on efficient splicing provides the cell with an opportunity for regulation of ribosome biogenesis via pre-mRNA processing pathways. Coordination of ribosome biogenesis with other cellular metabolic pathways reflects a key 'resource allocation' decision for cells, and therefore would be expected to be subject to regulation at a number of levels (Warner, 1999). The regulatory effect need not be at the level of splicing per se. For example, in light of models discussed above, Yra1p recruitment and subsequent mRNA export might be the key regulatory event.

Several reports from other labs have implicated Tom1p in ribosome biogenesis, raising the possibility that a role for Tom1p in this process may underlie the genetic interactions between *TOM1* and splicing factors (Umen, 1995). Tom1p interacts in a 2-

hybrid assay with Krr1p, a highly-conserved nucleolar protein with a predicted KH RNA binding domain (Sasaki et al., 2000a). *krr1* mutants have defects in ribosomal subunit assembly, and Krr1p localization expands beyond the nucleolus to include a subregion of the nucleoplasm in *Δtom1* mutants (Sasaki et al., 2000a). Nucleolar-localized proteins that co-purify with a nucleolar ribosomal biogenesis complex (Fath et al., 2000) are high-copy suppressors of *Δtom1* (Davey et al., 2000). *TOM1* alleles show synthetic lethality with each of 2 redundant genes encoding components of the small ribosomal subunit, *RPS0A/B*, and these genes are also partial high copy suppressors of *Δtom* (Tabb et al., 2001). *RPS0A/B* are required for ribosome biogenesis and have extremely similar relatives in mammals that are implicated in tumorigenesis. *tom1* mutants have multiple conditional defects in ribosome biogenesis (Tabb et al., 2001). While many of these defects were assayed relatively late after a temperature shift, and could therefore be secondary to nuclear polyA RNA accumulation, Tabb et al. also observe a constitutive defect in 18S rRNA maturation which is presumably not a secondary defect (Tabb et al., 2001).

It is possible that Tom1p plays completely distinct roles in both ribosome biogenesis and Nab2p-mRNA export. In support of this view, other hect domain proteins have been shown to have multiple, apparently functionally unrelated substrates (Beaudenon et al., 1999; You and Pickart, 2001). However, an intriguing alternative is that Tom1p function relates to coupling of ribosome biogenesis with mRNA export and polyadenylation via Nab2p. How might one test this hypothesis? Obviously, identification of Factor X, and perhaps other Tom1p substrates would make both formulating and testing specific hypotheses about Tom1p's potential role as a coupling

factor much easier. In the absence of Factor X, the best strategy would probably be to use a two-pronged approach involving genetics and biochemistry. Key focal points for genetics would be open-ended synthetic lethal and/or suppressor screens with *rps0 A/B* and *Δtom1*. Biochemical connections between Nab2p and ribosome components should also be investigated in more detail. In addition, since Krr1p interacts with Tom1p by 2-hybrid and has a predicted RNA binding domain, this protein merits closer inspection.

To test the hypothesis that Krr1p might be an mRNA binding protein, I made a preliminary investigation of whether Krr1-ZZp could be UV cross-linked to polyA RNA *in vivo*. Surprisingly, although Nab2p behaved as expected in this experiment, co-purifying with polyA RNA in a crosslinking-dependent manner, Krr1-ZZp co-purified with polyA even in the absence of crosslinking (KD and CG, unpublished results). The profile appeared the same in *Δtom1* mutants. This experiment needs to be repeated, but suggests not only that Krr1-ZZp may associate with polyA RNA, but also that this interaction may be stable even in the presence of 1% SDS. Alternatively, Krr1-ZZ may have affinity for oligo-dT cellulose under the assay conditions. In the former case, Krr1p, would be the first example of a nucleolar protein affecting ribosome biogenesis that also interacts with polyA RNA, presumably mRNAs. Thus, Krr1p may be a particularly interesting focus for future studies, both to search for evidence of coupling of ribosome biogenesis with mRNA export, and for determining the nature of Tom1p's effects on these pathways. This case is bolstered by the recently reported finding that the *S. pombe* homolog of Krr1p may be a substrate for the *pombe* homolog of the S. *cerevisiae* Npl3p kinase Sky1p, and that this phenomenon is related to ribosome biogenesis (Kondoh et al., 2000).

Coupling between 3'-end processing and mRNA export

An additional area where Tom1p might play a role in coupling processes in mRNA metabolism might be through its effects on Nab2p, which has been shown to couple 3'-end processing to mRNA export, at least that mediated by Nab2p. Most of the ideas and future directions related to this possibility have already been covered in the discussion for Chapter 2; I will review them here with a focus on speculating about possible roles Tom1p might play.

Since Nab2p is a nuclear-localized hnRNP protein, and Pab1p is a cytoplasmic RNA binding protein, a transition from a Nab2p-associated mRNA to a Pab1p-associated mRNA somewhere in the mRNA export process is implied. However, recent results from the Swanson lab (Nykamp and Swanson, manuscript in preparation), coupled with my genetic results presented in Chapter 2 suggest a model where a Nab2p- to-Pab1p mRNP remodeling step is not simply a consequence of export, but rather is a key step affecting the efficiency of mRNA export. My observation was that recessive, inviable alleles of *NAB2* that lack the Kap104p binding domain interfere with the ability of high-copy *PAB1*

to suppress lethality associated with *Δnab2*. These alleles retain the CCCH domain that is necessary and sufficient for polyA RNA binding, suggesting that they bind to polyA RNA, but are defective at the Nab2p-Pab1p transition, possibly due to loss of Kap104p interaction. An additional observation is that the efficiency of *PAB1* bypass suppression of *Δnab2* is severely reduced when combined with *Δtom1*, indicating that Tom1p directly or indirectly affects this process as well.

What effect might Tom1p have and how might this relate to coupling of 3'end processing with mRNA export? *TOM1* seems to influence both the efficiency of Nab2p-mRNA export, and the efficiency of *PAB1* bypass of *Δnab2*. This suggests that the *Δtom1* defect may affect Nab2p via an effect on Pab1p. This hypothesis fits well with the idea of a Nab2p-to-Pab1p mRNP transition playing a key role in efficiency of export; presumably Tom1p ubiquitin ligase activity directly or indirectly affects this transition.

Curiously, bypass suppression of *Δnab2* by *2μPAB1* indicates that mRNAs with long polyA tails can be exported when there is enough Pab1p in the cell, and implies that long polyA tails are not inherently problematic for viability. This is consistent with results seen with some viable alleles of Nab2p (Nykamp and Swanson, manuscript in prep.). If long tails are not particularly problematic for yeast cell growth, then what might be the biological significance of coupling mRNA export to polyA tail length regulation via Nab2p? One intriguing possibility is that it may relate to a yeast version of the newly-discovered 'pioneer round' of translation observed in mammalian cells. In this view, Nab2p plays a role analogous to mammalian PABP2, interacting with the polyA tail in the nucleus during the pioneer round, presumably as part of an mRNA and/or ribosome quality control process. Thus, when Nab2p is not around, excess Pab1p may end up promoting export of mRNAs of 'low quality' and this may be the basis for slower growth in the *Δnab2 2μPAB1* strains. It will be very interesting to see whether Nab2p does indeed interact with the mRNA tail during a pioneer round in yeast and, if so, whether *Δnab2 2μPAB1* strains undergo an aberrant pioneer round mediated by Pab1p or another factor, or omit the pioneer round altogether. Recent data suggest that the nuclear cap-binding complex can mediate translation in yeast extracts through interaction with

eIF4G, and provide genetic evidence this may be important *in vitro* (Fortes et al., 2000). Whether Pab1p (or perhaps Nab2p) plays a role in this reaction *in vitro* or *in vivo* is not yet clear. However, one can imagine using these assay conditions as the basis for a 'pioneer round' assay in yeast and determining whether Nab2p mediates such a round and what distinguishes the first round functionally from the subsequent rounds of translation.

What might be the role of Tom1p in all this? While it is possible that Tom1p might play a direct role in promoting export via an mRNP associated factor, less direct- but equally interesting- effects can also be imagined. For example, Tom1p might conceivably assist in clearing of the 'low quality' complexes, perhaps by promoting degradation of a protein or protein that is associated. In one variation of this idea, export to the cytoplasm might precede degradation. It has long been hypothesized that nuclear translation might be involved in mRNA quality control (Maquat and Carmichael, 2001). Indeed, nuclear protein synthesis was recently demonstrated, apparently in association with nascent transcripts (Iborra et al., 2001), although these results remain somewhat controversial. Additional studies have also provided strong evidence for nuclear translation in the context of mRNA quality control (Brogna, 2001). Nuclear protein synthesis is also consistent with several aspects of the mammalian pioneer round, including the nuclear steady-state localization of several of the factors mediating it. Interestingly, the redistribution of Nab2p and polyA RNA observed in *Δtom1* mutants is not observed if the strains are shifted in the presence of cylcloheximide, implying that protein synthesis is required to see redistribution (KD and CG, unpublished results). One wild hypothesis is that accumulation of Nab2p and polyA RNA at the nuclear periphery

in *tom1* strains reflects accumulation of translation-dependent 'low quality' complexes that would be efficiently cleared by Tom1p in wild type cells.

One interesting aspect of such models is the question of how an mRNA that has been judged to be of sufficient quality moves from a pioneer round of translation to future rounds, and what happens to the low quality mRNPs and nascent proteins. Many will be substrates for nonsense mediated decay (NMD), but some may be degraded through alternate pathways. Tom1p is not implicated in NMD, but may nevertheless affect the proteins in the 'low quality complex'. A final intriguing aspect of mRNA quality control involving nuclear ribosomes and protein synthesis is the question it raises of whether ribosome quality control exists and whether ribosome quality control might be related to mRNA quality control. One wild idea is that both may occur simultaneously in the nucleus, in a process coupled to export of both molecules. As described in the previous section, Tom1p has multiple connections to ribosome biogenesis. Thus one appealing role for Tom1p is in a hypothetical quality control pathway involving both mRNAs and ribosomes that is coupled to export via a common factor, perhaps Krr1p. This role would presumably be intimately related to the Nab2-to-Pab1 transition; polyA tail length modulation by Nab2p might serve as a signal for 'quality.'

A challenge for the future: an integrated model of cellular mRNA metabolism

The previous sections have presented some recent evidence for coupling of pre-mRNA processing or other cellular processes with mRNA export and have discussed how Tom1p may be involved in related coupling phenomena. Currently, our understanding of

Tom1p's role in such processes can be called rudimentary, at best. However, in the broader view we know very little about such processes, recent progress notwithstanding. Indeed, one of the biggest challenges we now face will be to integrate what we know about mRNA export pathways with our understanding of other aspects of mRNA metabolism including not only splicing, but also transcription, polyadenylation, and mRNA turnover pathways, to produce a more integrated model of mRNA metabolism. An even more daunting challenge for molecular cell biologists of the future will be to integrate mRNA metabolism with other cellular processes, for example signal transduction and ribosome biogenesis. It seems inevitable that the cell will have a number of mechanisms by which to coordinate processes we currently imagine to be distinct. Indeed, is not integration of molecular processes 'under one roof' a defining characteristic of a cell? Why should cellular evolution care that our brains and culture have evolved to favor reductionist approaches? Nevertheless, given this constraint on our thinking, the trick will be to rigorously define 'coupling'in a manner that captures its biological significance, and to find suitable approaches to study it. On this last point, I expect that application of emerging technologies for real-time in situ analysis of macromolecular interactions and biochemical reactions will play an essential role in these investigations.

Conclusions and Perspective

My thesis work has made some progress toward achieving some of the objectives I defined at the outset. I have demonstrated a role for the nuclear protein Tom1p, and its predicted enzymatic activity as a ubiquitin-protein ligase, in a specific Nab2p-mediated mRNA export pathway. In addition, my results suggest a novel role for the Nab2p

import receptor,Kap104p, in this pathway. Finally, they suggest that a transition from a Nab2p associated mRNP to a Pab1p-associated mRNP may play an import role in promoting mRNA export via this pathway. Finally, the results implicate Kap104p and Tom1p in this transition. My thesis work provides novel molecular insights into mRNA export in *S. cerevisiae* and also provides genetic handles and a working mechanistic framework to guide future studies of this fundamental process in eukaryotic gene expression.

Despite the successes just claimed, and much learned by others in the field during the last few years, it is particularly humbling to realize that many of the questions that initially motivated this work remain unresolved. For example, as discussed in the sections above, we still don't know how many export pathways there actually are, the extent to which hnRNP proteins contribute to them, how or indeed whether these pathways relate to each other, and how they are coordinated with other mRNA metabolic pathways and cellular growth control mechanisms. Nor do we know the identity of the substrate for Tom1p, 'Factor X,' that affects mRNA export or the mechanism underlying this effect.

With hindsight, tackling some of these questions were overly ambitious goals for a reverse genetics project focused on *TOM1*. Moreover, if every Ph.D. thesis were to answer unequivocally all the big questions driving the field of study we might have run out of questions long before I arrived on the scene. Indeed, while answering some of these outstanding questions will likely just require additional time at the bench using standard approaches, others are actually difficult to imagine ever answering satisfactorily with existing methods. Nevertheless, it seems that scientists have an incredible

propensity for devising clever new approaches to perform what seemed only yesterday to be impossibly difficult, if not outright impossible. I am confident that those who bear the baton after me will have the fortitude and ingenuity to succeed where the rest of us have thus far not. I eagerly await their discoveries.

Appendix I

Identification of the Ubiquitin-conjugating enzyme, Cdc34p, as a candidate factor in Tom1p dependent-mRNA export

Abstract

To identify proteins in the ubiquitin pathway that might be additional components of the Tom1p-dependent mRNA export pathway, a panel of ubc and proteasome subunit mutants was screened to identify phenocopies of the *Δtom1* polyA RNA accumulation phenotype. Results from this approach indicate that most ubiquitin pathway mutants do not phenocopy *Δtom1* or show any effect on polyA RNA localization. However, temperature sensitive *ubc3/cdc34-1* mutants do show nuclear accumulation of polyA RNA in a punctate pattern at the nuclear periphery, i.e. they phenocopy *Δtom1*. These results underscore the specificity of the *Δtom1* phenotype within the ubiquitin-proteasome pathway. They also indicate that Cdc34/Ubc3p is currently the best candidate for the E2 that acts upstream of Tom1p to mediate ubiquitin transfer.

Introduction

The results described in Chapter 1 provided genetic, cytological and biochemical evidence for the existence of at least two mRNA export pathways. These pathways can be distinguished by the hnRNP proteins involved (Nab2p vs. Npl3p) and a requirement for the nuclear protein Tom1p, a putative ubiquitin ligase. To further dissect these separate mRNA export pathways, I took a genetic approach to identify additional components of the Tom1-dependent mRNA export pathway. Because many other investigators had already looked for suppressors of *tom1* growth defects and this approach had not clarified Tom1p's role in mRNA export (Davey et al., 2000; Sasaki et al., 2000b; Utsugi et al., 1995), I chose instead to focus on the *tom1* transport phenotypes themselves. The *tom1* nuclear transport phenotypes are quite robust, which makes them particularly suitable for visual screening in directed genetic approaches (or with mutant libraries). Redistribution of polyA RNA with Nab2p in a punctate pattern at the nuclear periphery is a striking phenotype that thus far has appeared to be unique to this mutant (Duncan et al., 2000). As a result, the *tom1* mutant is also highly amenable to directed screens for mutants that phenocopy *tom1* or modify its nuclear transport defects. In Chapter 2, I reported the use of genetic epistasis as a method for identifying candidates for additional molecular components of the Tom1-dependent mRNA export pathway. A complementary approach is to screen for other conditionally lethal mutants that phenocopy *Δtom1* transport defects. If the *Δtom1* mRNA export phenotype is relatively rare, as it seems to be, then this approach is likely to be very effective.

Of particular interest to screen were components of the ubiquitin pathway, primarily for two reasons. First, since hect proteins have been shown to receive ubiquitin from UBC/E2 enzymes prior to transfer to the substrate (Pickart, 2001a), it is predicted that a yeast Ubcp will transfer ubiquitin to Tom1p. Since specific Ubcp's interact with specific hect proteins (Pickart, 2001a), it seemed likely there might be a specific Ubc that would interact with Tom1p. Loss of function alleles of this *UBC* should result in phenocopy of *Δtom1*. Second, it was of interest to see whether *Δtom1*-like effects on polyA RNA localization would be commonly associated with mutations of components of the ubiquitin-proteasome pathway, or whether the specificity observed with transport mutants would be observed among mutants in this pathway as well.

This appendix describes the testing of components of the ubiquitin-proteasome pathway for phenocopies of the *Δtom1* polyA RNA accumulation defect. Results from this approach indicate that most ubiquitin pathway mutants do not phenocopy *Δtom1* or show any effect on polyA RNA localization. *ubc3/cdc34-1* mutants (Tyers et al., 1992) do show nuclear accumulation of polyA RNA in a punctate pattern at the nuclear periphery in a pattern similar to *Δtom1*. Thus, Cdc34/Ubc3p is currently the best candidate for the E2 that acts upstream of Tom1p to mediate ubiquitin transfer.

Results

cdc34-1 mutants at 37° C phenocopy *tom1* polyA RNA mislocalization

To identify the Ubc/E2's that might interact with Tom1p I examined a panel of *ubc* mutants (gift of Mark Hochstrasser) for phenocopy of the *tom1* mRNA export phenotype. Exponentially growing *ubc* mutant strains and isogenic wild-type control strains were shifted from room temperature to 37° C. Aliquots from each culture were

removed at 90 and 180 minutes, fixed with formaldehyde and prepared for FISH with an oligo dT50 probe as previously described (Duncan et al., 2000).

The *ubc* mutant collection that was screened included mutant alleles of 10 of the 13 yeast UBCs as well as a double mutant strain with both *ubc4* and *ubc5* deleted. Interestingly, only one strain, *Δrad6/ubc2*, phenocopies *tom1* growth defects: wild type growth at room temperature, but failure to grow as temperature increases. Therefore, one might naively imagine this to be the most likely strain to phenocopy the *tom1* mRNA export defect. However, only two mutants showed an effect on mRNA localization, and *Δrad6/ubc2* was not one of them (Table2). *Cdc34-1*, a temperature sensitive allele of the essential gene *CDC34/UBC3* displayed nuclear accumulation of polyA RNA in about 50% of cells. Importantly, the polyA RNA signal appeared in a punctate pattern of 3-7 dots at the nuclear periphery that was strikingly similar to the *tom1* accumulation pattern, although staining intensity of these dots was substantially diminished relative to *tom1* strains (Figure 1). This result implies that temperature sensitive *cdc34-1* mutants at 37° C phenocopy the *tom1* nuclear polyA RNA mislocalization phenotype. Specific phenocopy of *tom1* by *cdc34-1*, in turn, raises the possibility that the phenotypes both result from decreased ubiquitination of the same target protein.

The only other mutant in the tested collection that showed nuclear accumulation of polyA RNA at 37° C was the temperature-sensitive mutant *ubc9-1*, which showed nuclear accumulation in approximately 2-5% of cells. In contrast to *cdc34-1* and *tom1* mutants, *ubc9* mutants accumulated polyA RNA in a whole-nuclear pattern rather than a punctate pattern at the nuclear periphery (data not shown). Ubc9p is an unconventional UBC, in that it acts as an E2 not for ubiquitin, but rather for the ubiquitin-like protein,

Smt3p (Johnson and Blobel, 1997; Schwarz et al., 1998). A temperature-sensitive allele of *SMT3* (gift of Sue Biggins and Andrew Murray) was essentially indistinguishable from *ubc9* in this assay, showing a very low-penetrance, whole-nuclear accumulation pattern (data not shown).

Cdc34-1 mutants do not phenocopy Nab2-GFP mislocalization seen in *Δtom1*

Tom1 mutants display mislocalization of Nab2p as well as polyA RNA in punctate nuclear-peripheral pattern. Thus, complete phenocopy of *tom1* would be expected to include mislocalization of Nab2p. We therefore assayed *cdc34-1* mutants for effects on Nab2-GFP localization. To our surprise, when *cdc34-1* mutants were shifted to 37° C for 90 minutes or 3 hours, no difference between the mutant and isogenic wild type strains could be detected. This was in contrast to *Δtom1*, which displayed its characteristic redistribution of Nab2-GFP in a punctate pattern at the nuclear periphery (data not shown). We do not believe this implies that the *cdc34-1* phenocopy is not real. Instead, we favor an alternative explanation. We noted in our studies of *kap104 tom1* double mutants (see Chapter 2) that we could detect mRNA mislocalization in many of these cells in a very faint punctate pattern at the nuclear periphery. However, examination of Nab2-GFP in this same strain displayed no difference in distribution from wild type, despite the fact that antibody staining with a monoclonal antibody against Nab2p in *kap104 tom1* double mutants revealed detectable mislocalization. Taken together, this suggests to us that the dT50 assay may be more sensitive than Nab2-GFP localization. As described above, although the pattern of mislocalized mRNA in *cdc34-1* cells at 37° C was strikingly similar to that observed with *tom1* mutants, the intensity of

FISH signal for the mislocalized mRNA was quite low (Figure 1). The reduction in intensity of the *cdc34-1* polyA FISH signal relative to *Δtom1* was similar to that observed with *kap104 tom1* double mutants. For this reason, we favor the idea that the *cdc34-1* phenotype represents a *bona fide tom1* phenocopy. It will be important to test this hypothesis using the anti-Nab2p antibody mAb 3F2.

Two general proteasome mutants *cim3* and *cim5* do not phenocopy *tom1*

An additional question of interest was whether the proteasome plays a role in the Tom1-dependent mRNA export pathway. If this were true, one would predict that a mutant that interfered with proteasome activity would phenocopy *tom1*. In a preliminary effort to test this hypothesis, I examined localization of polyA RNA at 37° C in two general proteasome mutants, *cim3-1* and *cim5-1*(Johnson and Blobel, 1997; Schwarz et al., 1998). These mutants contain temperature-sensitive loss of function alleles of two ATPases in the 19S regulatory subunit of the proteasome. Both have cell cycle defects at the restrictive temperature, among other pleiotropic defects resulting from the loss of protein degradation by the proteasome. In spite of these reported general defects in protein degradation, we observed no detectable difference in polyA RNA distribution between wild type cells and *cim3-1* or *cim5-1* mutants at any timepoint (Table 2). This was true even after cell growth was severely slowed and cell cycle arrest had appeared. These results indicate that two general components of the 26S proteasome are not required for efficient mRNA export and underscore again that the *tom1* defect is not due to general defects in protein turnover or cell cycle progression.

At face value, the results also suggest that the function of Tom1p ubiquitination may be proteasome-independent. However, such a conclusion would be highly

premature. The proteasome contains a variety of redundant factors both in the 20S peptidase subunit and the 19S regulatory subunit. Consequently, it is difficult to find mutants that show highly general defects with a variety of known substrates. Moreover, with existing assays it is impossible to know for sure whether a mutation that appears very general as *cim5* indeed does is actually truly comprehensive. In addition, a final potentially confounding possibility is that the defect in *tom1* could result from an imbalance due to hyper-accumulation of a Tom1p target that is normally degraded by the proteasome. Inactivation of the proteasome might cause a general accumulation of proteins, and could alleviate the imbalance, thereby leading to no apparent accumulation of mRNA in proteasome mutant. Such a scenario would not support the conclusion that the Tom1p function is proteasome independent. Thus, while it would be straightforward and of interest to examine a wider panel of proteasome mutants for *tom1* phenocopies, failure to detect an effect will never be conclusive evidence that the proteasome does not contribute to Tom1-dependent mRNA export. To obtain convincing evidence it would seem necessary to identify the relevant substrate for ubiquitination by Tom1p and to determine how modification with ubiquitin affects the function and/or stability of the substrate protein.

Conclusions

The results described above indicate that mutations that interfere with the function of several major ubiquitin pathway enzymes do not have detectable effects on mRNA export. Rather, punctate accumulation of mRNA at the nuclear periphery is a highly specific nuclear transport phenotype observed to date only in *tom1* and *cdc34-1*. The data also suggest that Cdc34p is an excellent candidate to be the E2 that acts upstream of

or perhaps cooperatively with Tom1p to promote ubiquitination of a target protein which must be modified for efficient export of mRNA. Conclusive demonstration of this will require the identification of a putative substrate and testing of Cdc34p with Tom1p and the substrate in an *in vitro* ubiquitination assay. This represents an exciting and challenging avenue for those who wish to dissect directly the role ubiquitination plays in Tom1p function in mRNA export.

Future directions

Several follow-up experiments are suggested by the results described above. On the genetics front it would be useful to examine other alleles of *CDC34* (Pitluk et al., 1995) for phenocopies of *tom1* in dT50 and other transport processes, in order to determine whether the phenotype is allele-specific, as seems the case now. The identification of additional specific alleles of *CDC34* that phenocopy *tom1* with previously characterized defects may help generate new hypotheses about potential targets and their cellular and molecular functions. As mentioned above, a more comprehensive panel of proteasome mutants would also be worth examining for phenocopy.

Since Cdc34p seems an attractive candidate for the E2 that transfers ubiquitin to Tom1p, it would be useful to try to develop biochemical assays with it. The first order experiment would be to try to detect ubiquitin thioester formation on HA-Tom1p *in vitro* using ^{32}P-labeled (Biederer, et al.) – or perhaps epitope-tagged (Myc) ubiquitin, ATP, purified E1 and purified Cdc34p. HA-tom1-C3235A can be used as a negative control.

Other E2's (e.g. Rad6/ubc2p) can be used as specificity controls in the assay. If Cdc34p-dependent thioester formation can be detected, the next step would be to try to expand the assay for ubiquitin transfer to a specific target protein. This approach has been discussed in detail in the Epilogue. While developing this assay is unlikely to be a trivial undertaking, it is undoubtedly a necessary step to fully understand how a specific branch of the ubiquitin pathway can influence mRNA export.

Materials and Methods

Yeast strains and manipulations

Yeast strains used in this study are listed in Table 1. Unless otherwise noted, standard yeast methods (Guthrie and Fink, 1991) were used for all manipulations. Transformations were performed according to the method of Itoh, et al.

Fluorescence microscopy

Localization of Nab2p using mAb3F2, polyA RNA by dT50 FISH, and Nab2-GFP by live fluorescence microscopy were all performed essentially as described (Duncan et al., 2000, Ch. 1 this volume). Exponentially growing strains in liquid cultures were used for all microscopy experiments. Relevant information on growth conditions and other manipulations are summarized in the text and/or figure legends.

Figure 1: *cdc34-1* mutants accumulate polyA RNA in a punctate pattern at the nuclear periphery

(A) PolyA RNA localization in wild type and *Δtom1* mutant cells at 37°C. Exponentially-growing YEPD cultures of the indicated genotype were shifted from 22° C to 37° C for 90 minutes. Cells were fixed with 5% formaldehyde at 37° C for 1 hour and processed for FISH with an oligo dT50 probe to detect the polyA tail. Cells were examined by epifluorescence microscopy to determine localization of polyA RNA. Images were collected with equivalent exposure times using a CCD camera and processed equivalently using IPLab imaging software. These images are from an independent experiment from those in (B) and (C), and are provided for qualitative comparison only. Cells of the same genotype used as controls for (B) and (C) looked equivalent (not shown).

(B) PolyA RNA localization in *cdc34-1* mutants at 37°C . Exponentially-growing cells were shifted to 37°C for 3 hrs, fixed with formaldehyde for 1 hour, and processed for FISH with an oligo dT50 probe to detect the polyA. tail. Images were collected and processed as in (A).

(C) Same as (B), but an independent field of cells is shown.

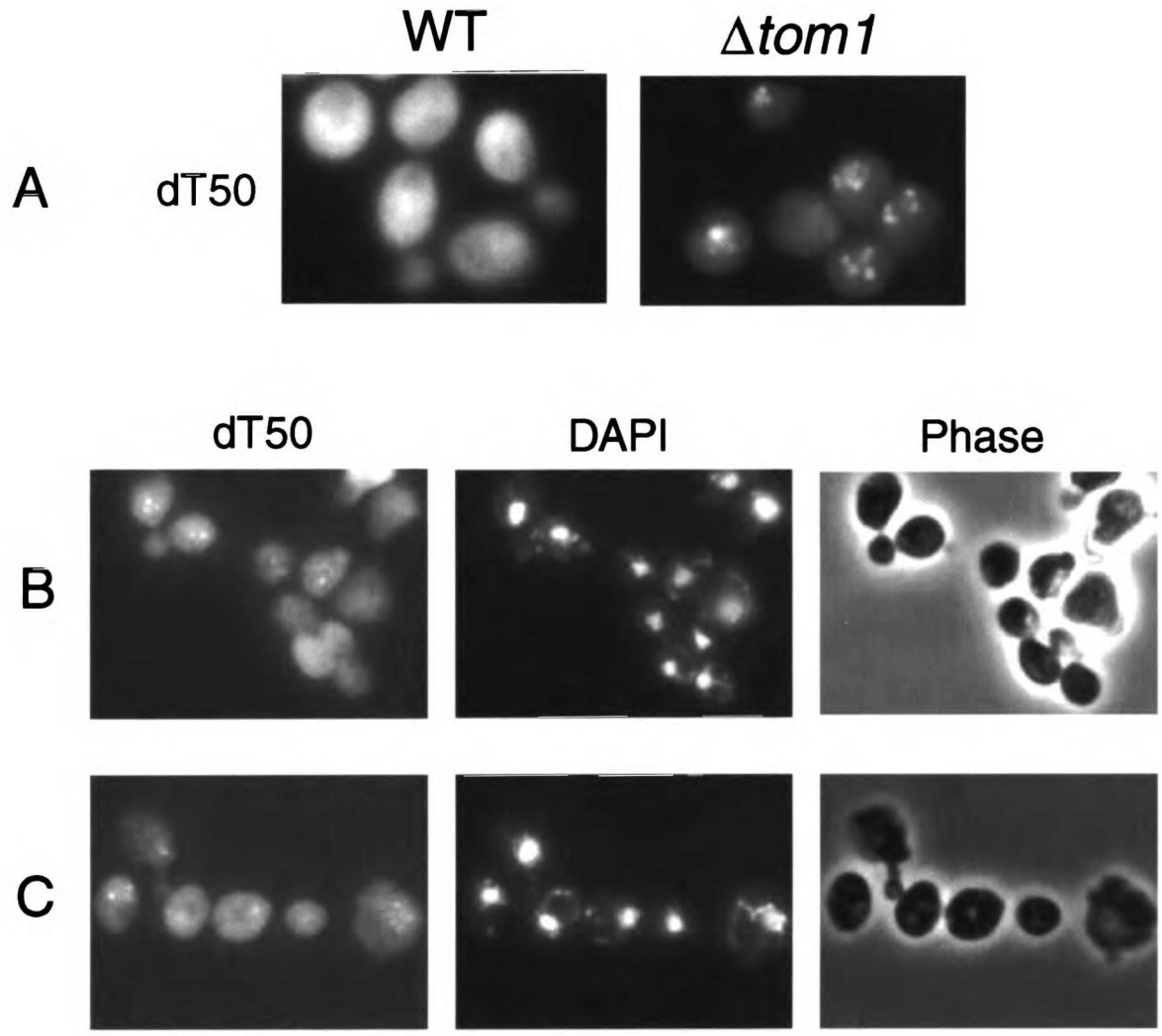

WT
Δtom1
A
dT50
dT50
DAPI
Phase
B
C

Table 1. Yeast strains used in this study.

strain	source	genotype
MHY500 Wild Type for all UBC strains	Mark Hochstrasser	*his3Δ, leu2,ura3-52,lys2,trp1, mat a*
MHY495	"	*Δubc6::HIS3*
MHY497	"	*Δubc1::HIS3*
MHY498	"	*Δubc4::HIS3*
MHY499	"	*Δubc5::LEU2*
MHY507	"	*Δubc7::LEU2*
MHY508	"	*Δubc4::HIS3, Δubc5::LEU2*
MHY599	"	*ubc10(pas2)*
MHY601	"	*Δubc8::URA3*
MHY612	"	*Δubc2/rad6::LEU2*
MHY678 –*CDC34*	"	*ura3-52, leu2-Δ1, gcn4Δ,GCN4,GAL2 Mata*
MHY679	"	Same as 678, but *cdc34-1*
MHY680	"	Same as 678, but *cdc34-2*
CIM3/5	Carl Mann	Same as S288C
cim3-1	"	", but also *cim3-1*
cim5-1	"	", but also *cim5-1*

Appendix II

In vivo crosslinking of a Nuclear Pore Complex Protein to polyA RNA: towards a biochemical assay for mRNA interaction with the Nuclear Pore Complex

Abstract

mRNA export from the nucleus requires interaction of ribonucleoprotein export substrates with the NPC. However, it remains unclear which nucleoporins are contacted during translocation and by which components of the export substrate. This problem is compounded by the dynamic nature of the RNP export substrates themselves, which must change both structure and composition during export. It has long been assumed that adapter proteins, either hnRNP proteins or hnRNP-interacting proteins (e.g. TAP/NXF family members) make protein-protein contacts with the nucleoporins. Alternatively, it is possible that nucleoporins interact directly with mRNA to promote export. In support of this possibility, several nucleoporins have conserved domains that bind RNA *in vitro*. However, in the absence of evidence for RNA binding *in vivo*, the significance of these findings has remained unclear. In this appendix, I describe the use of an *in vivo* crosslinking assay to show that the nucleoporin Nsp1p contacts polyA RNA directly *in vivo*, presumably in the context of mRNA export. This result indicates that this assay can be adapted to study interactions of polyA RNA with NPC components. Thus, examining the efficiency of Nsp1p UV-crosslinking to polyA RNA may enable semi-quantitative analysis of the flux of polyA RNA through the NPC under different conditions. Moreover, identification of a UV-crosslinking interaction between polyA RNA and Nsp1p *in vivo* raises the possibility that direct contact between mRNAs and nucleoporins may play an important mechanistic role in substrate docking and/or translocation during mRNA export.

Introduction

The precise nature and timing of interaction of ribonucleoprotein export substrates with the NPC during transport remains a poorly undertood aspect of mRNA export. Many simpler nuclear transport systems have been characterized in sufficient detail to provide general principles that may relate to the role of nucleoporins in mRNA export. For example, in most, if not all, pathways there is an initial 'docking' step involving formation of a ternary complex between the substrate, transport adapter and the NPC (Gorlich and Kutay, 1999). Docking is followed by translocation through the pore, while substrate release occurs in response to the Ran-nucleotide state. While the exact mechanism of translocation is not known, it is clear that FG-repeat domains of nucleoporins play central roles in the process (Gorlich and Kutay, 1999).

Several general models of translocation have been proposed to account for this fact and incorporate other data. For example, Rexach and co-workers have proposed an FG-affinity gradient model (Rexach and Blobel, 1995). The major caveat with this model is whether rates of transport can be rationalized with sequential series of high affinity interactions. As an alternative, Rout has proposed a 'Brownian Affinity Gate' model (Rout et al., 2000). This model, while physically reasonable, is inferred from structural studies of the NPC and currently lacks direct experimental evidence. Moreover, the Rout model provides a mechanism for selectivity, but little insight into the translocation process through the pore itself. A recent study measured actual rates of translocation in a simple, well-defined system (Ribbeck and Gorlich, 2001). This study indicated that low-affinity interactions underlie the transport pathway for several simple

transport substrates. The lower bound for transport substrate-NPC interactions would need to be in the micromolar range to be consistent with their data. This contrasts with the other models where high affinity interactions are proposed to be the basis of selective translocation.

mRNA presents an additional challenge to these kinds of studies because the precise composition of the transport substrate is not clear (Zenklusen and Stutz, 2001). Moreover, available evidence suggests that the substrate itself changes significantly during the transport process, probably at multiple stages before, during and after translocation itself (Daneholt, 1997; Daneholt, 1999; Daneholt, 2001). Data that I have presented in Chapter 1 suggests the existence of multiple mRNA export pathways in yeast. This idea has recently been supported in mammalian cells as well (Gallouzi, 2001). Thus, on top of the low-affinity and transient interactions proposed to mediate NPC translocation by Ribbeck and Goerlich, mRNP translocation is likely to vary depending on the preferred pathway for a given mRNA, and is likely to be intimately coupled to dynamic alterations in proteins associated with the export substrate RNP.

To address a problem featuring such dynamic rearrangements, it would be ideal to have a biochemical assay that could capture low affinity, transient interactions,and would not be sensitive to disruption of the cell and mixing of components during lysis. *In vivo* crosslinking has these attributes and therefore appears particularly attractive in this case. UV light-induced photochemical crosslinking is a time-honored technique for identification of proteins that are associated with RNA species of interest *in vivo*(Mayrand and Pederson, 1981; Mayrand et al., 1981; van Venrooij et al., 1981; Wagenmakers et al., 1980). The principle of photochemical crosslinking of protein to

RNA is that UV light of sufficient intensity will generate highly reactive RNA species that will react indiscriminately with whatever molecules with which the RNA is in stable, direct contact(Pinol-Roma et al., 1989a). Because the crosslinking distance for photochemical approach is extremely short, UV crosslinking is generally believed to be more stringent than the use of bifunctional crosslinking agents.

The assay developed by Adam et al. (Adam et al., 1986)and later used primarily by Swanson and coworkers can be used to measure the amount of a particular RNP protein bound to polyA RNA *in vivo* (Krebber et al., 1999). We have adapted this assay for use in our lab for this purpose and find it to be somewhat cumbersome, but generally robust (Duncan et al., 2000; Gilbert et al., 2001); WVG, KED and CG, unpublished results). Hurt and coworkers had previously attempted to identify nucleoporin interactions using this approach without success (Segref et al., 1997). Here I report my finding that the nucleoporin Nsp1p (Nehrbass et al., 1990) can be UV cross-linked to polyA RNA *in vivo*. I very briefly discuss implications of the result and propose a short list of follow-up experiments.

Result and Discussion

The nucleoporin Nsp1p interacts with mRNA *in vivo*

In the process of determining the levels of yeast hnRNP proteins associated with polyA RNA using a UV-crosslinking/oligo-dT selection method (Figure 1A), it occurred to me that this might be a useful assay to identify interactions with other proteins that may come in close contact with polyA RNA during during mRNA export, for example nucleoporins. Crosslinking to polyA RNA had been tested before for the nucleoporins Nup57 and Nup85 by Hurt and colleagues (Segref et al., 1997), who were unable to

detect crosslinking of these nucleoporins by western blotting using standard ECL reagents. However, when I performed the assay with standard ECL reagents to detect the hnRNP proteins, I realized that the assay was not particularly sensitive even with these known, highly-abundant RNA binding proteins. I therefore reasoned that the Hurt lab's inability to detect interactions between nucleoporins and polyA RNA could be due to lack of sensitivity of the assay rather than absence of such interactions. With this in mind, I performed the *in vivo* crosslinking assay (Figure 1A) and probed the western blot for the relatively abundant nucleoporin Nsp1p (Rout et al., 2000) using a well-characterized monoclonal antibody mAb32D6 (gift from John Aris). To boost sensitivity, I used a quaternary antibody sandwich (see methods) and the Super Signal West Femto reagent (Pierce), claimed by the manufacturer to be 500-1000x more sensitive than standard ECL.

With the enhanced sensitivity provided by optimizing the assay, I was able to detect signal with mAb 32D6 that co-purified with polyA RNA in a crosslinking-dependent manner (Figure 1B, compare lanes 5 and 6). The recognized protein migrates at the size expected to be Nsp1p based on previously published reports (109 kD, original Hurt paper). Three additional lines of evidence suggest that the recognized protein is Nsp1p, rather than a fortuitous cross-reacting species. First, the protein recognized in the crosslinked lanes co-migrated with the primary band recognized in the diluted flow-through samples of both crosslinked and non-crosslinked control samples (Figure 1B, lanes 1 and 2). Second, all other bands were smaller than the primary band, and were recognized significantly less well than the one that co-migrates with the crosslinked band. Third, the high degree of proteolysis observed for the flowthrough lanes has been noted

for Nsp1p in several previous studies (Nehrbass et al., 1990). Thus, these data suggest that Nsp1p contacts mRNA directly *in vivo*, presumably during mRNA export. A similar assay in oocytes suggested that the vertebrate homolog of Nsp1p, p62 was a significant UV crosslinking species in this system (Dargemont et al., 1995). Thus, interaction of this class of nucleoporin with mRNA during export may be a conserved aspect of translocation.

A transient interaction of an NPC component with polyA RNA during export would be expected to give a lower crosslinking efficiency than would be seen for an abundant hnRNP protein that interacts with nuclear mRNAs. From the data described here it is clear that the the crosslinking yield is 100-200x lower than that for Npl3p or Nab2p. Crosslinking efficiencies can vary because of the proteins involved rather than the abundance of complexes (Pinol-Roma et al., 1989a). Nevertheless, the reduction in crosslinking yield for Nsp1p is consistent with expectation.

The Nsp1p-polyA crosslinking efficiency appears unaffected in *Δtom1*

We have previously reported that *Δtom1* inhibits mRNA export and causes polyA RNA to redistribute to the nuclear periphery (Duncan et al., 2000, Chapter 1). This suggested that *Δtom1* might affect the level of polyA RNA interacting with Nsp1p. For example, if mRNA export is inhibited upstream of Nsp1p interaction in *Δtom1*, the level of Nsp1p crosslinked to polyA RNA would be expected to decrease. Conversely, accumulation at the nuclear periphery in *Δtom1* might reflect accumulation of mRNA in association with Nsp1p, in which case the level of Nsp1p detected would be expected to

increase. We detected no difference in the level of Nsp1p in crosslinked eluates from wild type and Δ*tom1* strains (Figure 1B).

There are a number of possible interpretations for this result. One possibility is that there is no significant decrease in mRNA export in *Δtom1*, which seems inconsistent with our cytological observations (Duncan et al., 2000); Chapter 1 and 2). Alternatively, Nsp1p may be primarily involved in an mRNA export pathway that is distinct from the one that is affected in *Δtom1*. It is also conceivable that Δ*tom1* inhibits export at an Nsp1-associated step, but the Nsp1p binding sites were already saturated before inhibition. Finally, it may simply be that the assay is not able to measure changes in mRNA export rates for whatever reason. Before interpreting the absence of a difference between wild type and Δ*tom1* in this assay, it will be important to determine whether the assay is robust by testing conditions that would be expected to massively reduce mRNA export also reduce Nsp1p crosslinking (see below).

Future Directions

The detection of Nsp1p crosslinked to polyA-RNA suggests that this nucleoporin may be directly contacting mRNA during export. Initial follow-up experiments should aim to establish that the crosslink is dependent on ongoing mRNA synthesis and export using shifts of conditional mutants and assaying for decreased Nsp1p signal in the eluates from crosslinked samples. In a sense these are experiments, since mutants we imagine to affect all mRNA export may not affect all export and may not even target mRNA to an Nsp1p-mediated pathway. Next, specificity for Nsp1p should be examined. Protein-A tagged nucleoporins may prove useful for this analysis. Nucleoporins that do not show mRNA export phenotypes might be expected not to crosslink. With an understanding of

which nups crosslink, and demonstrated dependence on mRNA synthesis and export, mutants that might affect specific pathways can be analyzed to see how they affect the different crosslinks. While this last idea will be quite labor intensive, it seems of enough interest to warrant this effort.

An additional exciting prospect for this method would be to 'reverse' the assay: isolating protein A- (or otherwise tagged) Nsp1p and identifying the associated mRNA species for analysis with a genomic technology, e.g. microarrays. In principle, this would enable characterization of the mRNA 'Exportome'under any condition of interest. This approach might be better suited to a quantitative array technology, rather than the commonly used differential display approach, since the choice of an appropriate reference sample and significance threshold for Nsp1-crosslinked samples seems likely to be difficult, given the inefficiency of the protein crosslinking. A reversible crosslink such as formaldehyde that may not interfere with polymerase activity would presumably also be preferable for this assay.

Materials and Methods

Yeast strains

The construction of yKD99 (*TOM1*) and yKD100 (*Δtom1*) in the YPH399 strain background has been previously described ((Duncan et al., 2000); and Chapter 1 this volume). Standard techniques for manipulating yeast were used as previously described (Duncan et al., 2000).

In vivo UV cross-linking assay

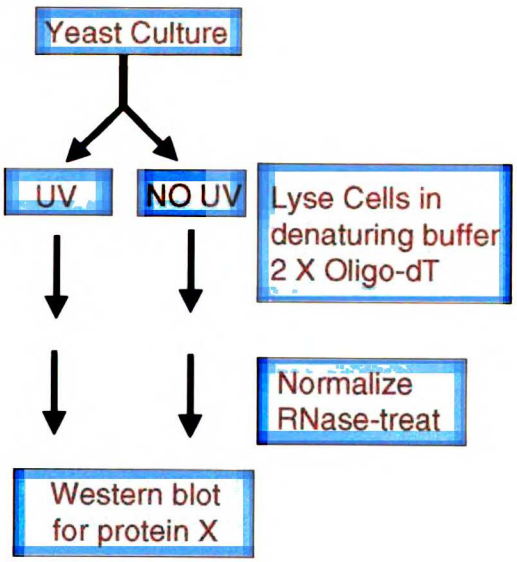
UV crosslinking *in vivo* and subsequent purification of proteins covalently attached to polyA RNA was performed essentially as described (Duncan et al., 2000); and Chapter1 this volume), with minor deviations. Importantly, a 'quaternary' antibody sandwich was used for the western blotting with mAb32D6 (anti-Nsp1p). Antibodies and the corresponding dilutions for the quaternary sandwich were: mab32D6 (1/10,000), rabbit-anti mouse unconjugated (Pierce, 1/5000), goat anti-rabbit-HRP and goat anti-mouse- HRP (Bio-Rad, 1/25,000), Peroxidase anti-Peroxidase (Sigma, 1/50,000). Check dilutions! A 1/50 dilution of the flow-through relative to immunoblots for crosslinked hnRNP proteins was also necessary so that flow-through lanes would be at a similar signal intensity to the crosslinked samples.

Figure 1. Nsp1p can be UV crosslinked to polyA RNA *in vivo*.

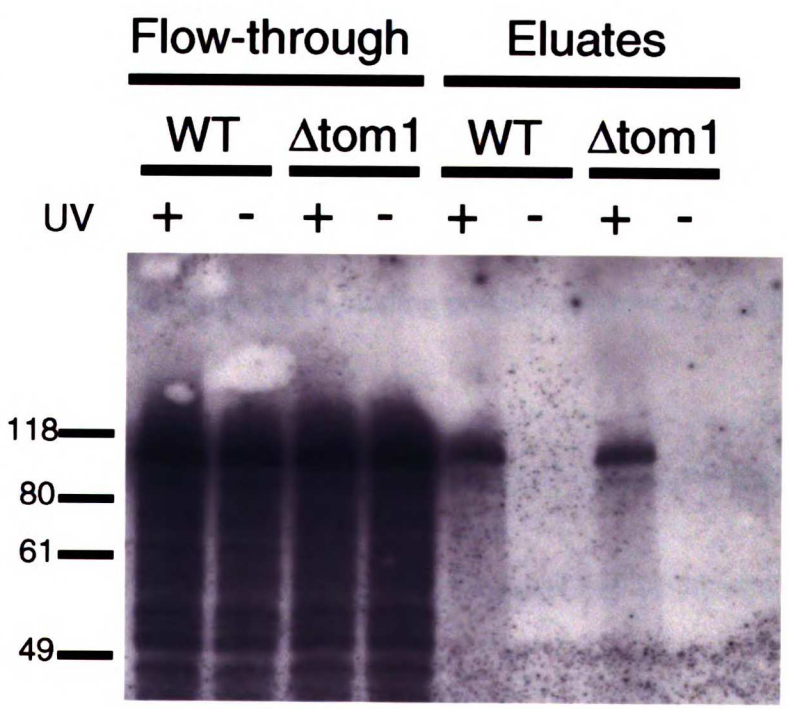
(A) Flowchart depicting the UV-crosslinked RNP purification protocol. See methods for details.

(B) Immunoblot detection of Nsp1p crosslinked to polyA RNA *in vivo*. 2x500ml of strains yKD99-2B (WT) or yKD100-2D (*Δtom1*) exponentially growing at room temperature (22° C) were rapidly mixed with 500ml each of 52° C YEPD medium and grown for 2 hours at 37° C on a shaker. Eluates from the second oligo-dT cellulose column (lanes 5-8, on the right) or ~$5x10^{-5}$ of the flow-through material from the first oligo-dT cellulose column (lanes 1-4, on the left) were subject to SDS-PAGE, blotted to a nitrocellulose filter, and probed with monoclonal antibody 32D6, which recognizes Nsp1p, followed by secondary, tertiary and quaternary antibodies. Note the presence of bands matching full-length Nsp1p in the eluates only for crosslinked samples. Note also the similar intensities of bands in the wild type and *Δtom1* lanes.

A


Yeast Culture
UV
NO UV
Lyse Cells in denaturing buffer 2 X Oligo-dT
Normalize RNase-treat
Western blot for protein X

B


Flow-through
Eluates
WT
Δtom1
WT
Δtom1
UV
+ - + - + - + -
118
80
61
49

REFERENCES

Abdul-Manan, N., and Williams, K. R. (1996). hnRNP A1 binds promiscuously to oligoribonucleotides: utilization of random and homo-oligonucleotides to discriminate sequence from base-specific binding. Nucleic Acids Res *24*, 4063-4070.

Adam, S. A., Nakagawa, T., Swanson, M. S., Woodruff, T. K., and Dreyfuss, G. (1986). mRNA polyadenylate-binding protein: gene isolation and sequencing and identification of a ribonucleoprotein consensus sequence. Mol Cell Biol *6*, 2932-2943.

Aitchison, J. D., Blobel, G., and Rout, M. P. (1996). Kap104p: a karyopherin involved in the nuclear transport of messenger RNA binding proteins. Science *274*, 624-627.

Amberg, D. C., Goldstein, A. L., and Cole, C. N. (1992). Isolation and characterization of RAT1: an essential gene of Saccharomyces cerevisiae required for the efficient nucleocytoplasmic trafficking of mRNA. Genes Dev *6*, 1173-1189.

Anderson, J. T., Wilson, S. M., Datar, K. V., and Swanson, M. S. (1993). NAB2: a yeast nuclear polyadenylated RNA-binding protein essential for cell viability. Mol Cell Biol *13*, 2730-2741.

Aparicio, O. M., Weinstein, D. M., and Bell, S. P. (1997). Components and dynamics of DNA replication complexes in S. cerevisiae: redistribution of MCM proteins and Cdc45p during S phase. Cell *91*, 59-69.

Attaix, D., Combaret, L., Pouch, M. N., and Taillandier, D. (2001). Regulation of proteolysis. Curr Opin Clin Nutr Metab Care *4*, 45-49.

Azad, A. K., Tani, T., Shiki, N., Tsuneyoshi, S., Urushiyama, S., and Ohshima, Y. (1997). Isolation and molecular characterization of mRNA transport mutants in Schizosaccharomyces pombe. Mol Biol Cell *8*, 825-841.

Beaudenon, S. L., Huacani, M. R., Wang, G., McDonnell, D. P., and Huibregtse, J. M. (1999). Rsp5 ubiquitin-protein ligase mediates DNA damage-induced degradation of the

large subunit of RNA polymerase II in Saccharomyces cerevisiae. Mol Cell Biol *19*, 6972-6979.

Bertolaet, B. L., Clarke, D. J., Wolff, M., Watson, M. H., Henze, M., Divita, G., and Reed, S. I. (2001). UBA domains of DNA damage-inducible proteins interact with ubiquitin. Nat Struct Biol *8*, 417-422.

Bochtler, M., Ditzel, L., Groll, M., Hartmann, C., and Huber, R. (1999). The proteasome. Annu Rev Biophys Biomol Struct *28*, 295-317.

Bonifaci, N., Moroianu, J., Radu, A., and Blobel, G. (1997). Karyopherin beta2 mediates nuclear import of a mRNA binding protein. Proc Natl Acad Sci U S A *94*, 5055-5060.

Bossie, M. A., DeHoratius, C., Barcelo, G., and Silver, P. (1992). A mutant nuclear protein with similarity to RNA binding proteins interferes with nuclear import in yeast. Mol Biol Cell *3*, 875-893.

Bousquet-Antonelli, C., Presutti, C., and Tollervey, D. (2000). Identification of a regulated pathway for nuclear pre-mRNA turnover. Cell *102*, 765-775.

Braun, I. C., Herold, A., Rode, M., Conti, E., and Izaurralde, E. (2001). Overexpression of TAP/p15 heterodimers bypasses nuclear retention and stimulates nuclear mRNA export. J Biol Chem *276*, 20536-20543.

Brodsky, A. S., and Silver, P. A. (2000). Pre-mRNA processing factors are required for nuclear export. Rna *6*, 1737-1749.

Brogna, S. (2001). Pre-mRNA processing: insights from nonsense. Curr Biol *11*, R838-841.

Burd, C. G., and Dreyfuss, G. (1994). RNA binding specificity of hnRNP A1: significance of hnRNP A1 high-affinity binding sites in pre-mRNA splicing. Embo J *13*, 1197-1204.

Buvoli, M., Cobianchi, F., Biamonti, G., and Riva, S. (1990). Recombinant hnRNP protein A1 and its N-terminal domain show preferential affinity for oligodeoxynucleotides homologous to intron/exon acceptor sites. Nucleic Acids Res *18*, 6595-6600.

Calado, A., Kutay, U., Kuhn, U., Wahle, E., and Carmo-Fonseca, M. (2000). Deciphering the cellular pathway for transport of poly(A)-binding protein II. Rna *6*, 245-256.

Carmeliet, P., Dor, Y., Herbert, J. M., Fukumura, D., Brusselmans, K., Dewerchin, M., Neeman, M., Bono, F., Abramovitch, R., Maxwell, P., *et al.* (1998). Role of HIF-1alpha in hypoxia-mediated apoptosis, cell proliferation and tumour angiogenesis. Nature *394*, 485-490.

Chen, C. Y., and Shyu, A. B. (1995). AU-rich elements: characterization and importance in mRNA degradation. Trends Biochem Sci *20*, 465-470.

Chen, C. Y., Xu, N., and Shyu, A. B. (1995). mRNA decay mediated by two distinct AU-rich elements from c-fos and granulocyte-macrophage colony-stimulating factor

transcripts: different deadenylation kinetics and uncoupling from translation. Mol Cell Biol *15*, 5777-5788.

Chen, Z. J., Parent, L., and Maniatis, T. (1996). Site-specific phosphorylation of IkappaBalpha by a novel ubiquitination-dependent protein kinase activity. Cell *84*, 853-862.

Choi, Y. D., and Dreyfuss, G. (1984). Isolation of the heterogeneous nuclear RNA-ribonucleoprotein complex (hnRNP): a unique supramolecular assembly. Proc Natl Acad Sci U S A *81*, 7471-7475.

Ciechanover, A. (1994). The ubiquitin-mediated proteolytic pathway: mechanisms of action and cellular physiology. Biol Chem Hoppe Seyler *375*, 565-581.

Ciechanover, A., Orian, A., and Schwartz, A. L. (2000). Ubiquitin-mediated proteolysis: biological regulation via destruction. Bioessays *22*, 442-451.

Conti, E., and Izaurralde, E. (2001). Nucleocytoplasmic transport enters the atomic age. Curr Opin Cell Biol *13*, 310-319.

Daneholt, B. (1997). A look at messenger RNP moving through the nuclear pore. Cell *88*, 585-588.

Daneholt, B. (1999). Pre-mRNP particles: From gene to nuclear pore. Curr Biol *9*, R412-415.

Daneholt, B. (2001). Assembly and transport of a premessenger RNP particle. Proc Natl Acad Sci U S A *98*, 7012-7017.

Dargemont, C., and Kuhn, L. C. (1992). Export of mRNA from microinjected nuclei of Xenopus laevis oocytes. J Cell Biol *118*, 1-9.

Dargemont, C., Schmidt-Zachmann, M. S., and Kuhn, L. C. (1995). Direct interaction of nucleoporin p62 with mRNA during its export from the nucleus. J Cell Sci *108 (Pt 1)*, 257-263.

Das, B., Guo, Z., Russo, P., Chartrand, P., and Sherman, F. (2000). The role of nuclear cap binding protein Cbc1p of yeast in mRNA termination and degradation. Mol Cell Biol *20*, 2827-2838.

Davey, M., Hannam, C., Wong, C., and Brandl, C. J. (2000). The yeast peptidyl proline isomerases FPR3 and FPR4, in high copy numbers, suppress defects resulting from the absence of the E3 ubiquitin ligase TOM1. Mol Gen Genet *263*, 520-526.

Davis, L. I. (1995). The nuclear pore complex. Annu Rev Biochem *64*, 865-896.

del Olmo, M., Mizrahi, N., Gross, S., and Moore, C. L. (1997). The Uba2 and Ufd1 proteins of Saccharomyces cerevisiae interact with poly(A) polymerase and affect the polyadenylation activity of cell extracts. Mol Gen Genet *255*, 209-218.

del Pozo, J. C., and Estelle, M. (2000). F-box proteins and protein degradation: an emerging theme in cellular regulation. Plant Mol Biol *44*, 123-128.

Deshaies, R. J. (1999). SCF and Cullin/Ring H2-based ubiquitin ligases. Annu Rev Cell Dev Biol *15*, 435-467.

Devaux, F., Marc, P., and Jacq, C. (2001). Transcriptomes, transcription activators and microarrays. FEBS Lett *498*, 140-144.

Doye, V., Wepf, R., and Hurt, E. C. (1994). A novel nuclear pore protein Nup133p with distinct roles in poly(A)+ RNA transport and nuclear pore distribution. Embo J *13*, 6062-6075.

Dreyfuss, G. (1986). Structure and function of nuclear and cytoplasmic ribonucleoprotein particles. Annu Rev Cell Biol *2*, 459-498.

Dreyfuss, G., Choi, Y. D., and Adam, S. A. (1989). The ribonucleoprotein structures along the pathway of mRNA formation. Endocr Res *15*, 441-474.

Dreyfuss, G., Matunis, M. J., Pinol-Roma, S., and Burd, C. G. (1993). hnRNP proteins and the biogenesis of mRNA. Annu Rev Biochem *62*, 289-321.

Duncan, K., Umen, J. G., and Guthrie, C. (2000). A putative ubiquitin ligase required for efficient mRNA export differentially affects hnRNP transport. Curr Biol *10*, 687-696.

Fabre, E., Boelens, W. C., Wimmer, C., Mattaj, I. W., and Hurt, E. C. (1994). Nup145p is required for nuclear export of mRNA and binds homopolymeric RNA in vitro via a novel conserved motif. Cell *78*, 275-289.

Fan, X. C., and Steitz, J. A. (1998). HNS, a nuclear-cytoplasmic shuttling sequence in HuR. Proc Natl Acad Sci U S A *95*, 15293-15298.

Fath, S., Milkereit, P., Podtelejnikov, A. V., Bischler, N., Schultz, P., Bier, M., Mann, M., and Tschochner, H. (2000). Association of yeast RNA polymerase I with a nucleolar substructure active in rRNA synthesis and processing. J Cell Biol *149*, 575-590.

Flach, J., Bossie, M., Vogel, J., Corbett, A., Jinks, T., Willins, D. A., and Silver, P. A. (1994). A yeast RNA-binding protein shuttles between the nucleus and the cytoplasm. Mol Cell Biol *14*, 8399-8407.

Fortes, P., Inada, T., Preiss, T., Hentze, M. W., Mattaj, I. W., and Sachs, A. B. (2000). The yeast nuclear cap binding complex can interact with translation factor eIF4G and mediate translation initiation. Mol Cell *6*, 191-196.

Frank, D. N. (1993) Genetic Analysis of Second-Step pre-mRNA Splicing Factors in *Saccharomyces cerevisiae*, PhD Dissertation, University of California, San Francisco.

Fridell, R. A., Truant, R., Thorne, L., Benson, R. E., and Cullen, B. R. (1997). Nuclear import of hnRNP A1 is mediated by a novel cellular cofactor related to karyopherin-beta. J Cell Sci *110 (Pt 11)*, 1325-1331.

Gallouzi, I. a. S., J (2001). Delineation of mRNA Export Pathways by the Use of Cell-Permeable Peptides. Science *294*, 1895-1901.

Gilbert, W., Siebel, C. W., and Guthrie, C. (2001). Phosphorylation by Sky1p promotes Npl3p shuttling and mRNA dissociation. Rna *7*, 302-313.

Gonzalez, C. I., Ruiz-Echevarria, M. J., Vasudevan, S., Henry, M. F., and Peltz, S. W. (2000). The yeast hnRNP-like protein Hrp1/Nab4 marks a transcript for nonsense-mediated mRNA decay. Mol Cell *5*, 489-499.

Gorlach, M., Burd, C. G., and Dreyfuss, G. (1994). The determinants of RNA-binding specificity of the heterogeneous nuclear ribonucleoprotein C proteins. J Biol Chem *269*, 23074-23078.

Gorlach, M., Burd, C. G., Portman, D. S., and Dreyfuss, G. (1993). The hnRNP proteins. Mol Biol Rep *18*, 73-78.

Gorlich, D. (1997). Nuclear protein import. Curr Opin Cell Biol *9*, 412-419.

Gorlich, D., and Kutay, U. (1999). Transport between the cell nucleus and the cytoplasm. Annu Rev Cell Dev Biol *15*, 607-660.

Gorlich, D., and Laskey, R. A. (1995). Roles of importin in nuclear protein import. Cold Spring Harb Symp Quant Biol *60*, 695-699.

Hamm, J., and Mattaj, I. W. (1990). Monomethylated cap structures facilitate RNA export from the nucleus. Cell *63*, 109-118.

Hartwell, L. H., McLaughlin, C. S., and Warner, J. R. (1970). Identification of ten genes that control ribosome formation in yeast. Mol Gen Genet *109*, 42-56.

Hecht, A., Strahl-Bolsinger, S., and Grunstein, M. (1999). Mapping DNA interaction sites of chromosomal proteins. Crosslinking studies in yeast. Methods Mol Biol *119*, 469-479.

Henriquez, R., Blobel, G., and Aris, J. P. (1990). Isolation and sequencing of NOP1. A yeast gene encoding a nucleolar protein homologous to a human autoimmune antigen. J Biol Chem *265*, 2209-2215.

Henry, M., Borland, C. Z., Bossie, M., and Silver, P. A. (1996). Potential RNA binding proteins in Saccharomyces cerevisiae identified as suppressors of temperature-sensitive mutations in NPL3. Genetics *142*, 103-115.

Hershko, A., and Ciechanover, A. (1998). The ubiquitin system. Annu Rev Biochem *67*, 425-479.

Hicke, L. (1999). Gettin' down with ubiquitin: turning off cell-surface receptors, transporters and channels. Trends Cell Biol *9*, 107-112.

Hicke, L. (2001). Protein regulation by monoubiquitin. Nat Rev Mol Cell Biol *2*, 195-201.

Hilleren, P., McCarthy, T., Rosbash, M., Parker, R., and Jensen, T. H. (2001). Quality control of mRNA 3'-end processing is linked to the nuclear exosome. Nature *413*, 538-542.

Hilleren, P., and Parker, R. (2001). Defects in the mRNA export factors Rat7p, Gle1p, Mex67p, and Rat8p cause hyperadenylation during 3'-end formation of nascent transcripts. Rna *7*, 753-764.

Hochstrasser, M. (1996). Ubiquitin-dependent protein degradation. Annu Rev Genet *30*, 405-439.

Hochstrasser, M., Johnson, P. R., Arendt, C. S., Amerik, A., Swaminathan, S., Swanson, R., Li, S. J., Laney, J., Pals-Rylaarsdam, R., Nowak, J., and Connerly, P. L. (1999). The Saccharomyces cerevisiae ubiquitin-proteasome system. Philos Trans R Soc Lond B Biol Sci *354*, 1513-1522.

Hofmann, K., and Bucher, P. (1996). The UBA domain: a sequence motif present in multiple enzyme classes of the ubiquitination pathway. Trends Biochem Sci *21*, 172-173.

Huibregtse, J. M., Scheffner, M., Beaudenon, S., and Howley, P. M. (1995a). A family of proteins structurally and functionally related to the E6-AP ubiquitin-protein ligase. Proc Natl Acad Sci U S A *92*, 2563-2567.

Huibregtse, J. M., Scheffner, M., Beaudenon, S., and Howley, P. M. (1995b). A family of proteins structurally and functionally related to the E6-AP ubiquitin-protein ligase. Proc Natl Acad Sci U S A *92*, 5249.

Huibregtse, J. M., Scheffner, M., and Howley, P. M. (1994). E6-AP directs the HPV E6-dependent inactivation of p53 and is representative of a family of structurally and functionally related proteins. Cold Spring Harb Symp Quant Biol *59*, 237-245.

Hurt, E., Strasser, K., Segref, A., Bailer, S., Schlaich, N., Presutti, C., Tollervey, D., and Jansen, R. (2000). Mex67p mediates nuclear export of a variety of RNA polymerase II transcripts. J Biol Chem *275*, 8361-8368.

Iborra, F. J., Jackson, D. A., and Cook, P. R. (2001). Coupled transcription and translation within nuclei of mammalian cells. Science *293*, 1139-1142.

Iovine, M. K., Watkins, J. L., and Wente, S. R. (1995). The GLFG repetitive region of the nucleoporin Nup116p interacts with Kap95p, an essential yeast nuclear import factor. J Cell Biol *131*, 1699-1713.

Ishigaki, Y., Li, X., Serin, G., and Maquat, L. E. (2001). Evidence for a pioneer round of mRNA translation: mRNAs subject to nonsense-mediated decay in mammalian cells are bound by CBP80 and CBP20. Cell *106*, 607-617.

Izaurralde, E., Jarmolowski, A., Beisel, C., Mattaj, I. W., Dreyfuss, G., and Fischer, U. (1997). A role for the M9 transport signal of hnRNP A1 in mRNA nuclear export. J Cell Biol *137*, 27-35.

Izaurralde, E., and Mattaj, I. W. (1995). RNA export. Cell *81*, 153-159.

Jackson, P. K., Eldridge, A. G., Freed, E., Furstenthal, L., Hsu, J. Y., Kaiser, B. K., and Reimann, J. D. (2000). The lore of the RINGs: substrate recognition and catalysis by ubiquitin ligases. Trends Cell Biol *10*, 429-439.

Jackson, V. (1978). Studies on histone organization in the nucleosome using formaldehyde as a reversible cross-linking agent. Cell *15*, 945-954.

Jackson, V. (1999). Formaldehyde cross-linking for studying nucleosomal dynamics. Methods *17*, 125-139.

Jarmolowski, A., Boelens, W. C., Izaurralde, E., and Mattaj, I. W. (1994). Nuclear export of different classes of RNA is mediated by specific factors. J Cell Biol *124*, 627-635.

Jensen, T. H., Boulay, J., Rosbash, M., and Libri, D. (2001a). The DECD box putative ATPase Sub2p is an early mRNA export factor. Curr Biol *11*, 1711-1715.

Jensen, T. H., Patricio, K., McCarthy, T., and Rosbash, M. (2001b). A block to mRNA nuclear export in S. cerevisiae leads to hyperadenylation of transcripts that accumulate at the site of transcription. Mol Cell *7*, 887-898.

Johnson, E. S., and Blobel, G. (1997). Ubc9p is the conjugating enzyme for the ubiquitin-like protein Smt3p. J Biol Chem *272*, 26799-26802.

Kadowaki, T., Chen, S., Hitomi, M., Jacobs, E., Kumagai, C., Liang, S., Schneiter, R., Singleton, D., Wisniewska, J., and Tartakoff, A. M. (1994). Isolation and characterization of Saccharomyces cerevisiae mRNA transport-defective (mtr) mutants. J Cell Biol *126*, 649-659.

Kadowaki, T., Zhao, Y., and Tartakoff, A. M. (1992). A conditional yeast mutant deficient in mRNA transport from nucleus to cytoplasm. Proc Natl Acad Sci U S A *89*, 2312-2316.

Kaiser, P., Flick, K., Wittenberg, C., and Reed, S. I. (2000). Regulation of transcription by ubiquitination without proteolysis: Cdc34/SCF(Met30)-mediated inactivation of the transcription factor Met4. Cell *102*, 303-314.

Katahira, J., Strasser, K., Podtelejnikov, A., Mann, M., Jung, J. U., and Hurt, E. (1999). The Mex67p-mediated nuclear mRNA export pathway is conserved from yeast to human. Embo J *18*, 2593-2609.

Kataoka, N., Yong, J., Kim, V. N., Velazquez, F., Perkinson, R. A., Wang, F., and Dreyfuss, G. (2000). Pre-mRNA splicing imprints mRNA in the nucleus with a novel RNA-binding protein that persists in the cytoplasm. Mol Cell *6*, 673-682.

Kessler, M. M., Henry, M. F., Shen, E., Zhao, J., Gross, S., Silver, P. A., and Moore, C. L. (1997). Hrp1, a sequence-specific RNA-binding protein that shuttles between the nucleus and the cytoplasm, is required for mRNA 3'-end formation in yeast. Genes Dev *11*, 2545-2556.

Kim, V. N., Yong, J., Kataoka, N., Abel, L., Diem, M. D., and Dreyfuss, G. (2001). The Y14 protein communicates to the cytoplasm the position of exon-exon junctions. Embo J *20*, 2062-2068.

Kipreos, E. T., and Pagano, M. (2000). The F-box protein family. Genome Biol *1*, REVIEWS3002.

Kistler, A. L., and Guthrie, C. (2001). Deletion of MUD2, the yeast homolog of U2AF65, can bypass the requirement for sub2, an essential spliceosomal ATPase. Genes Dev *15*, 42-49.

Kondoh, H., Yuasa, T., and Yanagida, M. (2000). Mis3 with a conserved RNA binding motif is essential for ribosome biogenesis and implicated in the start of cell growth and S phase checkpoint. Genes Cells *5*, 525-541.

Krebber, H., Taura, T., Lee, M. S., and Silver, P. A. (1999). Uncoupling of the hnRNP Npl3p from mRNAs during the stress-induced block in mRNA export. Genes Dev *13*, 1994-2004.

Krecic, A. M., and Swanson, M. S. (1999). hnRNP complexes: composition, structure, and function. Curr Opin Cell Biol *11*, 363-371.

Kretzner, L., Krol, A., and Rosbash, M. (1990). Saccharomyces cerevisiae U1 small nuclear RNA secondary structure contains both universal and yeast-specific domains. Proc Natl Acad Sci U S A *87*, 851-855.

Laroia, G., Cuesta, R., Brewer, G., and Schneider, R. J. (1999). Control of mRNA decay by heat shock-ubiquitin-proteasome pathway. Science *284*, 499-502.

Le Hir, H., Gatfield, D., Izaurralde, E., and Moore, M. J. (2001). The exon-exon junction complex provides a binding platform for factors involved in mRNA export and nonsense-mediated mRNA decay. Embo J *20*, 4987-4997.

Le Hir, H., Izaurralde, E., Maquat, L. E., and Moore, M. J. (2000). The spliceosome deposits multiple proteins 20-24 nucleotides upstream of mRNA exon-exon junctions. Embo J *19*, 6860-6869.

Lee, D. C., and Aitchison, J. D. (1999). Kap104p-mediated nuclear import. Nuclear localization signals in mRNA-binding proteins and the role of Ran and Rna. J Biol Chem *274*, 29031-29037.

Lee, D. H., and Goldberg, A. L. (1996). Selective inhibitors of the proteasome-dependent and vacuolar pathways of protein degradation in Saccharomyces cerevisiae. J Biol Chem *271*, 27280-27284.

Lee, D. H., and Goldberg, A. L. (1998). Proteasome inhibitors: valuable new tools for cell biologists. Trends Cell Biol *8*, 397-403.

Lee, M. S., Henry, M., and Silver, P. A. (1996). A protein that shuttles between the nucleus and the cytoplasm is an important mediator of RNA export. Genes Dev *10*, 1233-1246.

Lee, T. I., Causton, H. C., Holstege, F. C., Shen, W. C., Hannett, N., Jennings, E. G., Winston, F., Green, M. R., and Young, R. A. (2000). Redundant roles for the TFIID and SAGA complexes in global transcription. Nature *405*, 701-704.

Leser, G. P., Escara-Wilke, J., and Martin, T. E. (1984). Monoclonal antibodies to heterogeneous nuclear RNA-protein complexes. The core proteins comprise a conserved group of related polypeptides. J Biol Chem *259*, 1827-1833.

Libri, D., Graziani, N., Saguez, C., and Boulay, J. (2001). Multiple roles for the yeast SUB2/yUAP56 gene in splicing. Genes Dev *15*, 36-41.

Loflin, P., Chen, C. Y., and Shyu, A. B. (1999). Unraveling a cytoplasmic role for hnRNP D in the in vivo mRNA destabilization directed by the AU-rich element. Genes Dev *13*, 1884-1897.

Long, R. M., Elliott, D. J., Stutz, F., Rosbash, M., and Singer, R. H. (1995). Spatial consequences of defective processing of specific yeast mRNAs revealed by fluorescent in situ hybridization. Rna *1*, 1071-1078.

Luo, M. L., Zhou, Z., Magni, K., Christoforides, C., Rappsilber, J., Mann, M., and Reed, R. (2001). Pre-mRNA splicing and mRNA export linked by direct interactions between UAP56 and Aly. Nature *413*, 644-647.

Maki, C. G., Huibregtse, J. M., and Howley, P. M. (1996). In vivo ubiquitination and proteasome-mediated degradation of p53(1). Cancer Res *56*, 2649-2654.

Maquat, L. E. (1991). Nuclear mRNA export. Curr Opin Cell Biol *3*, 1004-1012.

Maquat, L. E., and Carmichael, G. G. (2001). Quality control of mRNA function. Cell *104*, 173-176.

Matunis, E. L., Matunis, M. J., and Dreyfuss, G. (1992a). Characterization of the major hnRNP proteins from Drosophila melanogaster. J Cell Biol *116*, 257-269.

Matunis, E. L., Matunis, M. J., and Dreyfuss, G. (1993). Association of individual hnRNP proteins and snRNPs with nascent transcripts. J Cell Biol *121*, 219-228.

Matunis, M. J., Michael, W. M., and Dreyfuss, G. (1992b). Characterization and primary structure of the poly(C)-binding heterogeneous nuclear ribonucleoprotein complex K protein. Mol Cell Biol *12*, 164-171.

Mayrand, S., and Pederson, T. (1981). Nuclear ribonucleoprotein particles probed in living cells. Proc Natl Acad Sci U S A *78*, 2208-2212.

Mayrand, S., Setyono, B., Greenberg, J. R., and Pederson, T. (1981). Structure of nuclear ribonucleoprotein: identification of proteins in contact with poly(A)+ heterogeneous nuclear RNA in living HeLa cells. J Cell Biol *90*, 380-384.

Michael, W. M. (2000). Nucleocytoplasmic shuttling signals: two for the price of one. Trends Cell Biol *10*, 46-50.

Michael, W. M., Choi, M., and Dreyfuss, G. (1995a). A nuclear export signal in hnRNP A1: a signal-mediated, temperature-dependent nuclear protein export pathway. Cell *83*, 415-422.

Michael, W. M., Eder, P. S., and Dreyfuss, G. (1997). The K nuclear shuttling domain: a novel signal for nuclear import and nuclear export in the hnRNP K protein. Embo J *16*, 3587-3598.

Michael, W. M., Siomi, H., Choi, M., Pinol-Roma, S., Nakielny, S., Liu, Q., and Dreyfuss, G. (1995b). Signal sequences that target nuclear import and nuclear export of pre-mRNA-binding proteins. Cold Spring Harb Symp Quant Biol *60*, 663-668.

Mili, S., Shu, H. J., Zhao, Y., and Pinol-Roma, S. (2001). Distinct RNP Complexes of Shuttling hnRNP Proteins with Pre-mRNA and mRNA: Candidate Intermediates in Formation and Export of mRNA. Mol Cell Biol *21*, 7307-7319.

Minvielle-Sebastia, L., Beyer, K., Krecic, A. M., Hector, R. E., Swanson, M. S., and Keller, W. (1998). Control of cleavage site selection during mRNA 3' end formation by a yeast hnRNP. Embo J *17*, 7454-7468.

Mizrahi, N., and Moore, C. (2000). Posttranslational phosphorylation and ubiquitination of the Saccharomyces cerevisiae Poly(A) polymerase at the S/G(2) stage of the cell cycle. Mol Cell Biol *20*, 2794-2802.

Moore, M. S., and Blobel, G. (1994). A G protein involved in nucleocytoplasmic transport: the role of Ran. Trends Biochem Sci *19*, 211-216.

Nakielny, S., and Dreyfuss, G. (1997). Nuclear export of proteins and RNAs. Curr Opin Cell Biol *9*, 420-429.

Nakielny, S., Siomi, M. C., Siomi, H., Michael, W. M., Pollard, V., and Dreyfuss, G. (1996). Transportin: nuclear transport receptor of a novel nuclear protein import pathway. Exp Cell Res *229*, 261-266.

Nefsky, B., and Beach, D. (1996). Pub1 acts as an E6-AP-like protein ubiquitiin ligase in the degradation of cdc25. Embo J *15*, 1301-1312.

Nehrbass, U., Kern, H., Mutvei, A., Horstmann, H., Marshallsay, B., and Hurt, E. C. (1990). NSP1: a yeast nuclear envelope protein localized at the nuclear pores exerts its essential function by its carboxy-terminal domain. Cell *61*, 979-989.

Nemeth, A., Krause, S., Blank, D., Jenny, A., Jeno, P., Lustig, A., and Wahle, E. (1995). Isolation of genomic and cDNA clones encoding bovine poly(A) binding protein II. Nucleic Acids Res *23*, 4034-4041.

Neumann, G., Hughes, M. T., and Kawaoka, Y. (2000). Influenza A virus NS2 protein mediates vRNP nuclear export through NES-independent interaction with hCRM1. Embo J *19*, 6751-6758.

Nichols, R. C., Wang, X. W., Tang, J., Hamilton, B. J., High, F. A., Herschman, H. R., and Rigby, W. F. (2000). The RGG domain in hnRNP A2 affects subcellular localization. Exp Cell Res *256*, 522-532.

Noble, S. M., and Guthrie, C. (1996). Identification of novel genes required for yeast pre-mRNA splicing by means of cold-sensitive mutations. Genetics *143*, 67-80.

Pickart, C. M. (2001a). Mechanisms Underlying Ubiquitination. Annu Rev Biochem *70*, 503-533.

Pickart, C. M. (2001b). Ubiquitin enters the new millennium. Mol Cell *8*, 499-504.

Pinol-Roma, S., Adam, S. A., Choi, Y. D., and Dreyfuss, G. (1989a). Ultraviolet-induced cross-linking of RNA to proteins in vivo. Methods Enzymol *180*, 410-418.

Pinol-Roma, S., and Dreyfuss, G. (1992). Shuttling of pre-mRNA binding proteins between nucleus and cytoplasm. Nature *355*, 730-732.

Pinol-Roma, S., Swanson, M. S., Gall, J. G., and Dreyfuss, G. (1989b). A novel heterogeneous nuclear RNP protein with a unique distribution on nascent transcripts. J Cell Biol *109*, 2575-2587.

Pioli, P. A., and Rigby, W. F. (2001). The von hippel-lindau protein interacts with heteronuclear ribonucleoprotein a2 and regulates its expression. J Biol Chem *276*, 40346-40352.

Pitluk, Z. W., McDonough, M., Sangan, P., and Gonda, D. K. (1995). Novel CDC34 (UBC3) ubiquitin-conjugating enzyme mutants obtained by charge-to-alanine scanning mutagenesis. Mol Cell Biol *15*, 1210-1219.

Pollard, V. W., Michael, W. M., Nakielny, S., Siomi, M. C., Wang, F., and Dreyfuss, G. (1996). A novel receptor-mediated nuclear protein import pathway. Cell *86*, 985-994.

Pringle, J. R., Adams, A. E., Drubin, D. G., and Haarer, B. K. (1991). Immunofluorescence methods for yeast. Methods Enzymol *194*, 565-602.

Reed, R., and Magni, K. (2001). A new view of mRNA export: separating the wheat from the chaff. Nat Cell Biol *3*, E201-204.

Rexach, M., and Blobel, G. (1995). Protein import into nuclei: association and dissociation reactions involving transport substrate, transport factors, and nucleoporins. Cell *83*, 683-692.

Ribbeck, K., and Gorlich, D. (2001). Kinetic analysis of translocation through nuclear pore complexes. Embo J *20*, 1320-1330.

Rose, M. D., Novick, P., Thomas, J. H., Botstein, D., and Fink, G. R. (1987). A Saccharomyces cerevisiae genomic plasmid bank based on a centromere-containing shuttle vector. Gene *60*, 237-243.

Rothstein, R. (1991). Targeting, disruption, replacement, and allele rescue: integrative DNA transformation in yeast. Methods Enzymol *194*, 281-301.

Rout, M. P., Aitchison, J. D., Suprapto, A., Hjertaas, K., Zhao, Y., and Chait, B. T. (2000). The yeast nuclear pore complex: composition, architecture, and transport mechanism. J Cell Biol *148*, 635-651.

Ryan, K. J., and Wente, S. R. (2000). The nuclear pore complex: a protein machine bridging the nucleus and cytoplasm. Curr Opin Cell Biol *12*, 361-371.

Saavedra, C., Tung, K. S., Amberg, D. C., Hopper, A. K., and Cole, C. N. (1996). Regulation of mRNA export in response to stress in Saccharomyces cerevisiae. Genes Dev *10*, 1608-1620.

Sachs, A. B. (1993). Messenger RNA degradation in eukaryotes. Cell *74*, 413-421.

Sachs, A. B., Bond, M. W., and Kornberg, R. D. (1986). A single gene from yeast for both nuclear and cytoplasmic polyadenylate-binding proteins: domain structure and expression. Cell *45*, 827-835.

Saleh, A., Collart, M., Martens, J. A., Genereaux, J., Allard, S., Cote, J., and Brandl, C. J. (1998). TOM1p, a yeast hect-domain protein which mediates transcriptional regulation through the ADA/SAGA coactivator complexes. J Mol Biol *282*, 933-946.

Sasaki, T., Toh, E. A., and Kikuchi, Y. (2000a). Yeast Krr1p physically and functionally interacts with a novel essential Kri1p, and both proteins are required for 40S ribosome biogenesis in the nucleolus. Mol Cell Biol *20*, 7971-7979.

Sasaki, T., Toh-e, A., and Kikuchi, Y. (2000b). Extragenic suppressors that rescue defects in the heat stress response of the budding yeast mutant tom1. Mol Gen Genet *262*, 940-948.

Scheffner, M. (1998). Ubiquitin, E6-AP, and their role in p53 inactivation. Pharmacol Ther *78*, 129-139.

Scheffner, M., Nuber, U., and Huibregtse, J. M. (1995). Protein ubiquitination involving an E1-E2-E3 enzyme ubiquitin thioester cascade. Nature *373*, 81-83.

Schwarz, S. E., Matuschewski, K., Liakopoulos, D., Scheffner, M., and Jentsch, S. (1998). The ubiquitin-like proteins SMT3 and SUMO-1 are conjugated by the UBC9 E2 enzyme. Proc Natl Acad Sci U S A *95*, 560-564.

Schwer, B., Saha, N., Mao, X., Chen, H. W., and Shuman, S. (2000). Structure-function analysis of yeast mRNA cap methyltransferase and high-copy suppression of conditional mutants by AdoMet synthase and the ubiquitin conjugating enzyme Cdc34p. Genetics *155*, 1561-1576.

Segref, A., Sharma, K., Doye, V., Hellwig, A., Huber, J., Luhrmann, R., and Hurt, E. (1997). Mex67p, a novel factor for nuclear mRNA export, binds to both poly(A)+ RNA and nuclear pores. Embo J *16*, 3256-3271.

Shen, E. C., Henry, M. F., Weiss, V. H., Valentini, S. R., Silver, P. A., and Lee, M. S. (1998). Arginine methylation facilitates the nuclear export of hnRNP proteins. Genes Dev *12*, 679-691.

Siebel, C. W., and Guthrie, C. (1996). The essential yeast RNA binding protein Np13p is methylated. Proc Natl Acad Sci U S A *93*, 13641-13646.

Sikorski, R. S., and Hieter, P. (1989). A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in Saccharomyces cerevisiae. Genetics *122*, 19-27.

Singleton, D. R., Chen, S., Hitomi, M., Kumagai, C., and Tartakoff, A. M. (1995). A yeast protein that bidirectionally affects nucleocytoplasmic transport. J Cell Sci *108 (Pt 1)*, 265-272.

Siomi, M. C., Eder, P. S., Kataoka, N., Wan, L., Liu, Q., and Dreyfuss, G. (1997). Transportin-mediated nuclear import of heterogeneous nuclear RNP proteins. J Cell Biol *138*, 1181-1192.

Siomi, M. C., Fromont, M., Rain, J. C., Wan, L., Wang, F., Legrain, P., and Dreyfuss, G. (1998). Functional conservation of the transportin nuclear import pathway in divergent organisms. Mol Cell Biol *18*, 4141-4148.

Smith, C. W., and Valcarcel, J. (2000). Alternative pre-mRNA splicing: the logic of combinatorial control. Trends Biochem Sci *25*, 381-388.

Snay-Hodge, C. A., Colot, H. V., Goldstein, A. L., and Cole, C. N. (1998). Dbp5p/Rat8p is a yeast nuclear pore-associated DEAD-box protein essential for RNA export. Embo J *17*, 2663-2676.

Solomon, M. J., and Varshavsky, A. (1985). Formaldehyde-mediated DNA-protein crosslinking: a probe for in vivo chromatin structures. Proc Natl Acad Sci U S A *82*, 6470-6474.

Stade, K., Ford, C. S., Guthrie, C., and Weis, K. (1997). Exportin 1 (Crm1p) is an essential nuclear export factor. Cell *90*, 1041-1050.

Strahl-Bolsinger, S., Hecht, A., Luo, K., and Grunstein, M. (1997). SIR2 and SIR4 interactions differ in core and extended telomeric heterochromatin in yeast. Genes Dev *11*, 83-93.

Strasser, K., and Hurt, E. (2000). Yra1p, a conserved nuclear RNA-binding protein, interacts directly with Mex67p and is required for mRNA export. Embo J *19*, 410-420.

Strasser, K., and Hurt, E. (2001). Splicing factor Sub2p is required for nuclear mRNA export through its interaction with Yra1p. Nature *413*, 648-652.

Stutz, F., Bachi, A., Doerks, T., Braun, I. C., Seraphin, B., Wilm, M., Bork, P., and Izaurralde, E. (2000). REF, an evolutionary conserved family of hnRNP-like proteins, interacts with TAP/Mex67p and participates in mRNA nuclear export. Rna *6*, 638-650.

Stutz, F., and Rosbash, M. (1998). Nuclear RNA export. Genes Dev *12*, 3303-3319.

Suyama, M., Doerks, T., Braun, I. C., Sattler, M., Izaurralde, E., and Bork, P. (2000). Prediction of structural domains of TAP reveals details of its interaction with p15 and nucleoporins. EMBO Rep *1*, 53-58.

Swanson, M. S., and Dreyfuss, G. (1988a). Classification and purification of proteins of heterogeneous nuclear ribonucleoprotein particles by RNA-binding specificities. Mol Cell Biol *8*, 2237-2241.

Swanson, M. S., and Dreyfuss, G. (1988b). RNA binding specificity of hnRNP proteins: a subset bind to the 3' end of introns. Embo J *7*, 3519-3529.

Tabb, A. L., Utsugi, T., Wooten-Kee, C. R., Sasaki, T., Edling, S. A., Gump, W., Kikuchi, Y., and Ellis, S. R. (2001). Genes encoding ribosomal proteins Rps0A/B of Saccharomyces cerevisiae interact with TOM1 mutants defective in ribosome synthesis. Genetics *157*, 1107-1116.

Terrell, J., Shih, S., Dunn, R., and Hicke, L. (1998). A function for monoubiquitination in the internalization of a G protein-coupled receptor. Mol Cell *1*, 193-202.

Truant, R., Fridell, R. A., Benson, E. R., Herold, A., and Cullen, B. R. (1998). Nucleocytoplasmic shuttling by protein nuclear import factors. Eur J Cell Biol *77*, 269-275.

Tseng, S. S., Weaver, P. L., Liu, Y., Hitomi, M., Tartakoff, A. M., and Chang, T. H. (1998). Dbp5p, a cytosolic RNA helicase, is required for poly(A)+ RNA export. Embo J *17*, 2651-2662.

Tyers, M., Tokiwa, G., Nash, R., and Futcher, B. (1992). The Cln3-Cdc28 kinase complex of S. cerevisiae is regulated by proteolysis and phosphorylation. Embo J *11*, 1773-1784.

Umen, J. G. (1995) The Role of Protein-RNA Interactions in 3' Splice Site Selection During pre-mRNA Splicing, PhD Dissertation, University of California, San Francisco.

Utsugi, T., Hirata, A., Sekiguchi, Y., Sasaki, T., Toh-e, A., and Kikuchi, Y. (1999). Yeast tom1 mutant exhibits pleiotropic defects in nuclear division, maintenance of nuclear structure and nucleocytoplasmic transport at high temperatures. Gene *234*, 285-295.

Utsugi, T., Toh-e, A., and Kikuchi, Y. (1995). A high dose of the STM1 gene suppresses the temperature sensitivity of the tom1 and htr1 mutants in Saccharomyces cerevisiae. Biochim Biophys Acta *1263*, 285-288.

van Venrooij, W. J., Wagenmakers, A. J., van den Oetelaar, P., and Reinders, R. J. (1981). In vivo cross-linking of proteins to mRNA in human cells. Mol Biol Rep *7*, 93-99.

Vasudevan, S., and Peltz, S. W. (2001). Regulated ARE-mediated mRNA decay in Saccharomyces cerevisiae. Mol Cell *7*, 1191-1200.

Verma, R., Chen, S., Feldman, R., Schieltz, D., Yates, J., Dohmen, J., and Deshaies, R. J. (2000). Proteasomal proteomics: identification of nucleotide-sensitive proteasome-interacting proteins by mass spectrometric analysis of affinity-purified proteasomes. Mol Biol Cell *11*, 3425-3439.

Vijayraghavan, U., Company, M., and Abelson, J. (1989). Isolation and characterization of pre-mRNA splicing mutants of Saccharomyces cerevisiae. Genes Dev *3*, 1206-1216.

Visa, N., Alzhanova-Ericsson, A. T., Sun, X., Kiseleva, E., Bjorkroth, B., Wurtz, T., and Daneholt, B. (1996). A pre-mRNA-binding protein accompanies the RNA from the gene through the nuclear pores and into polysomes. Cell *84*, 253-264.

Wagenmakers, A. J., Reinders, R. J., and van Venrooij, W. J. (1980). Cross-linking of mRNA to proteins by irradiation of intact cells with ultraviolet light. Eur J Biochem *112*, 323-330.

Wahle, E. (1991a). A novel poly(A)-binding protein acts as a specificity factor in the second phase of messenger RNA polyadenylation. Cell *66*, 759-768.

Wahle, E. (1991b). Purification and characterization of a mammalian polyadenylate polymerase involved in the 3' end processing of messenger RNA precursors. J Biol Chem *266*, 3131-3139.

Wahle, E. (1992). The end of the message: 3'-end processing leading to polyadenylated messenger RNA. Bioessays *14*, 113-118.

Wahle, E., Martin, G., Schiltz, E., and Keller, W. (1991). Isolation and expression of cDNA clones encoding mammalian poly(A) polymerase. Embo J *10*, 4251-4257.

Warner, J. R. (1999). The economics of ribosome biosynthesis in yeast. Trends Biochem Sci *24*, 437-440.

Weighardt, F., Biamonti, G., and Riva, S. (1996). The roles of heterogeneous nuclear ribonucleoproteins (hnRNP) in RNA metabolism. Bioessays *18*, 747-756.

Wente, S. R. (2000). Gatekeepers of the nucleus. Science *288*, 1374-1377.

Wente, S. R., and Blobel, G. (1994). NUP145 encodes a novel yeast glycine-leucine-phenylalanine-glycine (GLFG) nucleoporin required for nuclear envelope structure. J Cell Biol *125*, 955-969.

Wilkinson, C. R., Seeger, M., Hartmann-Petersen, R., Stone, M., Wallace, M., Semple, C., and Gordon, C. (2001). Proteins containing the UBA domain are able to bind to multi-ubiquitin chains. Nat Cell Biol *3*, 939-943.

Wilson, S. M., Datar, K. V., Paddy, M. R., Swedlow, J. R., and Swanson, M. S. (1994). Characterization of nuclear polyadenylated RNA-binding proteins in Saccharomyces cerevisiae. J Cell Biol *127*, 1173-1184.

Wimmer, C., Doye, V., Grandi, P., Nehrbass, U., and Hurt, E. C. (1992). A new subclass of nucleoporins that functionally interact with nuclear pore protein NSP1. Embo J *11*, 5051-5061.

Withers-Ward, E. S., Mueller, T. D., Chen, I. S., and Feigon, J. (2000). Biochemical and structural analysis of the interaction between the UBA(2) domain of the DNA repair protein HHR23A and HIV-1 Vpr. Biochemistry *39*, 14103-14112.

Wurtz, T., Kiseleva, E., Nacheva, G., Alzhanova-Ericcson, A., Rosen, A., and Daneholt, B. (1996). Identification of two RNA-binding proteins in Balbiani ring premessenger ribonucleoprotein granules and presence of these proteins in specific subsets of heterogeneous nuclear ribonucleoprotein particles. Mol Cell Biol *16*, 1425-1435.

You, J., and Pickart, C. M. (2001). A HECT domain E3 enzyme assembles novel polyubiquitin chains. J Biol Chem *276*, 19871-19878.

Zenklusen, D., and Stutz, F. (2001). Nuclear export of mRNA. FEBS Lett *498*, 150-156.

Zenklusen, D., Vinciguerra, P., Strahm, Y., and Stutz, F. (2001). The yeast hnRNP-Like proteins Yra1p and Yra2p participate in mRNA export through interaction with Mex67p. Mol Cell Biol *21*, 4219-4232.

Zhang, M., and Green, M. R. (2001). Identification and characterization of yUAP/Sub2p, a yeast homolog of the essential human pre-mRNA splicing factor hUAP56. Genes Dev *15*, 30-35.

Zhou, Z., Luo, M. J., Straesser, K., Katahira, J., Hurt, E., and Reed, R. (2000). The protein Aly links pre-messenger-RNA splicing to nuclear export in metazoans. Nature *407*, 401-405.