Title
Multiple modes of regulation of transcription factor activity in signaling pathways

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Publication Date
2001

Peer reviewed|Thesis/dissertation

"Multiple Modes of Regulation of Transcription Factor Activity in Signaling Pathways"

by

Arash Komeili

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

Cell Biology

in the

GRADUATE DIVISION

of the

UNIVERSITY OF CALIFORNIA SAN FRANCISCO

Date University Librarian

Degree Conferred: .....

This thesis is dedicated to my parents, sister and my fiancee Sara for their unconditional love and support througout the years.

Acknowledgements:

I would like to thank my parents for sacrificing their life's work to provide a better future for me and my sister and teaching me how to be patient and dedicated to my work. I would like to thank my sister for being one of my best friends and supporting me during my undergraduate and graduate years. I would also like to thank my fiancee Sara for being an amazing source of love, encouragement, friendship and fun. Meeting her has been the most significant part of my life at UCSF. I would like to thank my undergraduate advisor Maury Fox for being a role model and mentor in teaching me how to be a good scientist. My graduate advisor Erin O'Shea has also been an amazing role model and has taught me how to think about problems critically and devise the simplest approach to solving them. Erin has also provided me with invaluable advice regarding my graduate work as well as future career plans. I would like to thank Christine Guthrie, Jonathan Weissman, Karsten Weis, and Carol Gross for all their advice on my work and other issues in my life. I would like to thank my friends and the members of Team O'Shea for making the last five years an unforgettable experience. Finally, I would like to acknowledge the following journals for giving me permission to reprint my work in this thesis: Science, Journal of Cell Biology, Current Opinion in Cell Biology, and Annual Review of Genetics.

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Komeili & O'Shea, Roles of Phosphorylation Sites in Regulating Activity of the Transcription Factor Pho4. Science 284, 977 (1999).

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Abstract:

Many signaling pathways rapidly and reversibly convert extracellular signals into changes in gene expression by modulating the activity of transcription factors. To study how transcription factor activity is regulated in a signal-dependent fashion, we focused on the regulation of Pho4, a transcription factor in budding yeast that activates expression of genes induced in response to phosphate starvation. When yeast cells are grown in phosphate-rich conditions, Pho4 is phosphorylated by the Pho80/Pho85 cyclin-cyclin-dependent kinase (CDK) complex on five serines and inactivated, thereby terminating expression of phosphate-responsive gene. We showed that the phosphorylation sites on Pho4 have distinct roles in regulating its activity. Phosphorylation of Pho4 at two sites promotes the factor's nuclear export by the export factor Msn5, and phosphorylation at a third site inhibits its nuclear import by the import receptor Pse1. Phosphorylation of a fourth site blocks the interaction of Pho4 with the transcription factor Pho2. Therefore, phosphorylation regulates the nuclear localization of Pho4 as well as its ability to activate transcription when in the nucleus.

To investigate the generality of this phenomenon we studied the regulation of the glutamine response transcription factors Rtg1 and Rtg3. In response to limiting levels of glutamine, these factors activate the transcription of genes whose products are required for intracellular glutamine biosynthesis. We found that the localization of these factors to the nucleus is regulated by the availability of glutamine, that Rtg3 is a phospho-protein and that they are exported by Msn5. Furthermore, our work implicated Rtg2 and the Tor1 and Tor2 kinases in glutamine signaling and regulation of Rtg1 and Rtg3 activity. Most surprisingly, we found that in addition to controlled localization to the nucleus,

Rtg1 and Rtg3 are governed by nuclear regulatory events. Thus, similar to Pho4, multiple modes of regulation are used to control the activity of Rg1 and Rtg3.

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New Perspectives On Nuclear Transport

Arash Komeili and Erin K. O'Shea

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Keywords: nuclear import, nuclear export, import receptor, export receptor, RNA transport

Shortened Title: Nuclear Transport

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Abstract:

A central aspect of cellular function is the proper regulation of nucleocytoplasmic transport. In recent years significant progress has been made in identifying and characterizing the essential components of the transport machinery. Despite these advances, some facets of this process are still unclear. Furthermore, recent work has uncovered novel molecules and mechanisms of nuclear transport. This review focuses on the unresolved and novel aspects of nuclear transport and explores issues in tRNA, snRNA, and mRNA export that highlight the diversity of nuclear transport mechanisms.

Introduction:

A distinguishing feature of eukaryotic cells is the compartmentalization of genetic information within a nucleus. The nucleus and the cytoplasm are separated by a nuclear envelope and all macromolecular exchange between them takes place through large protein channels termed the nuclear pore complex (NPC). The size and complexity of molecules that are exchanged between these two compartments ranges from ions and other small molecules to large complexes such as the ribosome. In contrast to ions and small proteins which diffuse across the NPC, transport of macromolecules is an active process. Active nucleocytoplasmic transport allows for the proper regulation and trafficking of nuclear proteins involved in transcription, DNA replication and chromatin remodeling as well as mRNAs, tRNAs and rRNAs that are transcribed in the nucleus but function in the cytoplasm.

In recent years, there has been a significant amount of progress in identifying the components and understanding the mechanics of nucleocytoplasmic transport. The

development of an *in vitro* import assay using digitonin permeabilized cells has led to the identification of the soluble factors required for nuclear import (2). This biochemical approach has been complemented by extensive genetic studies in *S. cerevisiae* where multiple components of the nuclear pore complex and the machinery for nuclear export of proteins and mRNAs have been identified (17, 88). In addition, the large size of *Xenopus laevis* oocytes has provided a powerful cell biological tool for studying transport processes.

As a result of this work a general model for nuclear transport has been established (25). As will be described later, active nuclear transport is mediated by a family of transport receptors that recognize targeting sequences on their cargoes and traverse the NPC through interactions with nuclear pore proteins. The small Ras-like GTPase Ran and its effector molecules establish the directionality of transport. Remarkably, it appears the components of the nuclear transport machinery are well conserved in a variety of organisms.

The specifics of this model and the history behind the discoveries that have fueled this explosion in the understanding of nucleocytoplasmic transport have been discussed in many reviews (22, 25, 55, 64). Despite the progress in the study of nuclear transport, certain aspects of this process have not been elucidated. In this review, we will examine the most recent findings regarding nucleocytoplasmic transport. We will focus on energetics and mechanisms of translocation through the NPC as well as recent work that has put aspects of the classical model for nuclear transport in question. Finally, in order to highlight the variety of nuclear transport mechanisms we will provide a detailed view

of recent progress in understanding tRNA, snRNA, and mRNA transport. Where possible, we will emphasize the role of genetics in the study of nuclear transport.

Classical Nuclear Transport:

General model for nuclear transport:

The soluble components of the nuclear transport machinery and the basic mechanisms of nuclear transport were identified through a combination of biochemical and genetic approaches (25, 55). The in vitro import assay led to the discovery of transport receptors that can bind to specific targeting sequences on their cargo and carry them across the NPC. Importin β, the first such transport receptor to be identified, mediates the import of proteins containing basic nuclear localization signals (NLS) (1, 24, 68). Importin β recognizes these targeting sequences using an adaptor molecule, importin α, which binds directly to the NLS (1, 26). Additionally, importin β can interact directly with a subset of cargoes and thus transport them independently of importin α (41). Other transport receptors that have been identified share significant homology to importin β in their N-terminal regions. Based on this sequence similarity, a group of 14 importin β-related transport receptors have been identified in *S. cerevisiae* (23). Interestingly, mutants in these homologues display a variety of defects in the import or export of proteins and RNAs, lending further support to their central role in nuclear transport (25).

Whereas the importin β family of receptors is responsible for recognition of transport cargoes, the GTPase Ran appears to be the key to establishing the directionality of transport in vivo (25). The RanGAP (GTPase Activating Protein) is localized to the

cytoplasm and stimulates Ran's intrinsic GTPase activity, resulting in conversion of RanGTP to RanGDP (5, 6, 57). RCC1, the Ran guanine nucleotide exchange factor (RanGEF), is chromatin-associated and stimulates release of the bound nucleotide on Ran (7, 66). The asymmetric distribution of RanGEF and RanGAP implies the existence of a RanGTP gradient within the cell such that there are high levels of RanGTP in the nucleus and low levels of RanGTP in the cytoplasm. This asymmetry is thought to be essential in establishing the directionality of transport since export receptors of the importin β family require RanGTP bind to their cargo whereas import receptors are dissociated from their cargoes in the presence of RanGTP (25). In addition to RanGAP and RanGEF, two other soluble factors are components of the RanGTP cycle and are required for efficient nuclear transport. The Ran binding protein RanBP1 helps RanGAP in dissociation of export complexes and the small protein NTF2 is responsible for the active import of RanGDP into the nucleus where it is converted to RanGTP (4, 71, 83).

The study of the soluble components of the nuclear transport machinery has led to the following model for the import and export of macromolecules (25) (figure 1). An import receptor will bind to its cargo in the cytoplasm and traverse the NPC. In the nucleus RanGTP binds to the import receptor and releases the cargo into the nucleus. Export of macromolecules begins by the formation of a trimeric complex consisting of the receptor, cargo and RanGTP in the nucleus. Subsequently, this complex translocates through the NPC and upon reaching the cytoplasm the combined action of RanBP1 and RanGAP lead to GTP hydrolysis by Ran and release of the cargo in the cytoplasm.

Energetics of transport:

Nuclear transport of macromolecules can occur against a concentration gradient, highlighting the need for energy in this process. Early studies in vivo and with the in vitro import assay showed that nucleotides and GTP hydrolysis by Ran were necessary for the import of NLS cargoes by importin α/β (2, 93). Although, these experiments show that energy is needed for nuclear transport they do not elucidate the exact step where this energy requirement manifests itself.

A long standing model suggested that energy and GTP hydrolysis by Ran would be used in the translocation of receptor-cargo complexes through the nuclear pore (93). However, several recent studies show that energy and GTP hydrolysis by Ran are not required for translocation of import or export complexes through the nuclear pore and might only be needed at the terminal steps of transport. One such study examined the energy requirements in transportin-mediated nuclear import (70). Transportin is an importin β homologue that acts as the import receptor for the M9 sequence of hnRNA1 (62). In an *in vitro* import assay Ran and energy are required for nuclear import of M9 when transportin levels are lower than M9 levels (70). However, when transportin and M9 are added in equimolar quantities, there is significant import of M9 into the nucleus independent of Ran, GTP, ATP, or an energy regenerating system (70). These results suggest that when an import receptor is present in substoichiometric amounts, RanGTP is needed for multiple rounds of transport. Thus, energy and Ran are not required for translocation through the pore but are important in the release of cargoes and recycling of receptors. This model is further supported by the observation that import of the importin β binding (IBB) domain of importin α by importin β is not dependent on nucleotides or GTP hydrolysis on Ran (60). Also, in the absence of RanGTP, importin β devoid of

cargo can translocate to the nuclear side of the pore (46). Interestingly, similar energy requirements are observed for nuclear export processes. Crm1 is an importin β-like transporter that mediates the export of proteins containing a leucine-rich nuclear export signal (LRNES) (20, 85). Although RanGTP is required for the formation of a Crm1-LRNES export complex, nucleotide hydrolysis is not needed for nuclear export of this complex in an *in vitro* export assay (16).

Collectively, these results imply that energy and Ran are not required for translocation but are involved in the terminal steps of nuclear transport. In the nucleus, RanGTP ensures the release of import receptors from their cargoes, allowing them to return to the cytoplasm for further rounds of transport. For export, GTP hydrolysis by Ran in the cytoplasm leads to dissociation of export complexes, thereby freeing the export receptor to return to the nucleus. Although it is clear from these results that translocation of simple transporter-cargo complexes through the NPC is energy-independent, it remains possible that larger and more complex cargoes will have different energy requirements.

Mechanisms of translocation through the NPC:

As can be seen, a somewhat complete picture of the soluble components and the energetics of transport has emerged in recent years. The events that initiate and terminate nuclear transport have been characterized in detail and examination of multiple transport pathways has confirmed most aspects of this general model. However, many questions remain regarding the translocation of transport complexes through the nuclear pore complex.

This is not a trivial issue since a transport complex must travel through 200nm of the NPC before it can be dissociated in its target compartment. The NPC is a large structure, varying in size from 60 MDa in *S. cerevisiae* to 125 MDa in vertebrates, that contains multiple copies of 30-50 different proteins (17, 75, 86). The size and the complexity of the NPC have made biochemical reconstitution of the translocation process very difficult. Genetic and biochemical studies with isolated nuclear pore proteins (Nups or nucleoporins) have shown that they interact directly with import and export receptors, leading to the hypothesis that these interactions form the basis for translocation through the NPC (21, 69, 74). Thus, identifying the protein components of the NPC and their localization within this large complex is crucial to understanding the translocation process.

The complete protein composition of the *S. cerevisiae* NPC has recently been elucidated (73). Rout et al. identified *S. cerevisiae* proteins that were enriched in nuclear envelope preparations. The localization of each protein was determined by immunofluorescence and those that displayed characteristic nuclear pore complex localization were categorized as members of the NPC. The results of this study indicate that each NPC in *S. cerevisiae* is composed 16 to 32 copies of only 30 proteins, a relatively low number given the size and architectural complexity of this structure. Immunoelectron microscopy experiments were carried out to determine the distribution of these proteins within the NPC. Remarkably, all but 5 of these proteins are present on both sides of the pore. Of these 25, 4 proteins were more abundant on one side of pore and the rest were distributed equally across the nuclear envelope. The largely symmetric distribution of NPC proteins is surprising because the NPC has an asymmetric

architecture consisting of a central channel, fibrils that extend into the cytoplasm and a basket-like structure within the nucleus. It is possible that the few nucleoporins that have a biased distribution are the constituents of the nuclear basket and the cytoplasmic fibrils. In addition to providing a full catalogue of NPC proteins, this study provides a framework for examining the mechanisms of translocation through the pore.

Given the symmetric distribution of the pore proteins it is tempting to hypothesize that directionality of transport relies more on the soluble transport components of transport rather than the actual protein composition of the NPC. In fact, the directionality of transport through the nuclear pore can be reversed (60). If a pre-formed Crm1-LRNES-RanQ69L export complex is added to the cytoplasmic face of permeabilized cells, the NES substrate can equilibrate between the two compartments. This "import" of an export complex relies on a viable interaction between Crm1 and the LRNES and requires functional NPCs. These results indicate that the directionality of transport may largely be determined by the Ran system and that the location of specific nucleoporins within the NPC does not play a dominant role in translocation.

These results have led to the suggestion that once an export complex has been formed in the nucleus it moves through the NPC through successive interactions between transport receptors and nucleoporins (60, 73). The cargo-receptor complex is thought to move in both directions until it reaches the cytoplasmic side in a stochastic manner. Once the complex nears the cytoplasm the concerted action of RanBP1 and RanGAP leads to GTP hydrolysis on Ran and dissociation of the complex into the cytoplasm. According to this model translocation of import complexes would proceed in a similar manner until their dissociation by RanGTP in the nucleus.

However, there is evidence disputing the above model. First, although the distribution of the majority of NPC proteins is symmetric, there are some that display a biased localization (73). Second, biochemical evidence has pointed to the existence of subcomplexes within the NPC that show differential localization (75). Third, certain mutants in yeast Nups display defects in either import or export processes, but not both (58). Lastly, experiments with reconstituted nuclear pores have indicated that the direction of transport across the NPC cannot be reversed (45). These results suggest that import and export receptors may display differential interactions with nucleoporins such that movement through the NPC is vectorial. In such a model the interactions between an export receptor and NPC components would increase in affinity as the complex approaches the cytoplasmic side of the pore. Similarly, an import complex would interact more strongly with nuclear nucleoporins than cytoplasmic ones. Further support for this model comes from examination of receptor-nucleoporin interactions in *S. cerevisiae* (10). During transport *in vivo*, the export receptor Msn5 and the import receptor Pse1 show differential interactions with nucleoporins suggesting that they use different routes for translocation through the NPC.

How can the largely symmetric composition of the NPC be reconciled with a model where translocation is driven by differential interactions between receptors and nucleoporins? Perhaps, despite their symmetric distribution, nuclear pore proteins interact asymmetrically with each other within the pore to create tracks that would be specific to either import or export complexes. This idea is supported by the observation that the yeast Nup53, Nup59 and Nup170 form a subcomplex that binds specifically to the import receptor Pse1/Kap121, but fails to bind to the closely related Kap123 or

importin β/Kap95 (54). In addition to the existence of subcomplexes that bind to a subset of transport receptors, certain Nups contain domains that display specificity in their interactions with transport receptors. Nup153 in humans contains one domain that is required for importin β-dependent import and another that mediates transportin-dependent import (81). Therefore, subcomplexes and subdomains of nucleoporins might create microenvironments within the NPC that are specific for different transporters.

Despite extensive progress in defining the architecture and composition of the NPC and the abundance of models to describe translocation, our view of this process is still rather unclear. Further characterization of the subset of nucleoporin-transport receptor interactions that promote translocation through the NPC is needed to arrive at a more unified theory regarding this crucial step in nuclear transport.

Non-classical Transport:

Due to the generality and the remarkable evolutionary conservation of the model described in the previous section, most studies have relied heavily on reverse genetics and homology-based techniques for identification of factors involved in transport of specific macromolecules. Although most cases follow this simple model, several examples of non-classical nuclear transport have been identified. In this section we will examine some of these cases which have introduced novel mechanisms of nuclear transport (figure 2).

Ran-independent transport:

The RanGTP cycle is at the center of the classical model for transport. Ran is required at three distinct points in nuclear transport (25). Without RanGTP in the nucleus import complexes would not dissociate and export complexes would not form. Also, GTP hydrolysis by Ran is required for the dissociation of export complexes. Therefore, it has been surprising to find that several cargoes can be transported independently of RanGTP (9, 33, 90).

The most striking example of Ran-independent transport is the import of cyclin B1-Cdc2 by importin β (88). In an in vitro import assay, the import of this complex depends on importin β but does not require of Ran. In contrast, the import of basic NLS-containing cargoes by importin β in the same assay is dependent on Ran. These results indicate that in contrast to classical importin β-mediated import, RanGTP is not required for the release of cyclin B1-Cdc2 from importin β in the nucleus. Accordingly, RanQ69L, a hydrolysis-deficient Ran mutant, inhibits import of basic NLS-containing cargoes by dissociating them in the cytoplasm but does not affect cyclin B1-CDC2 import. Thus, an importin β-dependent but Ran-independent mechanism is used in the import of cyclin B1-CDC2.

If RanGTP is not required for these transport processes then alternative mechanisms must exist for the formation of export complexes and dissociation of import and export complexes. One possibility is that direct modification of cargoes in the target compartment leads to dissociation of the transport complex.

Alternative roles of Ran:

It has been known for some time that mutations in the Ran system lead to a variety of phenotypes such as defects in nuclear morphology and the cell cycle (37). Given the importance of nuclear transport, it has been difficult to assign a direct role for Ran in these processes. Recently, several reports have identified a direct role for the RanGTPase cycle in regulation of the mitotic spindle (31). Addition of demembranated sperm nuclei to *Xenopus* oocyte mitotic extracts induces the formation of a mitotic spindle around the DNA. Addition of high levels of RanQ69L can also induce the formation of microtubule asters that resemble those formed in the presence of sperm nuclei (29, 31, 59). Interestingly, RanGTP uses the components of the nuclear transport machinery in inducing spindle assembly (12). Mitotic *Xenopus* egg extracts that have been depleted of RanQ69L binding proteins form microtubule asters spontaneously (59). Addition of importin β to these depleted extracts inhibits their spontaneous aster formation, suggesting that RanGTP promotes spindle assembly by overcoming an inhibitory effect imposed by importin β. In support of this hypothesis, addition of an excess of SV40 NLS or IBB domain of importin α to mitotic egg extracts also induces aster formation (29, 59). Thus, proteins required for an aster promoting activity (APA) are sequestered by importin α/β during interphase, resulting in a block to spindle assembly. Presumably in interphase, APA proteins and other factors required for spindle assembly are kept in separate compartments. After nuclear envelope breakdown during mitosis, RanGTP releases these proteins from importin α/β allowing them to interact with other factors to build the mitotic spindle. This mechanism ensures that the spindle forms in the proximity of the DNA where high levels of RanGTP are present as a result of the action of the chromatin-bound RanGEF.

Two potential components of APA have been identified. The nuclear mitotic apparatus protein (NuMA) is a known mitotic spindle protein and it is able to induce aster formation when added at very low concentrations to extracts that are depleted of both RanQ69L and importin β-associated proteins (59, 94). NuMA is able to bind to importin α/β and it is released in the presence of RanGTP. Another protein, TPX2, targets the motor protein Xklp2 to microtubules (29). Recombinant TPX2 is sufficient to induce aster formation in mitotic extracts and this activity is inhibited by co-addition of importin α. NuMA and TPX2 are not the only components of APA. Addition of either importin α or amino acids 1-601 of importin β inhibits the spontaneous formation of asters in *Xenopus* egg extracts that have been depleted of RanGTP-binding proteins. Importin β 1-601 lacks the importin α binding domain but retains the ability to bind a group of importin α-independent cargoes (41). Since TPX2 and NuMA are importin α cargoes, other APA proteins must also exist.

The common theme emerging from these studies is that RanGTP is a biochemical marker for DNA throughout the cell cycle. In addition to its role in nuclear transport during interphase and spindle formation in metaphase, RanGTP is also required for reformation of the nuclear envelope in late mitosis (32, 98). Furthermore, perturbations of RanGTP concentrations in the nucleus appear to alter chromatin structure, suggesting that Ran may play a role in DNA maintenance (37, 59). More work is needed to know if other components of the nuclear transport machinery are also involved in these Ran-dependent processes.

It is not clear if RanGTP plays a similar role in spindle assembly in *S. cerevisiae*. Yeast cells undergo a closed mitosis where the nuclear envelope does not breakdown. If

a similar system is used by yeast, then import of APA or other spindle formation factors must be regulated across the cell cycle. The study of yeast also provides the opportunity to learn about the evolutionary origins of RanGTP function in nuclear transport and spindle assembly. It is possible that the original function of Ran and importin α/β was in mitotic spindle assembly (59). As eukaryotic cells acquired nuclear envelopes this system may have evolved to have multiple functions including a role in nuclear transport. Alternatively, nuclear import of APA might have been necessary for spindle formation before the evolution of nuclear envelope breakdown (94). Once cells adopted nuclear envelope breakdown then the transport machinery was used to locally release APA proteins.

Transport receptors dedicated to both import and export:

One prevailing hypothesis regarding importin β-like transporters has been that transport receptors are dedicated to either import or export of cargoes. Accordingly, import receptors have been termed importins and export receptors are called exportins.

Contrary to this hypothesis, the *S. cerevisiae* importin β homologue Msn5 has been implicated in both export and import processes (97). Msn5 was categorized as one of the 14 importin β homologues in *S. cerevisiae* (23). Msn5 is the export receptor for several *S. cerevisiae* proteins including the transcription factors Pho4 and Mig1 and the CDK inhibitor Far1 (8, 13, 42). Affinity chromotography with Msn5 identified Rpa1-3, a nuclear complex involved in the DNA damage response, as an Msn5-associated protein complex (97). When affinity chromotography is performed in the presence of RanGTP, the interaction between Rpa1-3 and Msn5 is lost. Additionally, there is a significant

mislocalization of Rpa1-3 to the cytoplasm in *msn5_* cells. These results suggest that Msn5 could be the import receptor for the Rpa complex. One question regarding these results is that the Rpa proteins are essential whereas Msn5 is not required for growth. In fact, in the *msn5_* cells, a portion of the Rpa proteins are still nuclear. It has been shown that Rpa proteins can interact with other import receptors, raising the possiblity that the Rpa complex can utilize multiple import pathways (97). Furthermore, these results do not show that RanGTP affects the Msn5-Rpa complex directly. To confirm this, it is important to assess the effects of RanGTP on a preformed Msn5-Rpa1-3 complex in a purified system.

These experiments raise the possibility that the same transport receptor can direct the import and export of different proteins. This idea is not unexpected since the natural cycle for a transport receptor requires it to move in both directions through the nuclear pore (25). Additionally, some transport receptors use different domains for binding to different cargoes ADDIN ENRfu (41). If these two sites could be affected differentially by RanGTP binding then a transport receptor might be used in both import and export. It would be interesting to investigate the generality of this phenomenon for other receptors and in other systems.

Non-importin β receptors:

Importin β family members have been thought of as the only proteins capable of transporting cargoes across the nuclear pore. However, recent work suggests that other proteins can also use the RanGTP system to transport cargoes. Calreticulin (CRT), an ER-resident calcium binding protein, has been implicated in the nuclear export of

proteins with a LRNES (35). Recombinant CRT can stimulate export of the LRNES even in the presence of leptomycin B (LMB), a specific inhibitor of Crm1-dependent nuclear export (20), showing that this pathway is distinct from the Crm1 pathway. Additionally, CRT binds to a LRNES peptide only in the presence of RanGTP with kinetics very similar to the Crm1-RanGTP-LRNES interaction (35). CRT's possible role as an export receptor is further substantiated by the discovery of CRT-specific cargoes. The export of glucocorticoid receptor (GR) is independent of Crm1, as it is not inhibited by LMB, but CRT can stimulate its export *in vitro* and is required for GR export *in vivo* (35).

Although these results indicate that CRT behaves like known importin β-like export receptors, it is not an importin β homologue (35). This is the first report of a non importin β–like receptor being involved in RanGTP-dependent export of macromolecules. One effective method for finding the import or export receptor for proteins or RNAs in *S. cerevisiae* has been to screen a panel of strains bearing mutations in importin β homologues (42,43). Such a reverse genetics approach is not sufficient if receptors not related to importin β are involved in nucleocytoplasmic transport. These results highlight the importance of classical non-biased genetics approaches in studying nucleocytoplasmic transport.

One cautionary note regarding these results is that CRT has been characterized as an ER-resident protein with multiple cellular functions (47). Although biochemical fractionation studies imply that a fraction of the CRT is present in the nucleus, examination of CRT localization by immunofluorescence has produced conflicting results (47). Since the discovery of a non-importin β receptor will have major

implications in the study of nucleocytoplasmic transport, it is quite important to resolve these outstanding issues.

RNA Transport

In this section we will examine the export of tRNAs, snRNAs and mRNAs with the purpose of showcasing the diversity of classical and non-classical transport mechanisms that were discussed above (figure 3).

tRNA export:

In order to function in translation, tRNAs must be transcribed, modified and subsequently exported from the nucleus (28, 95). The export receptor, exportin-t (Xpo-t) is an importin β homologue that is responsible for tRNA export (3, 48). Similar to protein export by importin β homologues, Xpo-t binds directly to tRNAs with the help of RanGTP. En route to maturation, bases and sugar residues of tRNAs are modified, their 5' and 3' ends as well as introns are specifically removed, and a CCA sequence is added to the 3' end (95). The maturation of tRNAs appears to be required for their export since mature tRNAs are exported much more efficiently than pre-tRNAs (3, 51). This bias in export is achieved primarily through the specificity of the interaction of mature tRNAs with Xpo-t and RanGTP (3, 51). In vitro, Xpo-t can interact efficiently with mature tRNAs, whereas it fails to show significant binding to pre-tRNAs. However, differential binding to Xpo-t may not be the sole source of specificity for export of mature tRNAs (3). Intron-containing tRNAs interact with Xpo-t as well as spliced tRNAs but they are exported with very low efficiency in vivo. Injection of excess Xpo-t stimulates the

export of intron-containing tRNAs, suggesting that competition between export factors and tRNA processing enzymes within the nucleus might be another mechanism restricting the export of pre-tRNAs. It has also been proposed that 3' and 5' end processing occurs after splicing in vivo (52). Such a mechanism ensures that only spliced tRNAs are exported since mature ends are required for tRNA recognition by Xpo-t.

In *S. cerevisiae* tRNA export seems to utilize multiple pathways. The importin β homologue Los1 has been implicated in tRNA export since it is homologous to Xpo-t and displays many genetic interactions with the tRNA biogenesis pathway (23, 36, 77). For example, it was first isolated as a mutant displaying loss of suppressor activity (thus called LOS) (38). Additionally, strains lacking Los1 display accumulation of tRNAs within their nuclei (77). However, deletion of Los1 is not lethal suggesting that it is not the sole receptor for tRNA export (38). Recently, the elongation factor eEF-1A and some tRNA synthetases have been implicated in the Los1-independent export of tRNAs (27). Deletion of these genes results in synthetic lethality when combined with Los1 deletions. Mutants defective in EF-1A and some tRNA synthetases display nuclear accumulation of tRNAs. Thus, it is possible that components of the translation machinery are used as an alternate tRNA export pathway in yeast. However, these results do not point to a specific mechanism for how translation factors might be involved in tRNA export. Does eEF1-A target tRNAs to another importin β or does it export them directly? Furthermore, is the action of tRNA synthetases and eEF1-A indicative of a role for maturation in tRNA export or does it imply the existence of a pathway parallel to the Los1 export pathway?

snRNA export:

At the heart of the splicing machinery are specialized small nuclear RNAs (snRNAs) (56). These snRNAs associate with proteins to form ribonucleoprotein complexes (snRNPs) that catalyze splicing reactions. In metazoans, subsequent to their transcription, snRNAs are exported to the cytoplasm where they bind to snRNP proteins. The resulting snRNP complexes are then reimported into the nucleus by the import receptor Snurportin-1 so that they can participate in splicing reactions (67). The export of snRNAs from the nucleus is dependent on RanGTP since it is blocked by injection of RanGAP and RanBP1 into *Xenopus* nuclei (39). Furthermore, snRNAs use a Crm1-dependent export pathway since their export is blocked by the addition of LMB and can be competed by the addition of NES peptides (19, 20).

Unlike tRNA export, however, snRNAs do not bind directly to their export receptor. It has been known for some time that the cap-binding complex (CBC) proteins, CBC20 and CBC80, bind cooperatively to the m7G-cap of snRNAs and that this binding is required for efficient snRNA export (40). Other factors also appear to be required for the formation of an snRNA export complex since recombinant CBC, CRM1 and RanGTP do not form an export complex with snRNAs unless crude extract is also present (65). Recently, a 55 kDa protein, p55, has been purified as an activity responsible for formation of a complex containing CBC, snRNA, CRM1, and RanGTP (65). p55 binds cooperatively to the CBC-snRNA complex, shuttles between the nucleus and cytoplasm and contains an NES that is recognized by Crm1. In addition to its in vitro role in formation of an export complex, p55 is also required for snRNA export in vivo since injection of antibodies against p55 blocks snRNA export without affecting tRNA or mRNA export. Additionally, injection of p55 into *Xenopus* oocyte nuclei enhances the

export of snRNAs. Interestingly, p55's ability to form an snRNA-export complex is dependent on its phosphorylation state (65). Phosphorylated p55 mediates the formation of this export complex whereas dephosphorylated p55 cannot bridge the CBC-snRNA complex to Crm1 and RanGTP. Since dephosphorylation of p55 induces diassembly of the export complex it is possible that dephosphorylation of p55 is a mechanism used to release snRNAs into the cytoplasm. In support of this idea, p55 is mainly in the phosphorylated form in the nucleus whereas it is mostly dephosphorylated in the cytoplasm. Accordingly, p55 has been named PHAX, **ph**osphorylated **a**daptor for RNA **x**port (65).

Several aspects of this system are quite intriguing. First, every step of the formation of an snRNA export complex is regulated by cooperative binding events (65). The CBC complex binds cooperatively to the cap, PHAX binds cooperatively to CBC and the snRNA molecule, and finally CRM1 and RanGTP recognize the PHAX-CBC-snRNA complex cooperatively. Presumably these multiple layers allow for proper regulation of snRNA export. Second, the release of snRNAs by dephosphorylation of PHAX seems to be a redundant mechanism. It is known that Ran dissociates export complexes through RanGAP-stimulated GTP hydrolysis. Perhaps in snRNA export GTP hydrolysis is most important for recycling of the export receptor whereas dephosphorylation of PHAX is required for the release of the snRNA from CBC and PHAX. Third, snRNA export in *S. cerevisiae* seems to follow very different rules. Homologues of PHAX and Snurportin exist in other eukaryotes but are absent from *S. cerevisiae* (65). Thus, snRNP biogenesis in *S. cerevisiae* may be an exclusively nuclear event.

mRNA export:

mRNA export appears to be a much more complicated process than protein, tRNA, or snRNA export. Studies with the Balbiani ring particles of *Chironomus tentans* showed that multiple proteins bind to and are released from mRNAs as they exit the nucleus (11). Furthermore, mRNA processing events such as capping, polyadenylation and splicing are nuclear events and must precede mRNA export. This complexity, coupled with the lack of *in vitro* RNA export assays, has hampered the biochemical exploration of mRNA export. Thus, yeast genetics has been a prominent and fruitful approach in understanding mRNA export (88). Poly(A)+ mRNA can be visualized by fluorescence in situ hybridization (FISH) using labelled oligo dT probes. Poly(A)+ mRNA has a cytoplasmic localization in wild-type cells but it is localized to the nucleus in mRNA export mutants. Genetic studies have implicated a variety of proteins in mRNA export (88). A persistent hypothesis has been the involvement of shuttling heterogeneous ribonucleoprotein particle (hnRNP) proteins in mRNA export (14, 61). These proteins have many of the characteristics expected of mRNA transport proteins. They bind RNA directly, some can shuttle between the nucleus and cytoplasm, and mutations in them lead to a block in export of poly-(A)+ mRNA. It has been thought that these hnRNP proteins would link mRNAs to specific transport receptors and promote their passage through the nuclear pore. A variety of importin β family members have also been implicated in mRNA export (79, 85). Several studies have linked the Crm1 (Xpo1 in yeast) pathway to mRNA export. The Xpo1 temperature sensitive mutant, *xpo1-1*, rapidly accumulates poly-(A)+ mRNA in the nucleus at non-permissive temperatures (85). In human cells mRNA accumulates in the nucleus after long incubations with the Crm1 inhibitor LMB (92).

However, many studies have put the role of Crm1 in mRNA transport in doubt. Neville et al. constructed LMB-sensitive versions of *S. cerevisiae* Crm1 and showed that LMB blocked mRNA export only partially and significantly later than a complete block to LRNES protein export, suggesting that Crm1 plays an indirect or redundant role in mRNA export (63). Furthermore, injection of NES proteins into *Xenopus* nuclei can compete for snRNA export but it has no effect on mRNA export (19). Another indication that importin β-like receptors are not involved in this process is that mRNA export does not require RanGTP (9). The export of snRNAs and tRNAs is rapidly blocked if nuclear pools of RanGTP are depleted by the injection of RanBP1 and RanGAP into *Xenopus* nuclei (9, 39). In contrast, mRNA transport is unaffected by these treatments. Furthermore, no detectable levels of RanGTP are found in purified mRNP complexes that are competent for export (9). The simplest interpretation of these results is that in some cases, RanGTP is not directly involved in mRNA transport. These results also imply that in contrast to protein, tRNA, and snRNA export, export of some classes of mRNAs might rely on non-importin β-like export receptors.

Recently, several lines of evidence have pointed to Mex67 as a possible export receptor for mRNA. Although not an importin β homologue, Mex67 localizes to the NPC, binds mRNA and is required for mRNA export in yeast (80). In yeast, Mex67 dimerizes with Mtr2 and this complex can bind to several different nucleoporins (76, 87). This pathway is well conserved from yeast to humans. TAP, the human version of Mex67, is a shuttling protein that localizes to the nuclear pore complex as well as the nucleoplasm (44). TAP is involved in export of viral messages by directly binding to the constitutive transport elements (CTE) of viral mRNAs (30). Analogous to the Mex67-

Mtr2 interaction, TAP has been shown to interact with p15, an NTF2 homologue. Remarkably, TAP can functionally replace yeast Mex67 (44). When expressed in yeast, TAP localizes to the nuclear pores in an Mtr2-dependent manner and coexpression of TAP and p15 in yeast can partially suppress the growth defect of strains carrying deletions of Mex67 or Mtr2 as well as strains lacking both proteins (44). Since Mex67/TAP can interact with mRNA cargoes as well as nucleoporins, a possible model is that Mex67/TAP fulfills a role similar to importin β family members in protein export.

Although Mex67 can bind directly to some mRNAs, other factors seem to be required for Mex67-dependent nuclear transport. One such protein, Yra1, was identified in a synthetic lethal screen with a temperature sensitive allele of Mex67 (89). Yra1 is a shuttling nuclear protein, is required for RNA export and can directly interact with RNA and Mex67. Yra1 is a member of the REF family of proteins that are conserved from yeast to humans. Interestingly, ALY, the mouse homologue of REF/Yra1, can complement the lethality of a Yra1 deletion in yeast (89). These proteins are essential for mRNA export as injection of antibodies against REF proteins has no effect on splicing but leads to a block in mRNA export (72).

Recently, Yra1/ALY/REF has been implicated in linking mRNA processing to its export (49, 53, 99). mRNAs derived from microinjected intron-bearing transcripts are exported much more efficiently from Xenopus oocyte nuclei than the same mRNAs produced from cDNAs lacking introns (53). It is important to note that the main difference between these two messages is that one has to go through the process of splicing whereas the other never encounters the splicing machinery. When these messages are spliced in vitro, the spliced mRNA is part of a different mRNP particle than

the message produced from intron-deleted cDNA. Interestingly, when the protein contents of these mRNPs are examined, REF becomes part of the mRNP complex after the completion of splicing suggesting that REF/Yra1 might couple the maturation of mRNAs to their export (99). Additional evidence for the role of REF/Yra1 in linking splicing to mRNA export has come from the discovery of a protein complex that marks spliced mRNAs 20-24 bases upstream of exon-exon junctions in a sequence-independent manner (49). REF is part of this protein complex whereas TAP is not. These observations imply that the completion of splicing is coupled to the deposition of REF proteins on the mature mRNA. REF then directs the message to TAP at the nuclear pores and this complex moves through the NPC as a result of the interactions of TAP with nucleoporins.

Although quite elegant, it is unclear if this model applies to export of all mRNAs. In yeast particularly, many messages do not contain introns but are still exported efficiently. It is possible that Mex67 and Yra1 participate in mRNA export differently in yeast and metazoans. However, even in metazoans some naturally occurring intronless messages are exported efficiently, suggesting that other processing events might be important for efficient mRNA export (72). Furthermore, if splicing is sufficient for making a message competent for export then messages containing multiple introns might be targeted for export after removal of only one intron. Perhaps splicing is restricted to certain locations in the cell such that messages are released only after splicing has occurred. And finally, in addition to REF, many other proteins are part of the complex marking exon-exon junctions after splicing. The role that these and other hnRNP proteins might play in mRNA export is not clear. Possibly REF and other hnRNPs such

as Npl3 and Nab2 represent different export pathways devoted to specific classes of transcripts. In support of this model, mutations in the ubiquitin ligase-like protein Tom1 result in mislocalization of Nab2 but do not affect the localization or shuttling of Npl3 (15).

Since it appears that RanGTP is not involved in mRNA export, the mechanism by which mRNAs are released from export complex remains to be elucidated. Screens for mRNA export mutants led to the discovery of Dbp5, an RNA helicase that localizes to the cytoplasm and to nuclear pores (84, 91). Studies with the human homologue of Dbp5 have shown that it localizes to the cytoplasmic fibrils of the nuclear pore complex and that its ATPase activity is required for mRNA export (78). An attractive hypothesis is that Dbp5 acts at the terminal steps of RNA transport by rearranging the mRNA-protein complex and releasing the mRNA into the cytoplasm. Interestingly, overexpression of Dbp5 can suppress the temperature-sensitive phenotype of the *xpo1-1* mutant, suggesting that the proposed Crm1 function in mRNA export might be linked to the localization of Dbp5 (34).

In addition to its role in identifying specific protein components of the mRNA transport machinery, yeast genetics has been crucial in uncovering global signaling events that may regulate mRNA export. Recently, inositol signaling has been implicated in regulation of mRNA export (96). Gle1 is a nucleoporin that appears to have a specific function in mRNA export (58). A synthetic lethal screen with a *GLE1* temperature sensitive mutant identified three mutants which displayed specific defects in mRNA export with no apparent perturbation of protein import or export (96). Interestingly, all three genes have distinct roles in the maintenance of phosphoinositol levels within the

cell. These genes encode phospholipase C, Plc1, and two novel inositol phosphate kinases, Ipk1 and Ipk2/Arg82. Plc1 cleaves PIP2 into diacylglycerol and IP3 which is then phosphorylated to form IP4, IP5, and IP6 (50, 82). Ipk1 and Ipk2 appear to phosphorylate IP5 to form IP6 (96). In accordance with their known functions, mutations in any of the three genes lead defects in IP6 production. Interestingly, Ipk1 is a nuclear protein with a distinct nuclear pore localization, lending further support for a direct role in mRNA export (96). Inositol phosphates may also have a role in regulating mRNA export in mammalian cells (18). Ectopic expression of SopB, a bacterial inositol phosphatase, causes a defect in mRNA export in cultured mammalian cells. Interestingly, when SopB is targeted to the nucleus via an NLS fusion the mRNA export defect is more severe.

These results imply a role for inositol phosphate signaling in mRNA export although the exact targets of inositol phosphates in this process are unknown. The defect in mRNA export in yeast strains unable to produce IP6 is probably not due to gross rearrangements of the NPC or the nuclear envelope since these structures appear normal in yeast strains containing mutations in *PLC1*, *IPK1*, or *IPK2* (96). However, it is possible that inositol phosphates modulate the activity of specific NPC or mRNA export proteins. This idea is supported by the synthetic lethal interactions between *PLC1*, *IPK1*, and *IPK2* and *GLE1* mutants. Furthermore, it is unclear if inositol signaling is required for constitutive or regulated mRNA export. For example, it is possible that the mutations in *PLC1*, *IPK1*, and *IPK2* mimic conditions that would lead to a down regulation of mRNA export in wild-type cells. Further work is needed to understand the specific role that inositol phosphates play in regulating mRNA export.

Conclusions and Future Directions:

Our knowledge of nucleocytoplasmic transport has progressed rapidly in recent years. The basic proteins involved in import and export of macromolecules are conserved in all eukaryotic systems studied thus far. However, recent results point to the existence of alternative machineries and unique modes of transport. Also, the understanding of essential aspects of transport such as translocation through the nuclear pore is still limited.

The diversity of mechanisms used in nuclear transport are clearly demonstrated by an examination of tRNA, snRNA, and mRNA nuclear export. tRNAs and snRNAs are exported in a Ran-dependent manner whereas mRNAs use a Ran-independent pathway. Importin β-like receptors are involved in tRNA and snRNA export but no such receptor appears to be involved in mRNA export. A simple and direct interaction with Xpo-t and RanGTP is the sole requirement for tRNA export whereas snRNA export requires the involvement of CBC20, CBC80 and PHAX for the formation of an export complex with Crm1 and RanGTP.

The existence of novel receptors and pathways of export also highlights the central role that genetic approaches can take in the study of nuclear transport. For example, many components of mRNA export were originally identified in genetic screens (88). The future study of nuclear transport will benefit greatly from development of genetic approaches that can be used to dissect the components of a given transport pathway. The development of small molecule inhibitors of transport receptors or specific nucleoporins will be useful in understanding transport pathways in organisms

that cannot readily be studied by classical genetics-based approaches. The combination of such "biochemical genetics" approaches and classical genetics approaches could have a significant impact in uncovering novel nuclear transport molecules and pathways.

Acknowledgments:

We would like to thank A Belle, W Gilbert, C Guthrie, and K Weis for helpful discussions and comments on the manuscript. AK is a Howard Hughes Medical Institute Predoctoral Fellow. EKO is supported by the Howard Hughes Medical Institute and by the NIH (GM59034)

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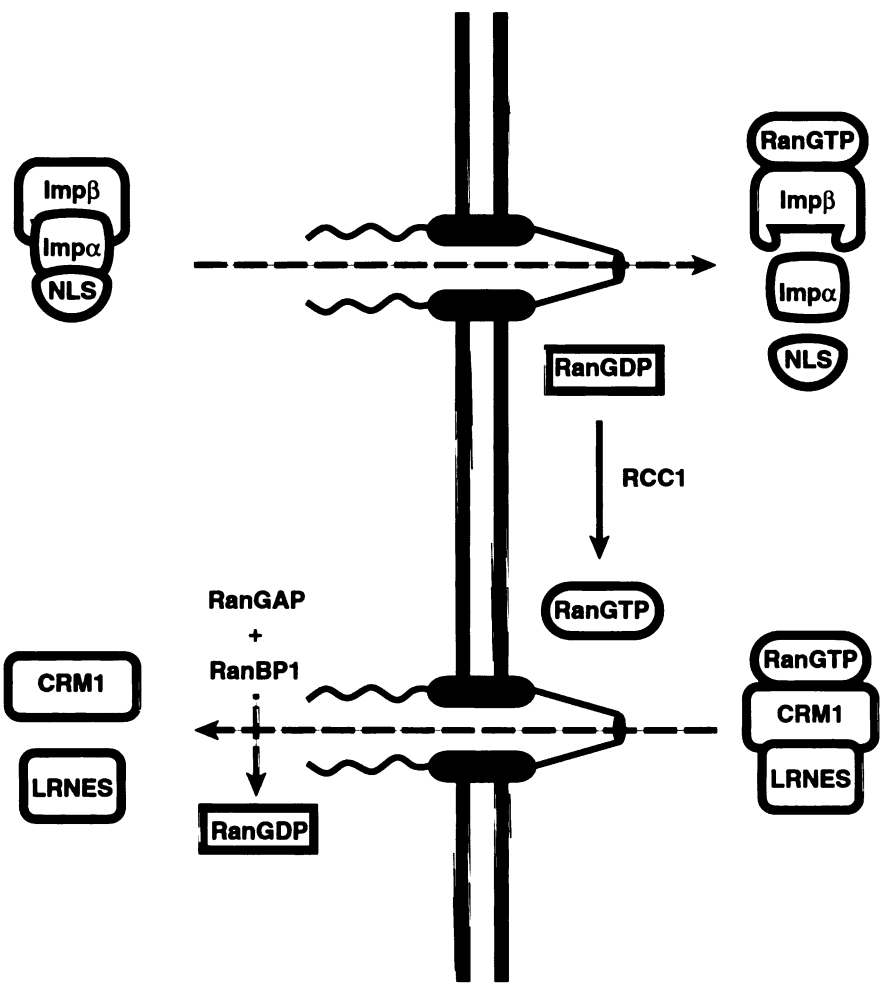
Figure Legends

Figure 1. **Classical model for nuclear transport**.

Figure 2. **Non-classical nuclear transport**. (A) Ran-independent tranport. (B) Msn5 acts as an import and an export receptor. (C) CRT, which is not related to importin β, is an export receptor.

Figure 3. **RNA export**. (A) tRNA export. (B) snRNA export. (C) mRNA export.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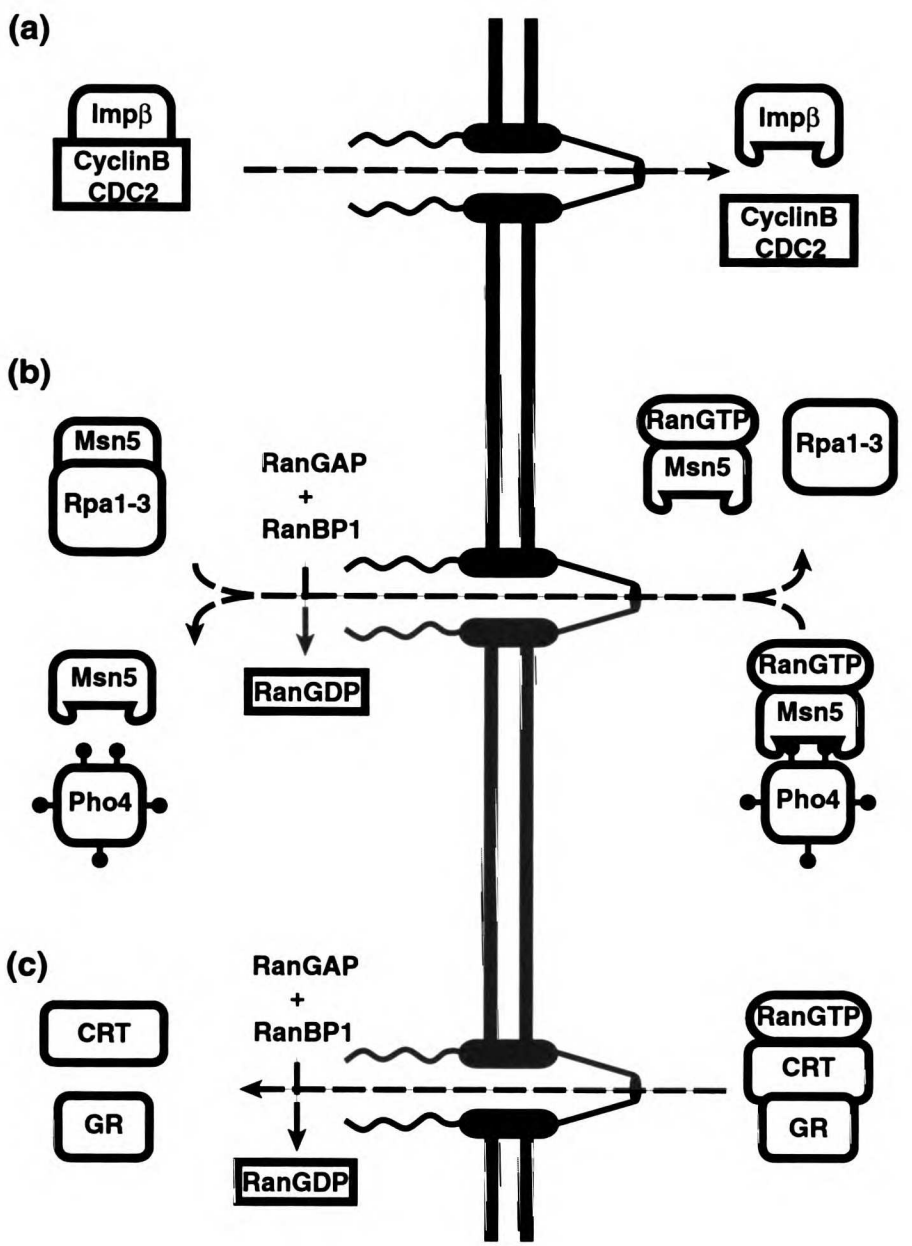
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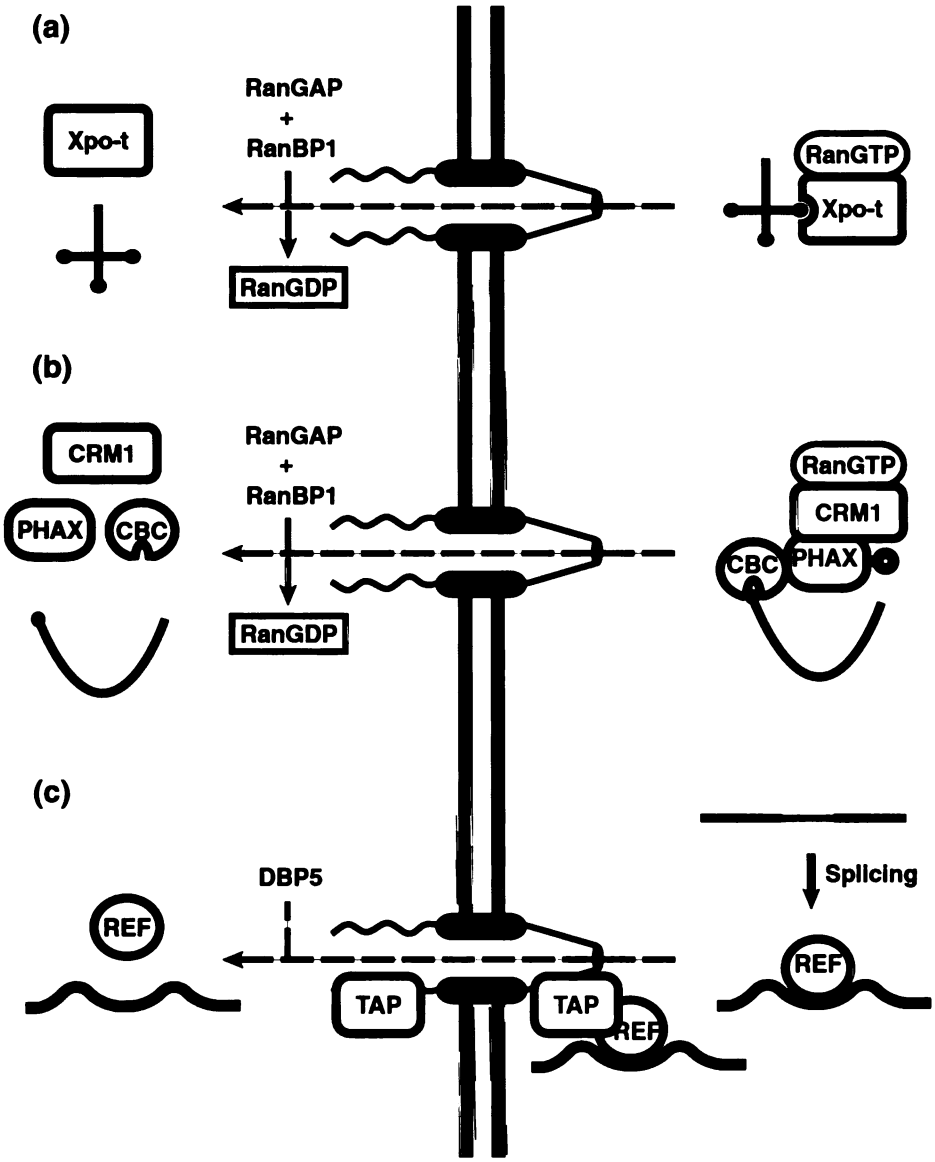
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Nuclear Transport and Transcription

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Abstract:

The compartmentalization of DNA in the nucleus of eukaryotic cells establishes an obligatory connection between the nuclear transport machinery and the transcriptional apparatus. General transcription factors, as well as specific transcriptional activators and repressors, need to be imported into the nucleus subsequent to their translation. In addition, nuclear transport plays a crucial role in regulating the activity of many transcription factors.

Introduction:

Most proteins are transported into and out of the nucleus by members of the β-importin family of nuclear transport receptors **[1*]**. These receptors, which are also known as karyopherins or Kaps, bind to their cargoes and, with the small GTPase Ran, direct their nuclear import and export. Although Ran is found in both the nucleus and the cytoplasm, its effectors are asymmetrically distributed within the cell; the Ran GTPase activating protein (RanGAP) is localized to the cytoplasm and the Ran GTP exchange factor (RanGEF)is bound to chromatin and localized to the nucleus. As a result, nuclear Ran is in the GTP-bound state, whereas cytoplasmic Ran is GDP-bound. The directionality of nuclear transport depends on this RanGTP gradient. Binding of RanGTP to an import receptor results in the release of its cargo. In contrast, an export receptor can only form a complex with its cargo in the presence of RanGTP. Thus, RanGTP acts to release import cargoes into the nucleus and the hydrolysis of GTP on Ran, promoted by RanGAP, acts to release export cargoes in the cytoplasm **[1*]**.

Recent advances in the field of nuclear transport have allowed for more detailed studies of the transport behavior of many transcription factors **[2]**. It is now accepted that localization of a protein can be regulated by controlling its rate of import into the nucleus and/or its rate of export from the nucleus. This realization, coupled with the identification of transport receptors for many factors, has prompted more mechanistic investigations into the regulation of nuclear localization. For example, the nuclear export of many transcription factors can be examined in more detail by use of leptomycin B (LMB), a drug which is a specific inhibitor of the nuclear export receptor Crm1 **[3]**.

This review will focus on recent work that highlights the role of nuclear transport in transcriptional regulation. The first section focuses on the transport of general transcription factors. In the second section, we focus on three examples of specific transcription factors whose activity is controlled at least in part by localization. These examples detail newly discovered mechanisms used to regulate the localization and activity of transcription factors.

General Transcription Factors

TBP and TFIIA

Recently, there has been a substantial amount of progress in uncovering the transport mechanisms used by general transcription factors **[4*, 5, 6]**. Two different approaches identified Kap114 as the nuclear import receptor for TATA Binding Protein (TBP) in *S. cerevisiae***[4*, 5]**. Pemberton et al. used affinity chromatography to identify Kap114 as a TBP-interacting protein **[4*]**. Additionally, Kap114 was identified as a high copy suppressor of TBP temperature-sensitive mutations **[5]**. Kap114 has all the properties

required of an import receptor for TBP. First, its deletion leads to a substantial mislocalization of TBP to the cytoplasm **[4*, 5]**. Second, TBP and Kap114 interact in vitro and in vivo, and this complex can be dissociated by addition of RanGTP **[4*, 5]**. RanGTP dissociation of an import complex is thought to reflect the termination step of nuclear import in vivo **[1*]**. Interestingly, Pemberton et al. found that RanGTP-mediated dissociation of TBP from Kap114 is greatly stimulated by the addition of double-stranded TATA-containing DNA and TFIIA **[4*]**. These results imply that TBP import by Kap114 may be a targeted event terminating at promoters within the nucleus.The import receptor for the general transcription factor TFIIA has also been identified recently in yeast **[6]**. Kap122/Pdr6 binds to the Toa1 and Toa2 proteins, which comprise the *S. cerevisiae* TFIIA complex. Deletion of Kap122 leads to the cytoplasmic accumulation of TFIIA. The binding of TFIIA to Kap122 is also sensitive to RanGTP **[6]**.

TBP and TFIIA are both essential for growth in *S. cerevisiae*. Deletions of Kap114 and Kap122 lead to severe mislocalization of TBP and TFIIA, but deletion of Kap114 or Kap122 is not lethal **[4*, 5, 6]**. These results suggest that cells can survive with very little TBP and TFIIA in the nucleus. Perhaps transient presence of these factors in the nucleus provides sufficient transcriptional activity required for cell survival. Additionally, large amounts of TBP and TFIIA might only be required under certain stress conditions where a large increase in bulk transcription is needed for survival.

These results also imply that although Kap114 and Kap122 account for the majority of TBP and TFIIA imported into the nucleus, alternate mechanisms must exist for targeting of these general transcription factors to the nucleus where they carry out their essential functions. One model is that multiple transport receptors might be used to

import TBP and TFIIA. Consistent with this model, TBP is seen to interact with three other karyopherins. However, it is also possible that these transcription factors can enter the nucleus through interactions with other nuclear proteins that possess nuclear localization signals.

Specific Transcription Factors

p53

The tumor suppressor p53 appears to have evolved a unique method of regulating its localization. In non-stressed cells, p53 is continuously shuttling through the nuclleus and its subcellular distribution varies throughout the cell cycle **[7]**. Under stress condiions, however, p53 is localized to the nucleus where it aids in the transcription of stress response genes **[7]**. Stommel et al. have identified a leucine-rich nuclear export signal (NES) in p53 **[8**]**. Leucine-rich NESs are recognized by the nuclear export receptor Crm1 **[3]**. Addition of LMB to cells expressing p53 disrupts the interaction of the export receptor Crm1 with the leucine rich NES, resulting in localization of p53 to the nucleus. Mutation of the critical leucine residues contained in the p53 NES to alanine also results in p53 localization to the nucleus. Furthermore, this short leucine rich sequence is sufficient for the export of a heterologous protein **[8**]**. Interestingly, the NES coincides with the tetramerization domain of p53. p53 binds to DNA and activates transcription most efficiently as a tetramer. From x-ray crystallographic studies of the p53 tetramerization domain, the leucines of the NES are known to be involved in crucial interactions within the tetramer interface **[9]**. Using peptide crosslinking the authors show that the leucine to alanine mutations in the p53 NES that disrupt its export also

prevent its tetramerization **[8**]**. Tetramerization of p53 would likely mask its NES and block its export. Thus, tetramerization regulates p53 activity at two distinct levels. First, the tetrameric form is a potent activator that can bind DNA. Second, tetramerization masks the p53 NES and ensures that active p53 remains in the nucleus.

The efficient regulation of p53 localization is likely to be important for its function. A significant fraction of p53-related tumors appear to have a defect in p53 localization **[10, 11]**. The authors examine one such tumor, a neuroblastoma, in which p53 is constitutively cytoplasmic. Addition of LMB or coexpression of the tetramerization domain results in nuclear accumulation of p53 in these neuroblastoma cells, suggesting that the localization defect is due to an increase in the export rate of p53.

It is unclear if p53 localization is regulated exclusively by NES masking through tetramerization. It has been proposed that p53 export is mediated by an NES within MDM2, a protein which targets p53 for degradation in the cytoplasm **[12]**. However, Stommel et al. showed that p53 export occurs in a cell line lacking MDM2. It is possible that both tetramerization and interaction with MDM2 contribute to p53 export. The signals and modifications that trigger the dissociation of the p53 tetramer to promote its export have yet to be identified.

NF-AT

NES masking has also been found to be an important aspect of NF-AT regulation **[13**]**. NF-AT is a transcriptional activator whose localization is regulated in response to intracellular calcium levels **[14]**. Under resting conditions, calcium levels are low and NF-AT is phosphorylated and localized to the cytoplasm. When intracellular calcium

levels increase, the phosphatase calcineurin is activated and dephosphorylates NF-AT, resulting in its relocalization from the cytoplasm to the nucleus **[14]**. It had previously been shown that phosphorylation of residues near the NLS of NFAT can prevent its import under resting conditions **[15]**. Upon activation, calcineurin dephosphorylates these sites, allowing for unmasking of the NLS and import of NF-AT into the nucleus **[15]**. Zhou and McKeon have uncovered an additional role for calcineurin in regulating the localization of NF-AT **[13**]**. Using leptomycin B as an inhibitor they show that Crm1 exports NF-AT. The interaction between NF-AT and Crm1 is dependent on two leucine rich sequences within NF-AT. Deletion of these two putative NESs blocks nuclear export of NF-AT. Remarkably, the authors show that these two NESs overlap precisely with the calcineurin binding site on NF-AT, suggesting that calcineurin and Crm1 compete for binding to NF-AT. Consistent with this model, a constitutively active calcineurin prevents Crm1-mediated export of NF-AT. Furthermore, a catalytically inactive calcineurin can prevent the export of NF-AT, indicating that the phosphatase activity of calcineurin is not required for this competition with Crm1.. These results are further corroborated in vitro by direct competition experiments between Crm1 and calcineurin. Zhou and McKeon propose that upon a rise in calcium levels calcineurin binds and dephosphorylates NF-AT, thereby unmasking its NLS. When NF-AT enters the nucleus, its export by Crm1 is inhibited since its NES is masked by the bound, activated calcineurin **[13**]**.

How does nuclear localization contribute to NF-AT transcriptional activity? The authors examine the transcriptional activity of NF-ATΔZ, a mutant that lacks the NLS masking domain **[13**]**. Although NF-ATΔZ is primarily localized to the nucleus, it is

transcriptionally inactive. In contrast, when the NESs of NF-ATΔZ are deleted this mutant is highly active as a transcription factor. These results suggest that localization of NF-AT to the nucleus is not sufficient for inducing its transcriptional activity. The authors attribute the inactivity of the NF-ATΔZ to continual export of the protein by Crm1 **[13**]**. Although NF-ATΔZ can still be exported it is localized predominantly to the nucleus **[13**]**, suggesting that it could spend sufficient time in the nucleus to activate transcription. An alternate hypothesis is that the NESs directly or indirectly interfere with the activation domain of NF-AT. Binding of activated calcineurin to these sequences or their deletion relieves this inhibiton and activates NF-AT.

Pho4

Pho4 is a *S. cerevisiae* transcriptional activator involved in the phosphate starvation response **[16]**. Under limiting phosphate conditions Pho4 is unphosphorylated, localized to the nucleus and, along with a cotranscription factor Pho2, activates transcription of a set of phosphate-responsive genes **[16, 17]**. When cells are in a phosphate-rich environment Pho4 is phosphorylated on five serines by the cyclin-CDK (cyclin-dependent-kinase) complex, Pho80-Pho85, and is rapidly relocalized to the cytoplasm **[17, 18]**.

The import and export receptors for Pho4 have been identified **[19, 20]**. Pho4 is imported by Kap121/Pse1 and exported by Msn5 **[19, 20]**. Remarkably, in vitro, Pse1 associates preferentially with the unphosphorylated form of Pho4 [19]. Msn5, on the other hand, associates exclusively with the phosphorylated form of Pho4 [20]. This

mechanism of regulation ensures that upon phosphorylation, Pho4 is exported to the cytoplasm and its reimport is inhibited.

The role of phosphorylation in regulating Pho4 activity was elucidated further when studies were performed to determine the role of individual phosphorylation sites in regulating localization and transcriptional activity of Pho4 **[21**]**. Phosphorylation of Pho4 on two sites was necessary and sufficient to promote export of Pho4 in vivo and also to promote its interaction with Msn5 in vitro. Phosphorylation of a third site located within the nuclear localization signal (NLS) of Pho4 blocked its interaction with Pse1 and impeded its import into the nucleus. When the phosphorylation sites regulating localization of Pho4 were mutated, Pho4 was localized to the nucleus in high phosphate conditions, but still transcriptionally inactive. Under these conditions Pho4 is nuclear but phosphorylated on a fourth site. To determine if this fourth phosphorylation site plays a role in regulating Pho4 activity in the nucleus, these localization mutations were combined with a mutation in the fourth phosphorylation site. The resulting mutant Pho4 was localized to the nucleus and fully active under phosphate rich conditions. Biochemical studies indicate that phosphorylation of this fourth site prevents both interaction with Pho2 and presumably binding to phosphate-responsive promoters **[21**]**. Therefore, Pho4 is regulated by two distinct mechanisms: nuclear localization and interaction with Pho2.

Conclusions:

We have seen significant progress in the past year in understanding the mechanisms of import of general trasncription factors. The studies mentioned in this

review have identified the molecular components responsible for the nuclear import of TBP and TFIIA **[4**, 5, 6]**. One of the challenges ahead is to determine whether the nuclear transport of these proteins is regulated. There is very little evidence for regulated transport of general transcription factors but in recent work, Miska et al have shown that HDAC4, a human histone deacetylase, can shuttle between the nucleus and cytoplasm **[22]**.

The studies on p53 and NF-AT have established NES masking as a novel regulatory mechanism which might be used by many shuttling proteins **[8**, 13**]**. p53 uses tetramerization to mask its NES whereas binding of calcineurin to NF-AT masks its NES. Prior to the work mentioned in this review, there had been only one other potential example of NES masking as a regulatory mechanism **[23]**. Further characterization of other nuclear export receptors, as well as development of inhibitors of their activity, will likely uncover more examples and mechanistic variations of NES-masking.

Perhaps one of the most surprising results of recent research has been that nuclear localization is not sufficient to regulate the activity of many transcription factors **[13**, 21**]**. Multiple levels of regulation may be important for efficient regulation of transcription. Additionally, the use of multiple regulatory levels could allow for selective expression of a subset of the targets of a transcription factors. For example, Pho4 does not require Pho2 at certain promoters **[24]** and the expression of those genes might depend solely on the localization of Pho4 and not its ability to bind Pho2. Another possibility is that mutiple levels of regulation are important for timing of signal-dependent transcriptional responses. Regulating both localization of Pho4 and its DNA binding activity would allow for its rapid dissociation from DNA and nuclear export

upon a shift from low to high phosphate conditions. Examining the interplay between nuclear localization, the activity of transcription factors, and the timing of transcriptional responses is one of the challenges lying ahead for this field.

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This study dissects the role of phosphoyaltion in regulating the activity of Pho4. Pho4 is inactivated when it is phopshorylated by the Pho80-Pho85 cyclin-CDK complex. The phosphorylations on Pho4 have unique and separable roles in regulating the activity of Pho4. Two sites promote the export of Pho4 and a third site regulates its import. Nuclear localization is not sufficient to regulate Pho4 activity as the phosphorylation of a fourth site regulates its interaction with the cotranscription factor Pho2.

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24. Pho8 paper.

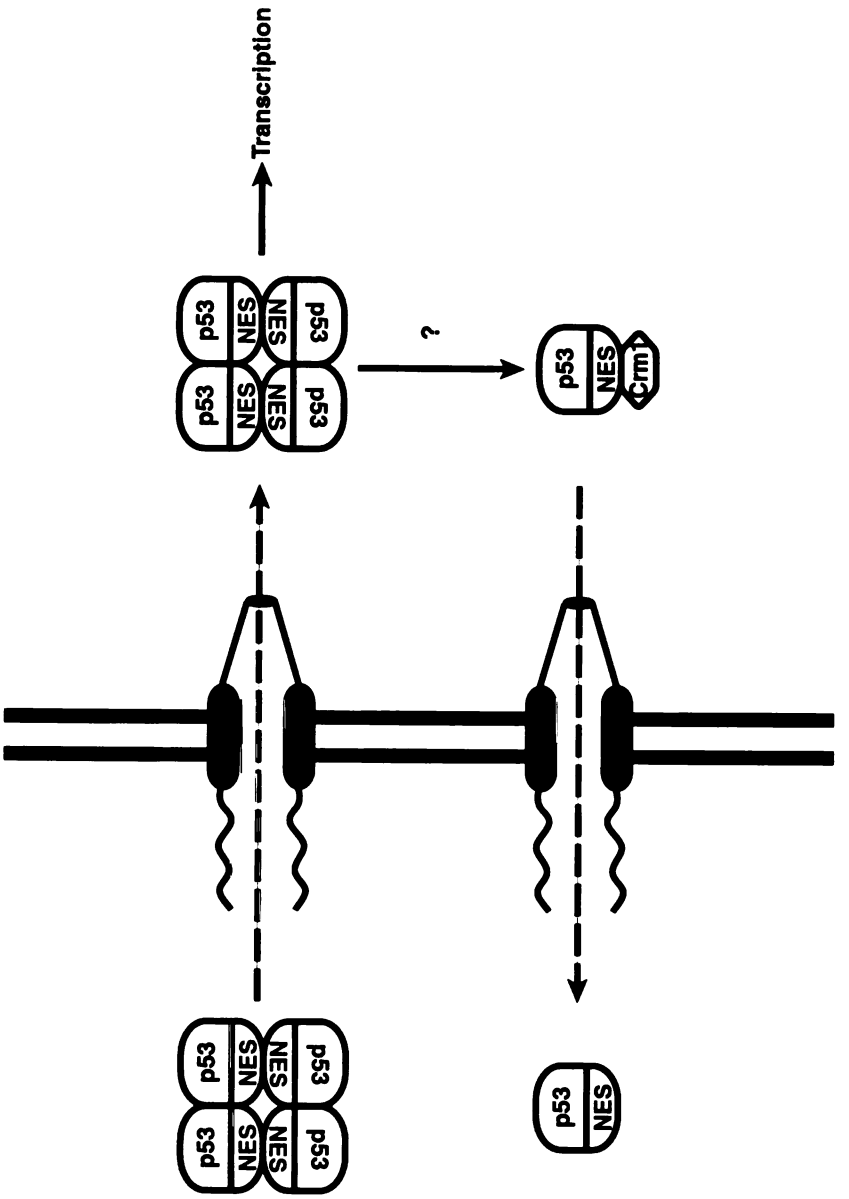
Figure Legends:

Figure 1. Tetramerization masks p53's NES and prevents its export. The p53 NES overlaps precisely with its tetramerization domain. The p53 tetramer is more active transcriptionally than the monomeric form. When p53 tetramerizes its NES is masked and it cannot bind to the export receptor, Crm1. Thus, tetramerization insures that the transcriptionally active form of p53 remains in the nucleus.

Figure 2. Calcineurin regulates NF-AT localization by masking its NES. 1. Under resting conditions calcium levels are low, NF-AT is phosphorylated and its NLS is masked. 2. A rise in calcium levels activates calcineurin which binds and dephosphorylates NF-AT. 3. Dephosphorylation unmasks the NLS leading to NF-AT's import into the nucleus. 4. Since calcineurin is bound to NF-AT's NES, Crm1 cannot export NF-AT and the protein is trasncriptionally active. 5. A drop in calcuium levels inactivates calcineurin leading to its dissociation from NF-AT. The NES is no longer masked and NF-AT is exported out of the nucleus by Crm1.

Figure 3. Phosphorylation regulates Pho4's import, export and ability to bind Pho2. Under low phosphate conditions Pho4 is unphosphorylated and transported into the nucleus by the import receptor Pse1. In the nucleus Pho4 binds to Pho2 and activates the transcription of phosphate responsive genes. A shift to high phosphate conditions activates the Pho80/Pho85 cyclin/CDK complex which phosphorylates Pho4 on five sites. Phosphorylated Pho4 does not bind to Pho2 and thus cannot activate trasncription

of phosphate responsive genes. The export receptor Msn5 recognizes only the phosphorylated form of Pho4 and, with the aid of RanGTP, exports Pho4 out of the nucleus. Phosphorylated Pho4 does not interact with Pse1 and cannot be reimported. Thus, phosphorylation inactivates Pho4 by dissociating it from Pho2, promoting its export, and preventing its reimport.


Transcription
p53
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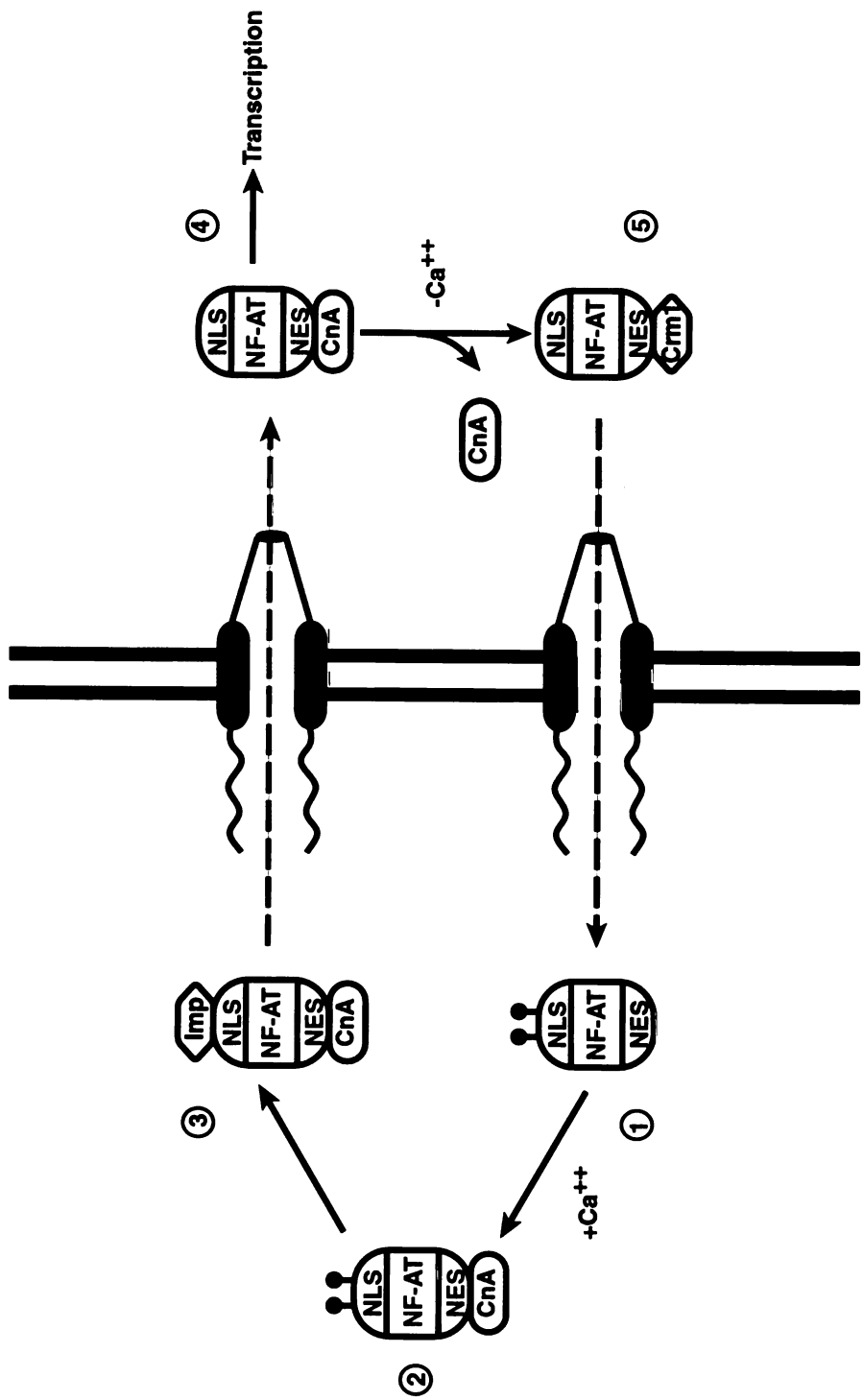
Transcription
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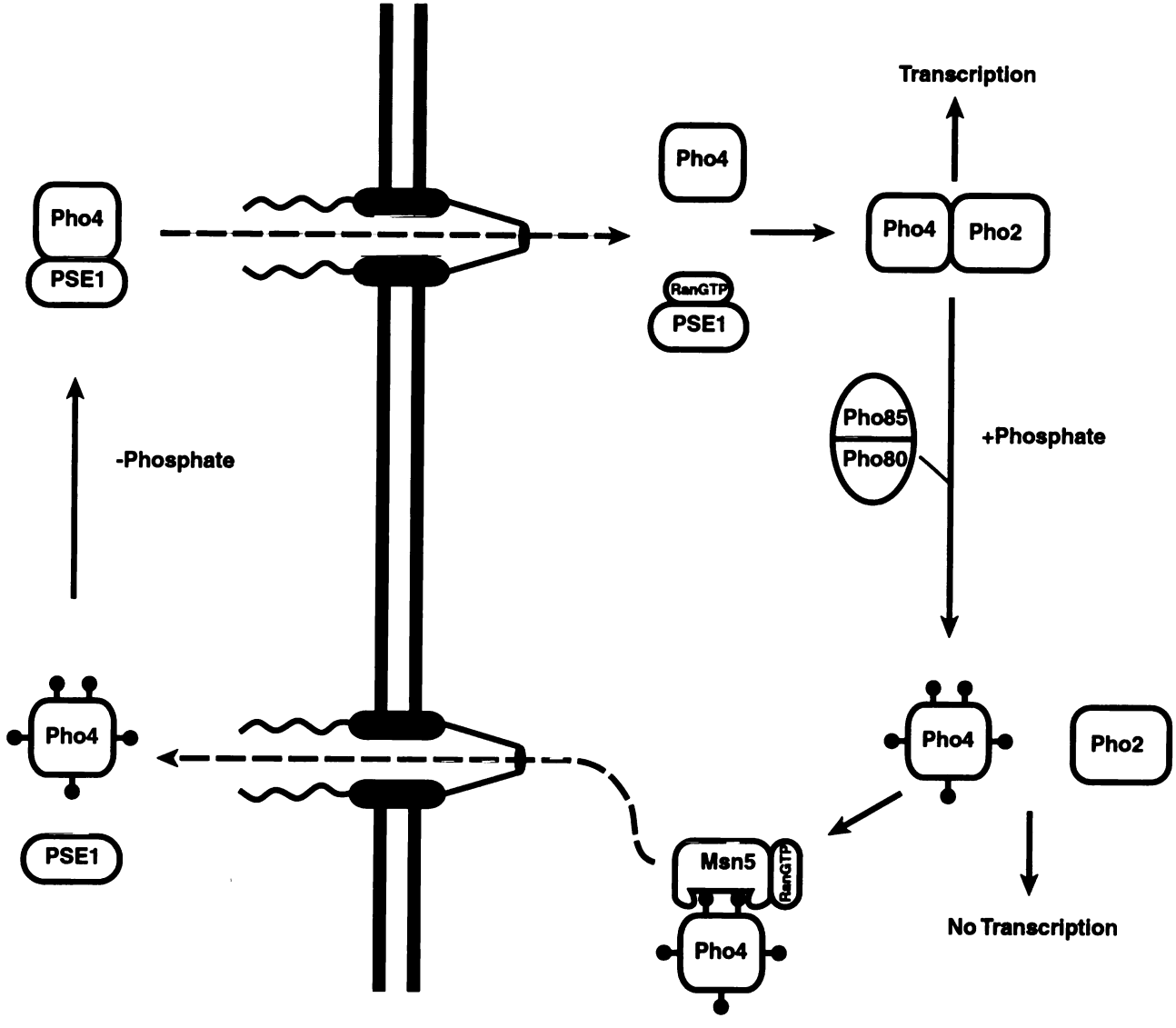
Transcription
Pho4
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Pho2
Pho4
PSE1
RanGTP
PSE1
Pho85
Pho80
+Phosphate
-Phosphate
Pho4
Pho4
Pho2
PSE1
Msn5
RanGTP
Pho4
No Transcription

Roles of Phosphorylation Sites in Regulating Activity of the Transcription Factor Pho4

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Credits:

Erin O'Shea constructed the $Pho4^{SN6}$, $Pho4^{SD6}$, $Pho4^{PA6}$, and $Pho4^{SA1234PA6}$ strains.

Transcription factors are often phosphorylated at multiple sites. Here it is shown that multiple phosphorylation sites on the budding yeast transcription factor Pho4 play distinct and separable roles in regulating the factor's activity. Phosphorylation of Pho4 at two sites promotes the factor's nuclear export, and phosphorylation at a third site inhibits its nuclear import. Phosphorylation of a fourth site blocks the interaction of Pho4 with the transcription factor Pho2. Multiple levels of regulation create a switch-like response that fully inactivates Pho4.

Many signaling pathways rapidly and reversibly convert extracellular signals into changes in gene expression. Phosphorylation of a transcription factor, often at multiple sites, is a common mechanism for responding to signaling events *(1)*. This modification can lead to changes in transcription factor concentration or activity in the nucleus *(2)*. However, the role of multiple phosphorylation sites in regulating the activity of a protein is not well understood.

To study how multiple phosphorylation sites control protein activity, we focused on the regulation of Pho4, a transcription factor in budding yeast that activates expression of genes induced in response to phosphate starvation *(3)*. When yeast cells are grown in phosphate-rich conditions, Pho4 is phosphorylated by the Pho80/Pho85 cyclin-cyclin-dependent kinase (CDK) complex *(4)* and exported to the cytoplasm *(5)*, thereby terminating expression of phosphate-responsive genes. The kinase Pho80/Pho85 phosphorylates Pho4 on five serine-proline (SP) dipeptides, referred to as SP1, SP2, SP3, SP4, and SP6 *(6)*. When yeast cells are starved for phosphate, the CDK inhibitor Pho81 inactivates Pho80/Pho85 *(7)*, leading to the accumulation of unphosphorylated Pho4 in the nucleus *(6)* and the subsequent transcription of phosphate-responsive genes.

Addition of phosphate to a phosphate-starved culture causes rapid phosphorylation and nuclear export of Pho4 fused to the green fluorescent protein *(5)* (Fig. 1A, Pho4-GFP). Export of Pho4 requires phosphorylation by Pho80/Pho85; Pho4 is localized to the nucleus and fully active transcriptionally in strains lacking Pho80 or Pho85 *(6)*. Additionally, the nonphosphorylatable mutant $Pho4^{SA12346}$ (containing serine to alanine substitutions at the five sites of phosphorylation) is constitutively localized to the nucleus and partially active transcriptionally *(6)*. To determine which of the five

phosphorylation sites are required for the export of Pho4, we tested the ability of Pho4 mutants to be exported from the nucleus. Pho4^{SA1}-GFP, Pho4^{SA4}-GFP, and Pho4^{SA6}-GFP, containing an individual serine to alanine substitution at phosphorylation site 1, 4, or 6, had no defect in nuclear export *(8)*. However, Pho4^{SA2}-GFP and Pho4^{SA3}-GFP, containing a serine to alanine substitution at sites 2 and 3, respectively, could not be exported (Fig. 1A). Additionally, Pho4^{SA146}-GFP, a mutant that can only be phosphorylated on sites 2 and 3, was exported from the nucleus upon addition of phosphate (Fig. 1A). Thus, phosphorylation of sites 2 and 3 is necessary and sufficient for nuclear export of Pho4.

Msn5, a member of the β-importin family of nuclear transport receptors, is the export receptor for Pho4 *(5)*. In vitro, Msn5 and the small GTPase Ran (in its GTP bound state) form a stable complex with phosphorylated Pho4, but not with unphosphorylated Pho4 *(5)*. We examined whether phosphorylation of sites 2 and 3 is also required for an interaction with Msn5 in vitro. Pho4^{SA146} and Pho4^{SA23} were tagged with two IgG binding "z" domains derived from Protein A (Pho4^{SA146} -zz and Pho4^{SA23}-zz), phosphorylated in vitro *(9)*, and assayed for Msn5 binding in the presence of Gsp1Q71L, a yeast Ran mutant locked in the GTP-bound form. Pho4^{SA23}-zz failed to interact with Msn5 in either its phosphorylated or unphosphorylated form (Fig. 1B). By contrast, Pho4^{SA146}-zz interacted with Msn5 only when phosphorylated (Fig. 1B). Thus, phosphorylation of Pho4 at sites 2 and 3 is necessary and sufficient to promote binding to Msn5 *(10)*.

Pse1, another member of the β-importin family of transport receptors, is the import receptor for Pho4 *(11)*. Phosphorylation of Pho4 inhibits its interaction with Pse1. Since phosphorylation site 4 is contained within the nuclear localization signal (NLS) of

Pho4 *(11)*, phosphorylation of this site might inhibit the interaction between Pho4 and Pse1. Pho4^{SA4}-zz (a mutant that can be phosphorylated on all sites except site 4) and Pho4^{SA1236}-zz (a mutant that can only be phosphorylated on site 4) were phosphorylated in vitro and assayed for binding to Pse1. Phosphorylated Pho4^{SA4}-zz bound to Pse1 whereas Pho4^{SA1236}-zz failed to bind Pse1 when phosphorylated (Fig. 2A). Thus, phosphorylation of Pho4 at site 4 is necessary and sufficient to disrupt the association of Pho4 and Pse1.

We examined the role of phosphorylation of site 4 in regulating import of Pho4 in vivo. We used a mutant that cannot be exported, because export of Pho4 and a block in its import both lead to its cytoplasmic accumulation. Since phosphorylation of Pho4 by Pho80/Pho85 occurs in the nucleus *(5)*, we attempted to mimic phosphorylation of site 4 by substituting serine with aspartic acid. Pho4^{SA1236SD4}-zz (containing serine to alanine substitutions at sites 1, 2, 3, and 6, and a serine to aspartic acid substitution at site 4) failed to bind Pse1 in vitro (Fig. 2A). To examine the effect of the aspartic acid substitution on import of Pho4 in vivo, expression of Pho4^{SA12346} and Pho4^{SA1236SD4} fused to three tandem copies of GFP (GFP_3) was induced *(12)*, and the localization of these proteins was monitored by fluorescence microscopy. One and one-half hours after induction, Pho4^{SA12346}-GFP_3 remained nuclear whereas Pho4^{SA1236SD4}-GFP_3 was mainly cytoplasmic *(13, 14)* (Fig. 2B). Thus, phosphorylation at site 4 inhibits nuclear import of Pho4.

If control of nuclear localization is the only mechanism by which phosphorylation regulates the activity of Pho4, then Pho4 that is localized to the nucleus should activate transcription of phosphate-responsive genes in both high and low phosphate conditions. Therefore, we measured production of the secreted acid phosphatase Pho5 in a strain

expressing Pho4^{SA1234}, a mutant containing serine to alanine substitutions at sites 1, 2, 3, and 4 *(15)* that was constitutively localized to the nucleus (Fig. 3A). Although expression of acid phosphatase was elevated in yeast expressing Pho4^{SA1234} grown in phosphate-rich medium *(16)*, it was further induced in response to phosphate starvation (Fig. 3B). Additionally, a *msn5*Δ strain, in which Pho4 is constitutively localized to the nucleus because it cannot be exported, produces high levels of acid phosphatase when starved for phosphate *(8)*, but not when grown in phosphate-rich medium *(5)*. Thus, another mechanism, distinct from control of its localization, regulates the activity of Pho4.

The only site that can be phosphorylated in the Pho4^{SA1234} mutant is site 6. We constructed a mutant Pho4 that could not be phosphorylated on site 6 by making a proline to alanine substitution in the serine-proline dipeptide corresponding to phosphorylation site 6 (Pho4^{PA6}) *(17)*. We did not use a serine to alanine substitution to prevent phosphorylation of site 6 because the Pho4^{SA6} mutant is not fully functional in activating transcription of acid phosphatase *(18)*. Localization of Pho4^{PA6}-GFP was regulated in response to phosphate levels (Fig. 3A) and Pho4^{PA6} was fully functional as a transcriptional activator (Fig. 3B). We combined the mutations that cause Pho4 to be constitutively localized to the nucleus with the proline to alanine mutation at site 6 to create Pho4^{SA1234PA6} (Fig. 3A). In contrast to a strain expressing Pho4^{SA1234}, a strain expressing Pho4^{SA1234PA6} produced acid phosphatase at nearly fully induced levels when grown in high phosphate medium *(19)* (Fig. 3B). Additionally, a strain lacking the export receptor Msn5 and expressing Pho4^{PA6} produced high levels of acid phosphatase when grown in phosphate-rich medium *(8)*. Thus, phosphorylation of site 6 provides an

additional mode for regulating the activity of Pho4. These observations suggest that phosphorylation by Pho80/Pho85 is the primary mode of regulating Pho4 in response to phosphate availability *(20)*.

Phosphorylation site 6 lies within a region of Pho4 involved in binding to the transcription factor Pho2 *(21)*. Pho2 is required for transcription of *PHO5 (3)*, interacts with Pho4, and binds cooperatively with Pho4 to the *PHO5* promoter *(22)*. To determine if phosphorylation of site 6 modulates the interaction between Pho4 and Pho2, we phosphorylated a Pho4-zz fusion protein in vitro and assayed for its binding to Pho2. Pho2 bound to unphosphorylated Pho4-zz, but not to phosphorylated Pho4-zz, indicating that phosphorylation of Pho4 inhibits its interaction with Pho2 (Fig. 3C). $Pho4^{SA1234}$-zz, which can only be phosphorylated on site 6, bound to Pho2 when unphosphorylated, but not when phosphorylated (Fig. 3C). Additionally, $Pho4^{PA6}$-zz, a mutant that can be phosphorylated on all sites except site 6, bound to Pho2 independent of its phosphorylation state (Fig. 3C). Thus, phosphorylation of site 6 is necessary and sufficient to inhibit interaction of Pho4 with Pho2 *(23)*.

Regulation of nuclear localization and regulation of the interaction with Pho2 act partially redundantly to control the activity of Pho4; yeast expressing either $Pho4^{PA6}$ (regulated only by nuclear localization) or $Pho4^{SA1234}$ (regulated only by control of the interaction with Pho2) induce transcription of the acid phosphatase Pho5 in response to phosphate starvation (Fig. 3B). Although overlapping, both levels of regulation are required for complete repression of Pho5 expression, since acid phosphatase expression is not completely repressed in yeast expressing $Pho4^{SA1234}$ or $Pho4^{PA6}$ (Fig. 3B). Therefore, multiple phosphorylation sites may exist to ensure complete shutoff of transcription.

The phosphorylation events that modify Pho4 have unique and separable roles in regulating the protein's export, import, and ability to activate transcription in the nucleus (Fig. 4). Multiple levels of regulation cooperate to create a switch-like response that fully inactivates Pho4. Many transcription factors, CDK inhibitors, and other regulatory proteins are phosphorylated on multiple sites, but the role of these phosphorylation events is not well understood. Phosphorylation may provide multiple levels of control that are important for efficient regulation of proteins other than Pho4.

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9. Previous studies demonstrate that Pho4 mutants containing serine to alanine substitutions of a subset of the phosphorylation sites can be efficiently phosphorylated in vitro *(6)*.

10. Phosphorylation of sites 2 and 3 cannot be mimicked by serine to aspartic acid mutations. $Pho4^{SD23}$-GFP, containing serine to aspartic acid substitutions at sites 2 and 3, is not exported from the nucleus *(8)*.

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12. Three tandem copies of GFP were used in order to: (i) enhance the fluorescence signal and (ii) slow down the import of the two proteins to facilitate detection of a difference in their nuclear import.

13. The following observations suggest that $Pho4^{SA1236SD4}$-GFP_3 is not cleaved by proteolysis to release cytoplasmic GFP and is a functional transcription factor. (i) Both

Pho4^{SA1236SD4}-GFP and Pho4^{SA12346}-GFP are localized to the nucleus at steady-state when expressed constitutively from the *PHO4* promoter (on a *CEN/ARS* plasmid) *(8)*. (ii) When grown in phosphate-rich medium, a *pho4Δ* strain containing Pho4^{SA1236SD4}-GFP or Pho4^{SA12346}-GFP expresses acid phosphatase at slightly elevated levels compared to a *pho4Δ* strain containing wild-type Pho4-GFP *(8)*. (iii) Western blotting analysis of extracts derived from strains expressing either Pho4^{SA1236SD4}-GFP$_3$ or Pho4^{SA12346}-GFP$_3$ indicate there is a similar amount of each full-length protein and a minor population of degraded protein *(8)*.

14. Substitution of site 4 with glutamic acid slowed import of Pho4 in response to phosphate starvation. Pho4^{SE4}-GFP expressed under the control of the *PHO4* promoter on a *CEN/ARS* plasmid was still cytoplasmic after two hours of phosphate starvation. In contrast, wild-type Pho4-GFP was imported into the nucleus in less than 30 min after phosphate starvation *(8)*. Additionally, a *pse1-1* strain, defective for import of Pho4, cannot induce expression of acid phosphatase in response to phosphate starvation.

15. In mapping the five sites of phosphorylation site 1 was found to be phosphorylated poorly in vitro *(6)*. Thus, this phosphorylation site may not play a physiologically relevant role in regulating Pho4 activity. Additionally, the Pho4^{SA1}-GFP mutant has no defect in localization or regulation of acid phosphatase expression *(8)*.

16. A *pho4Δ* strain expressing Pho4^{SA2}-GFP or Pho4^{SA3}-GFP, which cannot be exported from the nucleus, produced elevated levels of acid phosphatase in high phosphate conditions *(8)*. By contrast, yeast expressing Pho4^{SA4}-GFP, which can be exported but whose import cannot be inhibited by phosphorylation, regulated acid phosphatase expression appropriately *(8)*.

17. We believe that the proline to alanine mutation at site 6 prevents phosphorylation because: (i) phosphorylation of substrates by CDKs requires a proline preceding the residue of phosphorylation [R. B. Pearson and Bruce E. Kemp, *Meth. Enzymol.* **200**, 62 (1991)]; and (ii) Pho4^{SA1234PA6} does not undergo a shift in its electrophoretic mobility after incubation with Pho80/Pho85 and ATP, suggesting that it cannot be phosphorylated *(8)*.

18. The observation that Pho4^{SA6} is not fully functional in activating transcription of acid phosphatase helps to explain our previously published results that Pho4^{SA12346} is a weak activator of transcription *(6)*.

19. A *pho4*Δ *pho80*Δ strain expressing Pho4^{SA1234} also produces fully induced levels of acid phosphatase when grown in phosphate-rich medium (Fig. 3B). Thus, the additional mode of regulation requires Pho80, as expected if the regulation involves phosphorylation.

20. Our new studies of Pho4^{SA1234PA6} (Fig. 3B) and our previous studies of Pho4^{SA12346} *(6)* indicate that a phosphorylation-independent mode of regulation of Pho4 exists, but it makes a minor contribution to the regulation of Pho5. We estimate the contribution of the phosphorylation-independent regulation to be less than two-fold - this is the ratio of the level of acid phosphatase produced by a strain expressing Pho4^{SA1234PA6} (or Pho4^{SA12346} *(8)*) grown in low phosphate (or in a strain lacking Pho80), divided by the level of acid phosphatase produced when this strain is grown in high phosphate conditions. In contrast, the contribution of the phosphorylation-dependent regulation is ~70-fold - this is the ratio of the level of acid phosphatase produced in a strain expressing a Pho4^{SA1234PA6}, divided by the level of acid phosphatase produced in a strain expressing wild-type Pho4, both grown in high phosphate conditions. Our previous studies *(6)* underestimated the

phosphorylation-dependent contribution to Pho5 regulation for two reasons: (i) These studies were performed using a strain producing the constitutively-expressed acid phosphatase Pho3, which makes it difficult to accurately measure the levels of Pho4-dependent acid phosphatase production in high phosphate conditions. (ii) The previous studies measured the activity of $Pho4^{SA12346}$, which is not completely functional transcriptionally due to the SA6 mutation *(18)*. A source of phosphorylation-independent regulation has been proposed - it has been suggested that Pho80 can negatively regulate Pho4 in high phosphate conditions by directly binding to and masking its activation domain [P. S. Jayaraman, K. Hirst, C. R. Goding, *EMBO J.* **13**, 2192 (1994)].

Our conclusions rely on the assumption that the PA6 mutation does not affect the phosphorylation-independent mode of regulation. For the following reasons we do not believe that the PA6 mutation renders Pho4 defective in its ability to interact with and be masked by Pho80: (i) Export of $Pho4^{PA6}$-GFP occurs with the same kinetics as wild-type Pho4 *(8)*, suggesting that the ability of this mutant to interact with and be phosphorylated by Pho80 is similar to wild-type Pho4. (ii) Phosphorylation site 6 is in a domain involved in interaction with Pho2 *(21)*, not with Pho80 [P. S. Jayaraman, K. Hirst, C. R. Goding, *EMBO J.* **13**, 2192 (1994)].

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23. $Pho4^{SA6}$ and $Pho4^{SD6}$ did not bind efficiently to Pho2 and did not efficiently activate expression of acid phosphatase *(8)*. A *pho4*Δ strain expressing $Pho4^{SN6}$,

containing a serine to asparagine mutation at site 6, produced acid phosphatase and bound Pho2 at a level in between the $Pho4^{SA6}$ and $Pho4^{PA6}$ mutants *(8)*. Additionally, unphosphorylated $Pho4^{SA1234}$ and $Pho4^{SA1234PA6}$ interacted efficiently with Pho2 (Fig. 3C)*(8)* and activated transcription of acid phosphatase (Fig. 3B). Thus, alanine substitutions at serines 1, 2, 3, and 4 do not affect the interaction with Pho2 and the serine at site 6 is important for the interaction between Pho4 and Pho2.

24. Pho4-GFP *(11)* and the Pho4 mutants fused to GFP were expressed under the control of the *PHO4* promoter on the *CEN/ARS* plasmid pRS316 in a *pho4Δ* strain *(27)*. Phosphate starvation experiments and microscopy were performed as described *(11)*. For the phosphate feed assay KH_2PO_4 was added to a final concentration of 20 mM. Pictures were taken 5 to 10 min following the addition of phosphate.

25. Phosphorylation of Pho4-zz and binding to Msn5-His_6 was performed essentially 7as described *(5)* except that 5 μM His_6-Gsp1Q71L was used in place of 1 μM Gsp1. His_6-Gsp1Q71L was purified as described for Pse1-His_6 *(11)*.

26. A *pho4Δ* strain *(27)* expressing $Pho4^{SA12346}$-GFP_3 or $Pho4^{SA1236SD4}$-GFP_3 *(12)* under the control of the *GAL1-10* promoter was grown in synthetic raffinose medium at 30°C to log phase. No fluorescence is visible under noninducing conditions when cells are grown in raffinose. Expression of each fusion protein was induced by addition of galactose to a final concentration of 2% and localization was monitored as a function of time by fluorescence microscopy *(11)*. The photo was taken 1.5 hours after the addition of galactose.

27. All yeast strains are derived from K699 *MAT***a** [K. Nasmyth, G. Adolf, D. Lydall, A. Seddon, *Cell* **62**, 631 (1990)]. *PHO3* encodes an acid phosphatase expressed in

phosphate rich conditions [A. Toh-e, Y. Ueda, S.-I. Kakimoto, Y. Oshima, *J. Bacteriol.* **113**, 727 (1973)].

28. Pho4 and each Pho4 mutant (not fused to GFP) were expressed in *pho4Δpho3Δ* or *pho4Δ pho80Δpho3Δ* yeast strains *(27)* under the control of the *PHO4* promoter on the *CEN/ARS* plasmid YCp50 [M. Johnston and R.W. Davis, *Mol. Cell. Biol.* **4**, 1440 (1984)]. Five-milliliter cultures were grown in synthetic dextrose media lacking uracil for 12 to 16 hours and diluted to an OD_{600} of 0.1. For high phosphate experiments the diluted cultures were grown to an OD_{600} of ~1.0. For phosphate starvation experiments the diluted cultures were grown to an OD_{600} of 0.5, centrifuged, washed, resuspended in low phosphate synthetic medium *(11)* and grown for 6 hours. Liquid acid phosphatase assays were performed essentially as described [A. Toh-e, Y. Ueda, S.-I. Kakimoto, Y. Oshima, *J. Bacteriol.* **113**, 727 (1973)]. One-tenth and 1/20 of the cultures were used and the assay was performed in a volume of 820 μL. The units are the ratio of A_{420} to OD_{600} values.

29. Twenty microliters of Protein G-Sepharose beads were incubated with 30 μg of 12CA5 anti-HA antibodies for 45 min and then washed twice with PBS [150 mM NaCl, 10 mM Na phosphate (pH 7.4)] + 0.1% NP40. Antibody beads were incubated with 750 μg of yeast extract overexpressing HA-Pho80 *(4)* for 1 hour. The immunoprecipitates were washed three times with PBS + 0.1% NP40, once with PBS, and once with kinase buffer [20 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$]. Five micrograms of Pho4-zz, 5 μL of 10x kinase buffer, and 5 μL of 10 mM ATP were added to each reaction and the final volume was adjusted to 50 μL with IgG buffer [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.05% Tween 20, 5 mM $MgCl_2$]. ATP was omitted from the mock

phosphorylation reactions. Reactions were carried out at room temperature for 1 hour and phosphorylated or mock phosphorylated Pho4-zz was obtained by collecting the supernatant. Binding of Pho4-zz to Pho2-His_6 was performed essentially as described for Pse1-His_6 *(11)*. The plasmid expressing Pho2-His_6 and its purification from *E. coli* have been described elsewhere [R. M. Brazas and D. J. Stillman, *Mol. Cell. Biol.* **13**, 5524 (1993)]. Pho2-His_6 was used at a concentration of 6 nM for each reaction. Ten percent of the 1 M $MgCl_2$ elutions were run on 7.8% SDS polyacrylamide gels and transferred to nitrocellulose membranes. Pho2 was detected by Western blotting with polyclonal anti-Pho2 antibodies.

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31. We thank M. Maxon for the gift of purified Pho2-His_6 and K. Weis for the plasmid expressing His_6-Gsp1Q71L; A. Kaffman for particularly helpful discussions; and C. Gross, J. Weissman, R. Tjian, and members of the O'Shea lab for helpful comments on the manuscript. Supported by the David and Lucile Packard Foundation (E.K.O.). A.K. was supported by a Howard Hughes Medical Institute Predoctoral Fellowship.

Figure Legends:

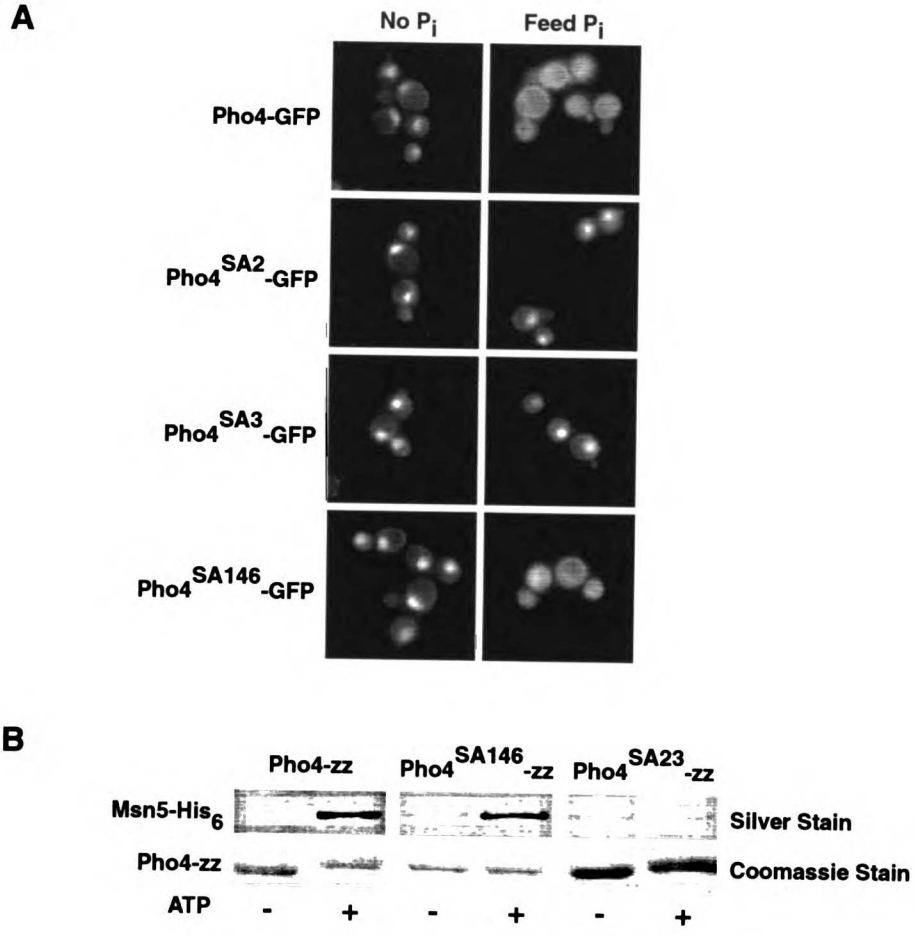
Fig. 1. Phosphorylation of sites 2 and 3 promotes nuclear export of Pho4. **(A)** Localization of wild-type Pho4-GFP, or the indicated Pho4 mutants fused to GFP, in cells grown in no phosphate medium. For the "feed P_i" sample, phosphorylation and nuclear export of Pho4-GFP were triggered by addition of phosphate to a culture that had been grown in no phosphate medium *(24)*. **(B)** Wild-type Pho4 and the indicated Pho4 mutants, joined to two IgG binding z domains derived from Protein A (Pho4-zz), were phosphorylated (+ ATP) or mock phosphorylated (- ATP) in vitro, immobilized on IgG Sepharose, and binding to Msn5-His_6 was measured *(25)*. The amount of bound Msn5-His_6 was analyzed on a Silver-stained SDS-PAGE gel. The amount of immobilized Pho4-zz was analyzed on a Coomassie-stained SDS-PAGE gel.

Fig. 2. Phosphorylation of site 4 inhibits nuclear import of Pho4. **(A)** Wild-type Pho4-zz and the indicated Pho4-zz mutants were phosphorylated (+ ATP) or mock phosphorylated (- ATP) in vitro, immobilized on IgG Sepharose, and binding to Pse1-His_6 was measured *(11)*. The amount of bound Pse1-His_6 was analyzed on a Silver-stained SDS-PAGE gel. The amount of immobilized Pho4-zz was analyzed on a Coomassie-stained SDS-PAGE gel. The band below Pse1-His_6 is an NH_2-terminally truncated form of the protein (*). **(B)** Expression of $Pho4^{SA12346}$-GFP_3 or $Pho4^{SA1236SD4}$-GFP_3 was induced and localization was monitored by fluorescence microscopy *(26)*.

Fig. 3. Pho4 is regulated by a mechanism distinct from control of its nuclear localization. **(A)** Localization of $Pho4^{PA6}$-GFP, $Pho4^{SA1234}$-GFP, and $Pho4^{SA1234PA6}$-GFP in cells grown

in no or high phosphate medium *(24)*. **(B)** Measurement of Pho5 acid phosphatase enzyme activity in either *pho4Δ pho3Δ* or *pho4Δ pho80Δ pho3Δ* yeast strains *(27)* transformed with a low-copy plasmid expressing the indicated Pho4 mutant *(28)*. The *pho4Δ pho3Δ* strain expressing the indicated Pho4 mutant was grown in high (black boxes) or low phosphate (white boxes) medium and the *pho4Δ pho80Δ pho3Δ* strain was grown in high phosphate medium (gray boxes). **(C)** Wild-type Pho4-zz and the indicated Pho4-zz mutants, were phosphorylated (+ ATP) or mock phosphorylated (- ATP) in vitro, immobilized on IgG-Sepharose, and binding to Pho2-His_6 was measured *(29)*. The amount of bound Pho2-His_6 was analyzed by SDS-PAGE followed by Western blotting using anti-Pho2 antibodies. The amount of immobilized Pho4-zz was analyzed on a Coomassie-stained SDS-PAGE gel.

Fig. 4. Phosphorylation events regulate Pho4 by distinct and separable mechanisms. Sites of phosphorylation consist of five serine-proline dipeptides labeled SP1, SP2, SP3, SP4, and SP6 (amino acids 100, 114, 128, 152, and 223) *(6)*. The activation and DNA binding domains are indicated *(30)*. Sites 2 and 3 regulate nuclear export, site 4 regulates import, and site 6 regulates the interaction with the transcription factor Pho2. We have not been able to determine a function for phosphorylation site 1 *(15)*.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Feed P_i
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B
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$Pho4^{SA146}$-zz
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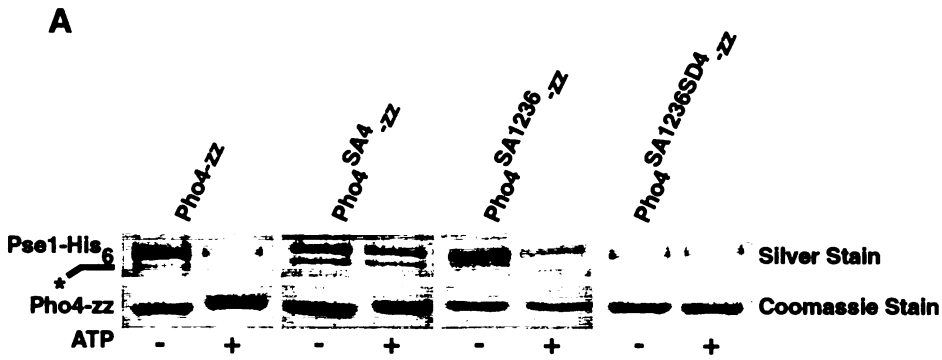
Coomassie Stain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Pho4 SA4 -zz
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Pse1-His6
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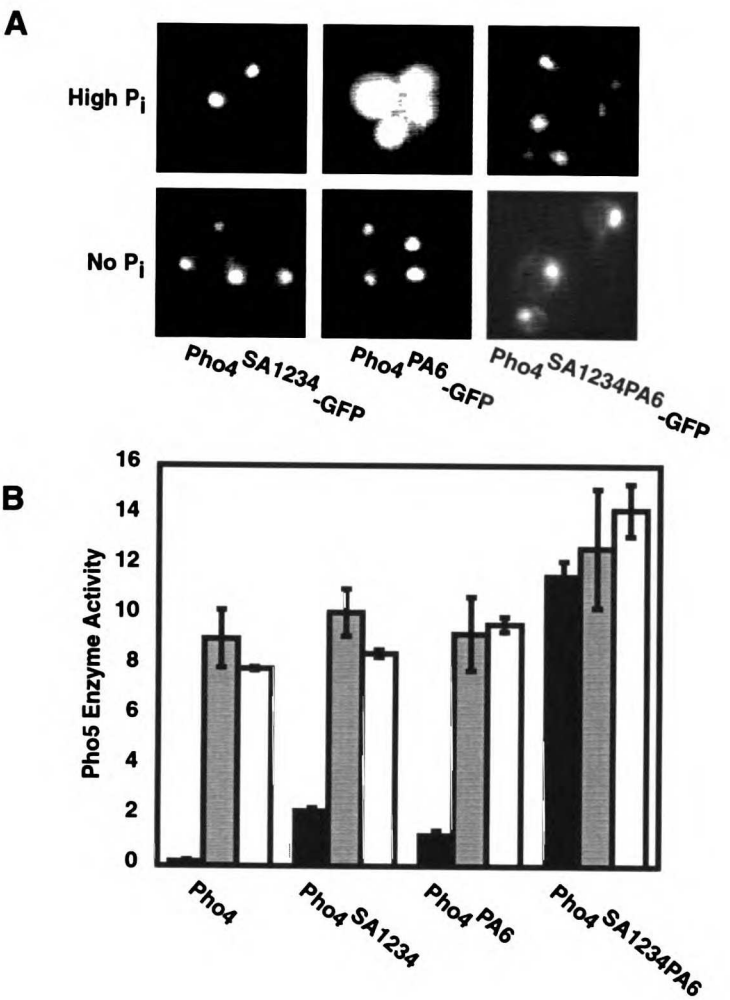
Pho4SA12346-GFP3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High P_i
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Pho4SA1234-GFP
Pho4PA6-GFP
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14
12
10
8
6
4
2
0
Pho4
Pho4SA1234
Pho4PA6
Pho4SA1234PA6
C
Pho4-zz
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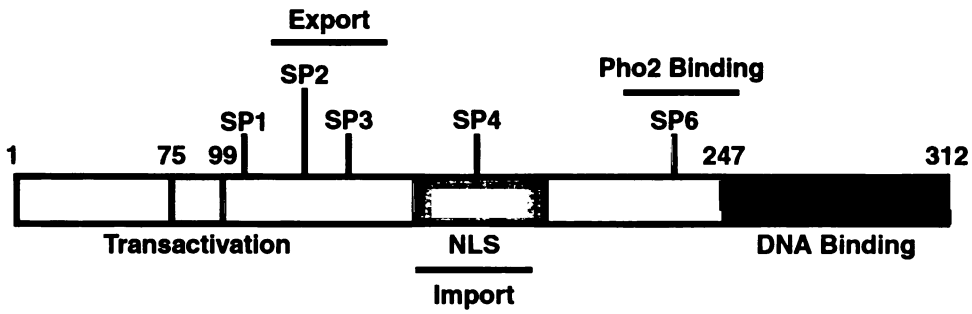
- + - + - +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SP2
Pho2 Binding
SP1
SP3
SP4
SP6
1
75
99
247
312
Transactivation
NLS
DNA Binding
Import

Mechanism of metabolic control: TOR signaling links nitrogen quality to the activity of the Rtg1 and Rtg3 transcription factors

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Running Title: Nitrogen-dependent control of Rtg1 and Rtg3 by TOR

Character count: 66,385

Keywords: gene expression, metabolism, phosphorylation, rapamycin, signal transduction

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This work is the result of a collaboration between our lab and the laboratory of Ted Powers at UC Davis. Ted Powers and Karen Wedaman performed the experiments depicted in figures 1, 2, 3, 6, and 8B. I performed the experiments shown in figures 4, 5, 7, and 8A. Erin O'Shea constructed several strains and performed many of the initial experiments.

Abstract

De novo biosynthesis of amino acids utilizes intermediates provided by the TCA cycle that must be replenished by anaplerotic reactions in order to maintain the respiratory competency of the cell. Genome-wide expression analyses in *S. cerevisiae* reveal that many of the genes involved in these reactions are repressed in the presence of the preferred nitrogen sources glutamine or glutamate. Expression of these genes in media containing urea or ammonia as a sole nitrogen source requires the heterodimeric bZip transcription factors Rtg1 and Rtg3 and correlates with a redistribution of the Rtg1p/Rtg3 complex from a predominantly cytoplasmic to a predominantly nuclear location. Nuclear import of the complex requires the cytoplasmic protein Rtg2, a previously identified upstream regulator of Rtg1 and Rtg3, whereas export requires the importin-β-family member Msn5. Remarkably, nuclear accumulation of Rtg1/Rtg3, as well as expression of their target genes, is induced by addition of rapamycin, a specific inhibitor of the TOR kinases. We demonstrate further that Rtg3 is a phosphoprotein and that its phosphorylation state changes following rapamycin treatment. Taken together, these results demonstrate that TOR signaling regulates specific anaplerotic reactions by coupling nitrogen quality to the activity and subcellular localization of distinct transcription factors.

Introduction

Normal cell growth requires that cells adjust their metabolic activity according to nutrient availability and other environmental cues. Specialized signal transduction mechanisms exist which enable cells to perceive and integrate these cues in order to establish and/or maintain appropriate patterns of gene expression. Understanding how these pathways function is thus important for understanding both normal cellular behavior as well as the underlying basis of many human diseases, including cancer.

One important signaling pathway used by all eukaryotic cells is the TOR (target of rapamycin) kinase pathway. This pathway was discovered through the action of the antibiotic rapamycin, a potent inhibitor of T-cell proliferation, which combines with the small immunophilin FKBP and targets the large, evolutionarily conserved TOR kinase (Thomas and Hall, 1997; Dennis et al., 1999). Rapamycin inhibits the growth of a wide variety of cell types and organisms, including the budding yeast, *S. cerevisiae*. Yeast contains two TOR genes, *TOR1* and *TOR2*, and the products of both are inhibited by the rapamcyin/FKBP complex (Heitman et al., 1991; Helliwell et al., 1994; Zheng et al., 1995). Treating yeast cells with rapamycin has several distinctive effects that mimic nutrient starvation, including inhibition of protein synthesis, cell cycle arrest at the G1/S boundary, onset of autophagy, and inhibition of ribosomal biogenesis (Heitman et al., 1991; Helliwell et al., 1994; Zheng et al., 1995; Barbet et al., 1996; Noda and Ohsumi, 1998; Zaragoza et al., 1998; Powers and Walter, 1999).

Recent studies have shown that TOR kinase activity is essential for the transcription of ribosomal RNA and ribosomal protein genes, as well as for the

modulation of r-protein gene expression in response to changes in nutrient sources (Zaragoza et al., 1998; Powers and Walter, 1999). These results demonstrate that control of gene expression at the level of transcription represents an important branch of TOR signaling. This conclusion has been extended dramatically by results of recent microarray studies where the expression of several hundred genes has been shown to be affected when TOR function is inhibited by rapamycin (Cardenas et al., 1999; Hardwick et al., 1999). In addition to genes involved in protein biosynthesis, many of the affected genes are involved in the glycolytic pathway, the TCA cycle, and nitrogen metabolism. Thus one important role of TOR appears to be the coordination of the transcription of genes involved in several distinct nutrient-responsive cellular pathways.

One of the most striking sets of genes affected by rapamycin treatment is composed of genes involved in the utilization of different sources of assimilable nitrogen (Cardenas et al., 1999; Hardwick et al., 1999; C. Kao, T. Powers, P. Walter, G. Crabtree, P. Brown, unpublished results). For example, a sharp decrease is observed in the expression of genes involved in the uptake and metabolism of preferred nitrogen sources, including glutamine and asparagine. In contrast, a corresponding increase is observed in the expression of genes involved in the uptake and utilization of alternative nitrogen sources, including urea, proline, and allantoin (a product of purine catabolism). These results establish a link between TOR signaling and nitrogen metabolism and suggest that one crucial role of this pathway is to couple nitrogen availability to continued cell growth. Moreover, they are consistent with results from studies of mammalian cells that demonstrate that TOR signaling is influenced by amino acid availability (Iiboshi et al., 1999b). Taken together, these results are particularly relevant given that levels of

preferred nitrogen sources in human cells (e.g. glutamine and asparagine) play an important role in the progression of several diseases, including childhood acute lymphoblastic leukemia (ALL) and these levels may be regulated, at least in part, by TOR (Iiboshi et al., 1999a).

The molecular mechanism by which TOR controls the expression of several genes involved in nitrogen metabolism, including *GLN1*, *MEP2*, and *GAP1*, has been shown recently to involve regulated changes in the subcellular localization of the Gln3 transcription factor (Beck and Hall, 1999). Thus, in the presence of preferred sources of nitrogen, Gln3 is sequestered in the cytoplasm by association with the Ure2 protein, a previously identified negative regulator of Gln3 function. TOR promotes formation of a Gln3-Ure2 complex by a mechanism that involves inhibition of the Sit4 phosphatase and that requires the TOR effector protein Tap42 (Di Como and Arndt, 1996; Beck and Hall, 1999; Jiang and Broach, 1999). Treating yeast cells with rapamycin, or alternatively, introducing them into nitrogen poor media, causes Gln3 to become dephosphorylated and to dissociate from Ure2, where it then moves into the nucleus to activate its target genes (Beck and Hall, 1999). Two additional studies suggest that TOR-dependent changes in the phosphorylation of Ure2 are also important for regulating the stability of the Gln3-Ure2 complex (Cardenas et al., 1999; Hardwick et al., 1999). Rapamycin treatment stimulates nuclear accumulation of two other transcriptional activators, Msn2 and Msn4, both of which respond to different sources of cellular stress, including carbon source limitation (Beck and Hall, 1999). Thus, it is likely that additional nutrient-responsive transcription factors will also turn out to be regulated by TOR.

In addition to permeases and degradative enzymes required for the utilization of specific nitrogen sources, distinct pathways involved in carbon metabolism are also responsive to nitrogen availability. For example, it has been observed that several genes encoding enzymes involved in the TCA and glyoxylate cycles are required for glutamate prototrophy (Ogur et al., 1964; Ogur et al., 1965; Kim et al., 1986; Gangloff et al., 1990). These genes include *ACO1*, which encodes mitochondrial aconitase, and *CIT1* and *CIT2*, which encode mitochondrial and peroxisomal forms of citrate synthase, respectively (Ogur et al., 1964; Kim et al., 1986; Rosenkrantz et al., 1986; Gangloff et al., 1990). Moreover, recent studies demonstrate that expression of these genes is subject to repression by glutamate (Liu and Butow, 1999). These results reflect the fact that in addition to providing reduced NADH and $FADH_2$ for mitochondrial respiration, the TCA cycle is also the source of many biosynthetic intermediates, including α-ketoglutarate, the primary precursor to glutamate (Stryer, 1995). Thus, in the absence of exogenously supplied glutamate (or glutamine), these intermediates must be replaced through additional anaplerotic reactions to keep the TCA cycle operational and to maintain the respiratory competency of the cell. Recent studies have demonstrated that glutamate-mediated inhibition of *ACO1*, *CIT1*, and *CIT2* involves regulation of the heterdimeric bHLH/Zip transcription factors Rtg1 and Rtg3 (Liu and Butow, 1999). The mechanism by which these transcription factors are regulated according to nitrogen availability, however, is not well understood.

Studies of both prokaryotic and eukaryotic cells emphasize glutamate and glutamine as important regulators of nitrogen metabolism (reviewed by Magasanik, 1992; Merrick and Edwards, 1995; Marzluf, 1997). Both are used in the biosynthesis of other

amino acids and, as discussed above, can be readily converted to -ketoglutarate for use in the TCA cycle. Glutamine is also an immediate precursor for the biosynthesis of nucleotides and other nitrogen containing molecules, including NAD^+, and thus represents a primary means by which nitrogen is assimilated into cellular material. Not surprisingly, cells have evolved elaborate mechanisms to sense the intracellular levels of these amino acids and to use this information to regulate their uptake and/or synthesis. Studies of enteric bacteria have revealed a complex signaling pathway involving a two component regulatory system that couples intracellular levels of glutamine to changes in gene expression (reviewed by Merrick and Edwards, 1995). Whether TOR signaling responds specifically to intracellular levels of glutamine and/or glutamate in eukaryotic cells is presently unknown.

We are interested in understanding further both the scope and mechanisms by which gene expression is modulated according to nitrogen availability in yeast. Toward this end, we have explored a novel use of genome-wide expression analysis by identifying genes that are expressed differentially when yeast cells are grown in the presence of two defined nitrogen sources, the primary source glutamine *versus* an alternative source, urea. We find that a surprisingly small number of genes (<40) show significant differences in their levels of expression under these two conditions, where each identified gene is either induced or repressed by glutamine. In addition to Gln3-dependent target genes, one of the most concise sets of genes subject to glutamine-mediated repression includes metabolic genes regulated by the transcription factors Rtg1 and Rtg3. We demonstrate that, like Gln3, Rtg1 and Rtg3 are regulated by changes in their subcellular localization according to available nitrogen and, moreover, that the TOR

kinase pathway plays an essential role in this regulation. Our data further suggest that glutamine-responsive transcriptional modulation defines a distinct branch of TOR signaling in yeast.

Materials and Methods

Strains, media, and general methods

All strains of *S. cerevisiae* used in this study are listed in Table I. The following culture media was used: YPD (1% yeast extract, 2% peptone, 2% dextrose); minimal dextrose (MD) (0.8% yeast nitrogen base without amino acids and ammonium sulfate (pH 5.5), 2% dextrose); synthetic complete dextrose (SCD) (0.7% yeast nitrogen base without amino acids (pH 5.5), 2% dextrose). MD media contained in addition one or more of the following nitrogen sources: glutamine, glutamate, ammonia, or urea, as indicated in the text, each at 0.2% final concentration. To supplement the auxotrophic requirements of strains used for the fluorescence microscopy experiment presented in Figure 4, required amino acids, adenine and uracil were added to MD media at concentrations described by Sherman (1991). SCD media was also supplemented with appropriate amino acids, adenine and uracil as described by Sherman (1991). Yeast cultures were grown at 30°C for all experiments. Yeast transformations were performed using a DMSO-enhanced lithium acetate procedure (Hill et al., 1991). Rapamycin (Sigma, St. Louis, MO) was dissolved in DMSO and added to a final concentration of 0.2 μg/ml unless stated otherwise.

Gene expression analysis using cDNA microarrays

Strain S288c was grown with vigorous shaking to 0.5 OD_{600}/ml in 1 liter of MD media containing appropriate nitrogen sources, as indicated in the text. Cells were

immediately harvested by centrifugation, flash frozen in liquid nitrogen, and stored at –80°C. Relative mRNA levels were determined by hybridizing fluorescently-labeled cDNAs to microarrays containing cDNAs representing virtually every yeast open reading frame (DeRisi et al., 1997; Lashkari et al., 1997). All procedures for RNA isolation, cDNA synthesis, fluorescent dye labeling, as well as construction of cDNA microarrays and array hydridization conditions were conducted as described on-line at www.microarrays.org. Arrays were produced under the auspices of J. Derisi at UCSF by a consortium of laboratories affiliated with the Department of Biochemistry and Biophysics. Primers for amplification of the yeast genome were purchased from Research Genetics (Birmingham, AL) and were provided by the laboratory of P. Walter at UCSF. Arrays were scanned using a GenePix 4000a Microarray Scanner (Axon Instruments, Redwood City, CA) and analyzed using software provided by the manufacturer. Data were also analyzed using software available through the web site affilitated with the laboratories of P. Brown and D. Botstein at Stanford University (http://rana.Stanford.EDU/software/). Scatterplots shown in Figure 2 were constructed using Excel software (Microsoft, Seattle, WA). The complete data set for the nitrogen source experiments presented here will be made available upon request.

Northern blots

Northern blot analyses was performed as described previously (Powers and Walter, 1999). DNA probes were generated by PCR using genomic DNA from strain S288c as template and specific primers (purchased from Research Genetics, Birmingham, AL) for individual genes (ORF names are listed in Table II). Quantitation of blots was

performed using a STORM 860 imaging system (Molecular Dynamics, Sunnyvale, CA) and analyzed using software provided by the manufacturer.

Plasmid construction

GFP-tagged plasmids were constructed by PCR amplification of the promoter and open reading frame (ORF) of *RTG1*, *RTG2*, and *RTG3*. Primers were designed such that 500 base pairs of upstream promoter region and the entire ORF of each gene were amplified. Each 5' upstream primer contained an XhoI restriction endonuclease site and each 3' downstream primer contained an EcoRI restriction endonuclease site immediately following the stop codon. Following digestion with XhoI and EcoRI, each fragment was introduced in the XhoI and EcoRI sites of pRS316-GFP, which contains GFP^{S65T} (Kaffman et al., 1998). The resulting plasmids, pRtg1-GFP, pRtg2-GFP, and pRtg3-GFP produced versions of Rtg1-Rtg3 that contained GFP^{S65T} fused to their C-termini. Plasmid pRtg1-GFP_3, which contains *RTG1* fused in frame with three tandem copies of GFP^{S65T}, was constructed by replacing the promoter and coding region of pPHO4-GFP_3 (Kaffman et al., 1998) with the corresponding promoter and coding region of *RTG1*. pRtg3-zz was constructed by replacing the GFP in pRtg3-GFP with two Protein A z domains (zz) as described (Kaffman et al., 1998). All plasmid-expressed, tagged genes were tested for their ability to complement the null phenotype of the appropriate deletion strains.

Construction of yeast strains

Strains derived from DBY7286 that were deleted for *RTG1* or *RTG3* were constructed using standard gene replacement techniques (Rothstein, 1991). The entire coding regions of both genes were replaced with the *URA3* gene from pRS306 (Sikorski and Heiter, 1989). These strains were used for the experiments presented in Figures 3 and 6.

Strains derived from DBY8943 that produced versions of Rtg1-Rtg3 tagged at their C-termini with three copies of the HA epitope were constructed using the PCR-based method described by Pringle and co-workers (Brachmann et al., 1998; Longtine et al., 1998). As template for PCR we used the plasmid pFA6a-3HA-HIS3MX6 that contained the *S. pombe* HIS3 homologue (Longtine et al., 1998). The tagged genes were determined to be functional based on the normal growth of each resulting strain, PLY047, PLY050, and PLY089, on MD-ammonia and MD-urea agar plates. These strains were used for the experiment presented in Figure 9A.

Strains derived from K699 that were deleted for *RTG1*, *RTG2*, or *RTG3* were made using the same PCR-based gene disruption technique described above, using the *TRP1* gene of *C. glabrata* as a selectable marker (Kitada et al., 1995). Primers used for PCR possessed 40 bases of homology that corresponded to the 5' and 3' ends of the open reading frame of each target gene. These strains, EY0733, EY0734, and EY0735, were transformed with an appropriate GFP fusion plasmid, described above, and used for the fluorescence microscopy experiments presented in Figures 4, 5A, and 7.

MSN5 was deleted from K699 using a *HIS3* marked disruption vector as described previously (Kaffman et al., 1998). The *rtg2Δ msn5Δ* strain was made by

mating the *rtg2Δ* and *msn5Δ* strains described above and selecting TRP+ HIS+ segregants following sporulation and tetrad dissection. The resulting *msn5Δ* and *rtg2Δ msn5Δ* strains, EY0736 and EY0744, respectively, were used for experiments presented in Figure 8.

Strains containing the *TOR1-1* allele combined with either *rtg1Δ*/pRTG1-GFP or *rtg3Δ*/pRTG3-GFP were constructed using the following approach. Strains EY0733 and EY0735 were transformed with pRTG1-GFP or pRTG3-GFP, respectively. The resulting transformants were mated to strain JH11-1c and diploids were selected for by their ability to grow on SCD agar plates lacking both adenine and uracil. Following sporulation, TRP^+, URA^+ segregants were isolated and tested for their ability to grow on plates containing 0.2 μg/ml rapamycin. The resulting selected strains, PLY079 and PLY083, were used for the experiment shown in Figure 5B.

All wild type parental strains used for construction of the above strains were examined by Northern blot analysis to confirm that expression of the *RTG*-dependent target genes *CIT2* and *DLD3* was (1) repressed by preferred nitrogen sources and (2) induced by rapamycin treatment.

Fluorescence microscopy

For all microscopy experiments, cells were freshly transformed with plasmids that expressed appropriate GFP-fusion proteins. Cells were first grown overnight in SCD media that lacked uracil to select for plasmid maintenance. To lower the background auto fluorescence of the parent strain, additional adenine and tryptophan were added to a

final concentration of 0.005%. Cells were then diluted to 0.005 OD_{600}/ml in media appropriate for each experiment, as indicated in the text, and were examined directly by fluorescence microscopy when they reached 0.5 OD_{600}/ml. Rapamycin was added to a final concentration of 1.0 μg/ml for microscopy experiments presented in the figures. Identical results were also obtained at the lower rapamycin concentration of 0.2 μg/ml. All images documenting GFP localization were collected on an Olympus BX60 microscope with a 100X objective and recorded with a CCD camera (Photometrics) using identical settings for each experiment and an average exposure time of 1.0-1.5 seconds.

Preparation of cell extracts and Western blot analysis

For detection of Rtg1-Rtg3 by immunoblotting, cells were grown in appropriate media, as described in the text, to 0.5 OD_{600}/ml, treated with rapamycin where indicated, and harvested directly. Extracts were prepared as described previously (Kaffman et al., 1998). As Rtg3 proved to be relatively unstable in cell extracts, even in the presence of protease inhibitors, fresh extracts were prepared for each experiment and were never frozen. To detect Rtg1-HA_3, Rtg2-HA_3, and Rtg3-HA_3, 1-2 mg of extract was separated by SDS-PAGE as described (Kaffman et al., 1998). Anti-HA westerns were performed using affinity purified 12CA5 antibodies (Babco). Rtg3-zz was immunoprecipitated using IgG-Sepharose beads as described (Amersham/Pharmacia). For phosphatase

experiments, immunoprecipitated Rtg3-zz was washed with IgG buffer and lambda phosphatase buffer (New England Biolabs). Samples were split in half and beads were resuspended in 100 μl of lambda phosphatase buffer with 2mM $MnCl_2$. Approximately 800 units of lambda protein phosphatase were added to one of the two samples and the reactions were incubated for 30-45 minutes at 30°C. Samples were eluted by boiling the beads in 2X SDS sample buffer for 3 minutes and analyzed using anti-zz rabbit polyclonal antisera as described (Kaffman et al., 1998).

Results

Identification of nitrogen regulated genes

We used genome-wide gene expression analyses to compare mRNA levels following the growth of yeast cells in the presence of two distinct sources of assimilable nitrogen, glutamine and urea. Whereas glutamine is used directly in the biosynthesis of several nitrogen containing compounds, urea must first be degraded, via a bi-functional enzyme encoded by the *DUR1,2* gene, to ammonia and carbon dioxide before the ammonium ion can be incorporated into glutamate and glutamine (Sumrada and Cooper, 1982). We chose urea in part because we observed that the prototrophic strain S288c grew with a similar doubling time of ~110 minutes in minimal dextrose media containing either glutamine (MD-glutamine) or urea (MD-urea) as a sole source of nitrogen. This was in contrast to several other alternative nitrogen sources that we tested, including arginine and proline, which resulted in reduced growth rates (data not shown). Thus we reasoned that any observed differences in gene expression would be restricted to the utilization of glutamine and urea as nitrogen sources, rather than secondary effects due to differences in growth rate. Accordingly, S288c was grown to mid-log phase in MD-glutamine or MD-urea and PolyA mRNA was isolated. Fluorescently-labeled cDNAs were then prepared and applied to DNA microarrays that contained nearly every yeast open reading frame (DeRisi et al., 1997; Lashkari et al., 1997).

Of the more than 6200 genes examined, a surprisingly small number (<40) displayed differences in expression three-fold or greater under these two nitrogen conditions; 12 were expressed preferentially in MD-glutamine and 24 were expressed

preferentially in MD-urea (Table II). We confirmed these results for a representative number of genes directly by Northern blot analysis (Figure 1). The majority of genes that were expressed better in MD-glutamine encoded permeases specific for amino acids associated with rich nutrient conditions, including *GNP1* and *TAT2*, which encode high affinity permeases specific for glutamine and tryptophan, respectively (Zhu et al., 1996; Beck et al., 1999). In contrast, genes expressed preferentially in MD-urea could be grouped into one of three general classes: (1) transport permeases specific for poor nitrogen conditions, including *DUR3*, which encodes urea permease; (2) the majority of the *DAL* genes, which are involved in the uptake and catabolism of allantoin (Cooper et al., 1979; Cox et al., 1999); (3) metabolic enzymes, including several associated with the citric acid and glyoxylate cycles (Table II). Given the relatively small number of genes identified in this experiment, these results suggest that a limited number of regulatory pathways are likely to be involved in the differential use of these two nitrogen sources.

To extend the above results, we determined the relative expression of a representative number of genes following the growth of cells in media containing one of two other preferred nitrogen sources, glutamate and ammonia. In general, the pattern of expression produced by cells in MD-glutamate was similar to that produced in MD-glutamine, particularly for the genes involved in the TCA and glyoxylate cycles (Figure 1B, compare lanes 1 and 2). Several differences were also observed, however. For example, *GLN1*, which encodes glutamine synthetase, was expressed in MD-glutamate but not in MD-glutamine (Figure 1C, compare lanes 1 and 2). This result demonstrates the extreme selectivity in gene expression that exists according to the precise nitrogen source provided (Magasanik, 1992).

Very few differences in gene expression were observed when ammonia and urea were compared, one of the most notable being *DUR3* (Figure 1C, compare lanes 3 and 4). Indeed, microarray analysis revealed that less than ten genes were expressed differently when cells were grown in media containing each of these two nitrogen sources (T. Powers, unpublished results). These results were somewhat surprising given that ammonia is known to display many of the same regulatory properties as glutamine and glutamate (Magasanik, 1992). One likely explanation for this result is that some strains of *S. cerevisiae* harbor mutations that affect ammonia-dependent regulation of certain genes (Courchesne and Magasanik, 1983). The precise molecular basis for this observation has yet to be clarified.

Demonstration of glutamine as a global regulator of gene activity

The results of the above microarray experiment revealed the scope of genes whose expression differed significantly in the presence of glutamine *versus* urea. This experiment could not distinguish, however, whether these differences resulted from the stimulation or repression of gene activity by either nitrogen source. We reasoned that we could address this issue using microarrays by pair-wise comparisons of cultures grown in the presence of one *versus* both nitrogen sources. The logic here was that a given gene should display the same level of expression (i.e., have a ratio ~1.0) if the activating (or repressing) nitrogen source was present in the two samples being compared. Accordingly, we compared mRNA levels from cells grown in the following media: MD-glutamine, MD-urea, and MD-glutamine+urea. The results of this experiment are shown in the form of scatter plots in Figure 2.

When mRNA levels from cells grown in MD-glutamine and MD-urea were compared, most genes appeared as points along a diagonal with a slope of ~1.0 and corresponded to genes expressed similarly under the two conditions (Figure 2A). A characteristic number of points fell both above and below this diagonal and corresponded to genes (reported in Table II) that were preferentially expressed in glutamine or urea, respectively (Figure 2A). This pattern of expression was remarkably similar when cells grown in MD-urea and MD-glutamine+urea were compared, indicating that the presence of urea in both cultures did not significantly change the relative expression of any gene (Figure 2, compare A and B; note that the plot in B appears as the reciprocal of the plot in A due to the arrangement of axes). In dramatic contrast, when MD-glutamine and MD-glutamine+urea samples were compared, essentially all points collapsed onto the diagonal, demonstrating the dramatic effect by glutamine on gene expression (Figure 1C). From these results we conclude that essentially all differences in gene expression observed in these experiments result from glutamine acting as both an activator as well as a repressor of gene activity. This conclusion was confirmed for a number of representative genes by Northern blotting (Figure 1, lanes 5-7) and is consistent with the demonstrated role of glutamine as an important regulator of nitrogen-dependent gene expression (Magasanik, 1992).

RTG-dependent gene expression: an interface between carbon and nitrogen metabolism

Many of the differences in gene expression observed in the preceding experiments were likely to reflect altered metabolic needs as cells utilize distinct nitrogen sources.

For example, *de novo* biosynthesis of amino acids requires intermediates provided by the TCA cycle, primarily oxaloacetate and α-ketoglutarate, that must be replaced through anaplerotic reactions in order to maintain the respiratory competency of the cell (Stryer, 1995). These reactions include production of succinate via the glyoxylate cycle and formation of oxaloacetate directly from pyruvate, a reaction catalyzed by pyruvate carboxylase, encoded by the *PYC1* gene in yeast (Stucka et al., 1991; Walker et al., 1991). Remarkably, it is precisely the genes encoding these enzymes, as well as several TCA cycle enzymes that catalyze steps leading to the formation of α-ketoglutarate, namely *ACO1*, *IDH1*, and *IDH2*, that we found to be repressed by glutamine (or glutamate) (Figure 1B; Figure 3A; data not shown). Thus, one physiological response of cells growing in the absence of these nitrogen sources was increased expression of genes involved in these anaplerotic reactions. We decided to explore this regulation in greater detail.

Two distinct transcriptional regulatory complexes, namely *HAP* and *RTG*, have been demonstrated to regulate expression of *ACO1*, *IDH1*, and *IDH2* (Forsburg and Guarente, 1989; McNabb et al., 1995; Liu and Butow, 1999). We reasoned that the *RTG* genes were most likely to be involved in nitrogen-regulated expression of these genes for the following reasons: (1) *HAP* gene control is subject to repression by glucose, which was used as a carbon source in all of our experiments; (2) the *RTG* genes are responsible for regulating several other glutamine-repressed genes identified in our microarray experiments, including *CIT2* and *DLD3*; (3) *rtg* mutants are reported to be glutamate auxotrophs, a phenotype that would be consistent with our observations (Liu and Butow, 1999). The *RTG* family consists of three genes, *RTG1-3*, where *RTG1* and *RTG3* both

encode members of the bZip family of transcription factors that form a heterodimeric complex (Jia et al., 1997; Rothermel et al., 1997). In agreement with results from a previous study (Liu and Butow, 1999), strains containing single deletions in either *RTG1* or *RTG3* grew very poorly on plates containing urea or ammonia as sole nitrogen sources, yet grew normally on plates containing glutamine or glutamate (data not shown).

To determine directly whether the *RTG* transcription factors were required for regulated expression of the above metabolic genes under our experimental conditions, we performed the following nutrient-shift experiment. Wild type, *rtg1*Δ, or *rtg3*Δ cells were grown in MD-glutamine to early log phase and were then transferred either to fresh MD-glutamine (as a control) or to MD-urea. Total RNA was isolated and mRNA levels of a representative number of these genes were analyzed by Northern blotting and normalized to actin mRNA levels (Figure 3B). As expected, each gene examined displayed increased expression, relative to actin, when wild type cells were transferred to MD-urea but not to MD-glutamine (Figure 3B, compare lanes 1 and 2 to lane 3). In contrast, no increased expression of *ACO1*, *IDH1*, *IDH2*, *CIT2*, or *DLD3* was observed upon transfer of either *rtg1*Δ or *rtg3*Δ cells to MD-urea (Figure 3B, lanes 6 and 9). Interestingly, *PYC1* expression was increased in each mutant strain in MD-urea by about half the extent observed in wild type cells (Figure 3B, compare lane 3 to lanes 6 and 9). In addition, similar levels of expression were observed for *MLS1* in both wild type and each *rtg* mutant strain in MD-urea (data not shown). These latter results demonstrate that factors in addition to the *RTG* genes are likely to be involved in the regulated expression of these two metabolic genes under these conditions and is consistent with previous biochemical and molecular genetic analyses of *RTG*-dependent control of *PYC1* expression (Small et

al., 1995; Menendez and Gancedo, 1998). Taken together, these results revealed a strong correlation between *RTG*-dependent expression for a number of metabolic genes and the ability of yeast to grow using urea as a nitrogen source.

Nitrogen-dependent changes in the subcellular localization of Rtg1 and Rtg3

We wanted to understand the mechanism by which Rtg1 and Rtg3 activity is regulated. No significant differences were observed in the steady state levels of either *RTG1* or *RTG3* mRNAs nor of Rtg1 or Rtg3 proteins in MD-glutamine *versus* MD-urea, suggesting that their activity was regulated post-translationally (data not shown; see below). Recently, a number of nutrient-responsive transcription factors have been shown to be regulated at the level of nuclear transport (reviewed in Kaffman and O'Shea, 1999; Beck and Hall, 1999). We therefore tested whether Rtg1 and/or Rtg3 might be similarly regulated in this manner. Toward this end, we fused the coding region of Green Fluorescent Protein (GFP) in frame to the 3' ends of both *RTG1* and *RTG3* to produce fusion proteins, termed Rtg1-GFP and Rtg3-GFP. Fluorescence microscopy was then used to examine the subcellular localization of Rtg1-GFP and Rtg3-GFP in cells grown under different nitrogen conditions. Control experiments demonstrated that each fusion protein complemented the glutamine auxotrophy of its respective *rtg1* or *rtg3* deletion strain (data not shown).

Both Rtg1-GFP and Rtg3-GFP appeared predominantly cytoplasmic when cells were grown in MD-glutamine (Figure 4, left panels). Similar results were obtained when glutamate was used instead as a nitrogen source (data not shown). In contrast, both

proteins were concentrated in the nucleus when cells were grown in MD-urea (Figure 4, right panels). Also, in close agreement with the transcriptional responses described above, both Rtg1-GFP and Rtg3-GFP remained cytoplasmic when cells were grown in media that contained both glutamine and urea (data not shown). From these results, we conclude that *RTG*-dependent gene activation involves changes in the subcellular distribution of the Rtg1/Rtg3 complex.

Rtg1 and Rtg3 activity and subcellular localization is regulated by the TOR pathway

We wished to identify the signaling pathway(s) that linked nitrogen quality to the localization of the Rtg1/Rtg3 complex. Here a clue was provided by the fact that many *RTG*-dependent target genes become induced when cells are treated with rapamycin, a specific inhibitor of the TOR kinases (Hardwick et al., 1999; C. Kao, T. Powers, P. Walter, G. Crabtree, P. Brown, unpublished observations). Accordingly, we localized Rtg1-GFP and Rtg3-GFP in cells grown in nitrogen rich media both in the absence or presence of rapamycin. As expected, both proteins remained in the cytoplasm in the absence of rapamycin (Figure 5A, left panels). In striking contrast, strong nuclear accumulation of both Rtg1-GFP and Rtg3-GFP was observed within five minutes of rapamycin addition (Figure 5A, right panels). Nuclear accumulation of these proteins by rapamycin was due specifically to inhibition of the TOR pathway, since little or no accumulation was observed when this experiment was repeated using a strain that contained the dominant rapamycin resistant *TOR1-1* allele (Figure 5B, right panels).

These results demonstrate that nuclear accumulation of Rtg1 and Rtg3 in rich nitrogen media is prevented by a functional TOR pathway.

A prediction of the above results was that inhibiting the TOR pathway might be sufficient to result in *RTG*-dependent gene activation. To test this directly, we performed a time course of rapamycin treatment of wild type, *rtg1*Δ, and *rtg3*Δ cells grown in MD-glutamine and then analyzed mRNA levels of several *RTG*-dependent targets by Northern blotting. In wild type cells, each target gene examined showed increased expression within 15 minutes following addition of rapamycin (Figure 6, compare lanes 1 and 2). The expression levels of these genes peaked at about 30 minutes and were comparable to the levels observed in cells grown in MD-urea (Figure 6, lane 3; compare to Figure 3B, lane 3).

In striking contrast, no induction was observed for any of these genes when rapamycin was added to *rtg1*Δ and *rtg3*Δ cells (Figure 6B, lanes 5-12). The sole exception was *PYC1*, which showed a level of induction in each mutant strain of about half that observed in wild type cells. This latter result is thus reminiscent of the behavior of *PYC1* in the nutrient shift experiment described above (Figure 4B) and indicates that an additional rapamycin-sensitive regulatory factor(s) is involved in the expression of this gene. As a control, we observed similar induction of two Gln3-dependent targets, *DUR3* and *DAL5*, in both wild type and the *rtg* deletion strains, demonstrating that the loss of induction in the *rtg* mutants is specific for *RTG*-dependent targets. The specificity of these results was also confirmed by an observed decrease in *RPL32* mRNA levels in all strains following rapamycin treatment, as reported previously (Powers and Walter, 1999).

Taken together, these results directly link TOR activity to both the subcellular localization as well as the activity of the Rtg1/Rtg3 complex.

Interestingly, deletion of the genes encoding the GATA transcriptional regulators Gln3 and Gat1, whose nucleocytoplamic transport is similarly regulated by TOR, confers weak resistance to rapamycin (Beck and Hall, 1999). By contrast, we observed no change in the sensitivity of *rtg1*Δ or *rtg3*Δ cells to this drug, in comparison to wild type cells (data not shown). Thus, we conclude that rapamycin-induced expression of *RTG*-dependent target genes does not contribute to the toxic effects of this drug on yeast cells.

Regulated nucleocytoplasmic transport of Rtg1 and Rtg3 requires all three RTG genes

The third member of the RTG gene family, *RTG2*, encodes a cytoplasmic protein that contains an HSP70-like ATP binding domain and displays homology to certain bacterial polyphosphatases and phosphatases (Liao and Butow, 1993; Koonin, 1994). Genetic evidence indicates that this gene functions upstream of *RTG1* and *RTG3* and is essential for expression of *RTG*-dependent target genes (Rothermel et al., 1997). We asked whether Rtg2 was involved in TOR-regulated nuclear accumulation of Rtg1 and Rtg3 by monitoring the localization of Rtg1-GFP and Rtg3-GFP in *rtg2*Δ cells, both in the absence as well as presence of rapamycin. As in wild type cells, both proteins were localized to the cytoplasm in the absence of drug (Figure 7A, left panels). In contrast, no nuclear accumulation of either protein was observed following addition of rapamycin (Figure 7A, right panels). Additional experiments demonstrated that Rtg2 was itself a cytoplasmic protein and that its localization did not change following rapamycin

treatment (data not shown). Thus, these results demonstrate that Rtg2 is essential for rapamycin-induced nuclear accumulation of the Rtg1/Rtg3 complex and that it carries out this function in the cytoplasm.

We next determined whether *both* Rtg1 and Rtg3 were required for TOR-regulated nuclear transport of the Rtg1/Rtg3 complex by monitoring the localization of Rtg1-GFP in *rtg3*Δ cells or, alternatively, Rtg3-GFP in *rtg1*Δ cells, both before and following rapamycin treatment. We observed that Rtg1-GFP remained exclusively cytoplasmic in *rtg3_* cells, even following rapamycin addition (Figure 7B, top panel and data not shown). Since Rtg1 is a relatively small protein of 177 amino acids, we wanted to exclude the possibility that the constitutive presence of Rtg1-GFP in the cytoplasm in *rtg3*Δ cells was not due simply to the diffusion of this protein out of the nucleus following rapamycin treatment. Toward this end, we fused three tandem copies of the coding region of GFP to the 3' end of the RTG1 gene to create a much larger protein, termed Rtg1-GFP_3. Control experiments confirmed that this fusion protein was functional (data not shown). We observed that Rtg1-GFP_3 also remained in the cytoplasm in rapamycin-treated cells, indicating that Rtg1 cannot accumulate in the nucleus in the absence of Rtg3 (data not shown).

In striking contrast, we observed that Rtg3-GFP was localized exclusively in the nucleus in *rtg1_* cells, both in the presence and absence of rapamycin (Figure 7B, bottom panel and data not shown). Thus, regulated transport of the Rtg1/Rtg3 complex requires that both proteins be present together. One potential explanation to account for the constitutive localization of these two proteins in different subcellular compartments is that Rtg1 contains a nuclear export signal (NES) whereas Rtg3 contains the nuclear

import signal (NLS) for the heterodimer. Consistent with this interpretation, a recent study has confirmed that the basic domain of the bHLH motif of Rtg3 contains a functional NLS (Sekito et al., 2000). Interestingly, however, we observed that Rtg3 was also localized constitutively to the nucleus when cells were deleted for both *RTG1* and *RTG2* (data not shown). Given that Rtg3 is localized exclusively in the cytoplasm when cells are singly deleted for *RTG2* (Figure 7A), together these results suggest that Rtg1 may play a more antagonistic role in regulating Rtg3 nuclear import. A similar conclusion has also been reached recently by Butow and co-workers (Sekito et al., 2000).

Export of Rtg1 and Rtg3 from the nucleus requires the β-importin homolog Msn5

Constitutive localization of Rtg3 in the nucleus in *rtg1*Δ cells suggested that export of the Rtg1/Rtg3 complex from the nucleus might play a role in the regulation of these transcription factors. Previous studies of another nutrient-responsive transcription factor, Pho4, has demonstrated that its export from the nucleus depends on the activity of Msn5, a member of the β-importin family of nuclear receptors (Kaffman et al., 1998). We therefore tested whether export of Rtg1 and Rtg3 might similarly be regulated by this protein. Indeed, we observed that both Rtg1-GFP and Rtg3-GFP were constitutively nuclear in *msn5*Δ cells, demonstrating that this factor is required, either directly or indirectly, for export of the Rtg1/Rtg3 complex from the nucleus (Figure 8A, left panels). Interestingly, when we examined the localization of Rtg1-GFP and Rtg3-GFP in *msn5*Δ *rtg2*Δ cells, both proteins remained in the cytoplasm, consistent with the above observation that Rtg2 is absolutely required for nuclear entry of the Rtg1/Rtg3 complex

(Figure 8A, right panels). Additional control experiments demonstrated that Rtg2-GFP remained in the cytoplasm in *msn5*Δ cells (data not shown).

If regulated access to the nucleus represents the primary mechanism by which the activity of the Rtg1/Rtg3 complex is controlled, then we expected to observe constitutive activation of their target genes in *msn5*Δ cells in the absence of rapamycin. However, no such increased expression of *RTG*-target genes was observed in *msn5*Δ cells compared to wild type (Figure 8B, compare lanes 1 and 3). This observation is consistent with studies of other regulated transcription factors, namely, that constitutive nuclear localization does not necessarily result in gene activation and that other regulatory mechanisms are involved (Komeili and O'Shea, 1999). Remarkably, however, we observed rapid induction of *RTG*-dependent target genes in *msn5*Δ cells following addition of rapamycin (Figure 8B, compare lanes 3 and 4). This latter result demonstrates that despite its steady state nuclear localization in *msn5*Δ cells, the Rtg1/Rtg3 complex remains responsive to changes in TOR signaling.

TOR-dependent changes in the phosphorylation state of Rtg3

Previous studies have demonstrated that changes in phosphorylation of a transcription factor can be important for regulating its activity as well as concentration in the nucleus (Beck and Hall, 1999; Komeili and O'Shea, 1999). We therefore wished to determine whether rapamycin-induced nuclear accumulation of Rtg1 and Rtg3 also correlated with changes in the phosphorylation state of either of these proteins. Toward this end, we used immunoblot analysis to monitor the electrophoretic mobility of these proteins, as well as Rtg2, from extracts in which each protein was genomically tagged at

its C-terminus with the HA_3 epitope (see Materials and Methods). Extracts were prepared from cells grown in rich nitrogen media that had been incubated with drug vehicle alone or with rapamycin and then subsequently prepared in either the presence or absence of phosphatase inhibitors. No change in the relative abundance or mobility of Rtg1-HA_3 or Rtg2-HA_3 was observed in untreated *versus* rapamycin-treated cells (Figure 9A, lanes 1-4). In contrast, a slower migrating form of Rtg3-HA_3 appeared following addition of rapamycin, which was observed only when extracts were prepared in the presence of phosphatase inhibitors (Figure 9A, compare lanes 5 and 6; data not shown). These results suggested that inhibition of TOR signaling resulted in a change in the phosphorylation state of Rtg3.

We wanted to confirm that Rtg3 was a phosphoprotein by treating purified Rtg3 with phosphatases *in vitro*; however, the HA-epitope tagged form of this protein was not efficiently immunoprecipitated from cell extracts. We therefore used a form of Rtg3 that was fused at its C-terminus to two z domains from Protein A, termed Rtg3-zz, which could be immunoprecipitated quantitatively from extracts using immobilized-IgG (data not shown). The results confirmed that rapamycin treatment resulted in the conversion of a portion of Rtg3-zz to a more slowly migrating form (Figure 9B, compare lanes 1 and 2). Moreover, this slower form was abolished following treatment with phosphatase, confirming that the rapamycin-induced change in electrophoretic mobility of Rtg3-zz is due to changes in phosphorylation (Figure 9B, compare lanes 2 and 4; the arrowhead denotes the slower migrating form). Interestingly, the rapamycin-induced shift in Rtg3

mobility was also observed in *rtg2*Δ cells, suggesting that TOR influences Rtg3 phosphorylation independently from Rtg2 function (Figure 9B, compare lanes 5 and 6).

While the above results demonstrate that TOR influences the phosphorylation state of Rtg3, several other results indicate that phosphorylation-dependent regulation of this protein is likely to be complex. First, we observed that Rtg3-zz was phosphorylated in the absence of rapamycin treatment, a conclusion based on its increased mobility following phosphatase treatment, suggesting that Rtg3 is likely to be phosphorylated on multiple sites (Figure 9B, compare lanes 1 and 3). The existence of multiple sites of phosphorylation could account for the relatively poor resolution of Rtg3 on SDS-PAGE gels in the absence of phosphatase treatment. Second, we were unable to detect a difference in the mobility of Rtg3-zz in cells grown in MD-glutamine versus MD-urea (Figure 9C, lanes 1 and 2). This latter result suggests either that rapamycin-induced changes are transient or that additional changes in the phosphorylation state of this protein occur during steady state growth of cells in media lacking glutamine. Finally, multiple levels of regulation, possibly mediated by distinct changes in the phosphorylation state of Rtg3, would be consistent with our above observation that the Rtg1/Rtg3 complex is concentrated in the nucleus in *msn5*Δ cells, yet their target genes remain uninduced. Identifying the residue(s) of Rtg3 that are phosphorylated under these different conditions will be required to resolve these issues.

Discussion

Genome-wide expression analysis represents a powerful approach for exploring the scope of transcriptional regulation associated with different biological processes. The appeal of this method is most often attributed to the enormous amount of information that it can yield, as exemplified by studies in yeast of the diauxic shift, sporulation, and the cell cycle, where each of these processes involves sequential changes in the expression of groups of co-regulated genes that number well into the hundreds (DeRisi et al., 1997; Chu et al., 1998; Spellman et al., 1998). By contrast, our results presented here demonstrate that by studying the effects of subtle changes in growth conditions, e.g., by varying the source of assimilable nitrogen, microarray analysis can help bring a specific biological problem into sharper focus by identifying a concise set of co-regulated genes.

Specifically, we have demonstrated that a surprisingly small number of genes are differentially expressed when yeast cells use glutamine *versus* urea as their sole source of nitrogen (Table II). All of these genes are either induced or repressed by the presence of glutamine, a fact that is consistent with its established importance as a major regulator of nitrogen metabolism (Magasanik, 1992; Marzluf, 1997). One prominent group of glutamine-repressed genes encodes proteins involved in the uptake and metabolism of alternative sources of nitrogen, including urea and allantoin, and whose expression is controlled by the Gln3 and Ure2 regulatory proteins (Coffman et al., 1997; Cox et al., 1999). Recent studies have demonstrated that these genes are negatively regulated by the TOR signaling pathway via a mechanism that involves sequestration of the transcription factor Gln3 in the cytoplasm (Beck and Hall, 1999).

A second prominent group of glutamine-repressed genes encodes enzymes involved in the TCA and glyoxylate cycles and whose expression in urea-containing media requires the heterodimeric transcription factors Rtg1 and Rtg3. Differential expression of these metabolic genes is consistent with the proposal by Liu and Butow (1999) that one important role of *RTG*-dependent gene expression is to provide adequate levels of α-ketoglutarate for use in the *de novo* biosynthesis of glutamate, which in turn is required for the synthesis of glutamine. In addition, our results extend involvement of *RTG*-dependent regulation to include *PYC1*, which provides an alternative route for the synthesis of oxaloacetate for use in both the TCA cycle and in amino acid biosynthesis (Figure 3). These results thus highlight the intimate relationship that exists between nitrogen and carbon metabolism as well as its importance to normal cell growth.

Remarkably, we find that TOR signaling also regulates *RTG*-dependent gene activity. Specifically, inhibition of the TOR kinases by rapamycin results in both rapid nuclear accumulation of the Rtg1/Rtg3 complex as well as induction of their target genes. In addition, rapamycin treatment correlates with changes in the phosphorylation state of Rtg3, indicating that nucleocytoplasmic transport of the complex is likely to be regulated by differential phosphorylation of Rtg3. These results thus contribute to a growing body of evidence demonstrating that one important role of TOR is to control the activity of specific nutrient-responsive transcription factors. Moreover, our findings suggest that TOR signaling may provide an important mechanism by which carbon and nitrogen utilization are linked. Whether Rtg3 phosphorylation is influenced by the activity of the Tap42-Sit4 phosphatase complex, as has been shown recently for Gln3 (Beck and Hall, 1999), remains to be determined.

Our results also shed light on the functional role of Rtg2 in regulating *RTG*-dependent gene expression, a previously identified upstream positive regulator of Rtg1 and Rtg3 (Liao and Butow, 1993; Rothermel et al., 1997). Specifically, we find that Rtg2 is required for rapamycin-induced entry of the Rtg1/Rtg3 complex into the nucleus (Figure 10). How Rtg2 acts mechanistically is unclear at present; however, we believe that Rtg2 is likely to be required for a step subsequent to the action of TOR, based upon the observation that rapamycin-induced changes in Rtg3 phosphorylation are observed in *rtg2_* mutant cells (Figure 8B). Furthermore, our examination of Rtg3 localization in *rtg1*Δ, *rtg2*Δ, and *rtg1*Δ *rtg2*Δ mutant cells suggests that Rtg2 may interact primarily with Rtg1 to regulate nuclear entry of the Rtg1/Rtg3 complex (Figure 7 and data not shown).

In contrast to the role played by Rtg2, we find that Msn5, a member of the importin-_-family of nuclear transport receptors, is required for nuclear export of the Rtg1/Rtg3 complex (Figure 10). Thus, in *msn5*Δ cells, both Rtg1 and Rtg3 are constitutively localized to the nucleus. We do not know yet whether Msn5 interacts directly with Rtg1 and/or Rtg3 to faciliate their export from the nucleus, as has been demonstrated for Pho4 (Kaffman et al., 1998). It is possible that Msn5 is instead required for the proper localization of another protein(s) that is involved directly in the export of the Rtg1/Rtg3 complex.

While nucleocytoplasmic transport of the Rtg1/Rtg3 complex is essential for regulated RTG-dependent gene activation, our results indicate that additional control mechanisms are involved. This conclusion is based on our analyses of *msn5*Δ cells, where both Rtg1 and Rtg3 are concentrated in the nucleus yet their target genes remain uninduced (Figure 9). As these genes can nevertheless be induced rapidly in *msn5*Δ cells

following rapamycin treatment, these results raise the intriguing possibility that TOR regulates Rtg1/Rtg3 activity in both the cytoplasm as well as in the nucleus.

RTG1-RTG3 were originally identified as genes required for increased expression of *CIT2* under conditions where mitochondrial respiratory function is impaired, as in rho^0 petite mutants that lack mitochondrial DNA (Liao et al., 1991; Liao and Butow, 1993). This process, termed retrograde regulation, is believed to involve intracellular signaling from the mitochondria to the nucleus (Liao and Butow, 1993). Subsequently, the *RTG* genes have been shown to be involved in the expression of several genes that encode peroxisomal proteins in addition to glyoxylate cycle enzymes (Chelstowska and Butow, 1995; Kos et al., 1995; McCammon, 1996; Liu and Butow, 1999). Indeed, the results of these studies suggest that both mitochondrial and peroxisomal functions are intimately linked via *RTG*-dependent gene expression. An important question prompted by our present results thus concerns the relationship between TOR signaling and retrograde regulation. While this manuscript was in preparation, Butow and co-workers reported that Rtg1 and Rtg3 are localized constitutively to the nucleus in rho^0 cells (Sekito et al., 2000). Moreover, this localization depends on a functional Rtg2 protein. Given these similarities to what we have presented here, one possibility is that TOR may be integrated with retrograde signaling.

At odds with this conclusion, however, is the finding that nuclear accumulation of the Rtg1/Rtg3 complex in rho^0 cells appears to correlate with a substantial decrease in phosphorylation of Rtg3 (Sekito et al., 2000). By contrast, our results suggest that nuclear import of this complex following rapamycin treatment may coincide with

increased phosphorylation of Rtg3. Moreover, and very importantly, we were unable to detect a difference in the mobility of Rtg3 on SDS-PAGE following the growth of cells in glutamine- *versus* urea-containing media (Figure 9C). Nevertheless, the Rtg1/Rtg3 complex is localized in different subcellular compartments, cytoplasm *versus* nucleus, respectively, under these different nitrogen conditions (Figure 4). Thus, the precise relationship between the phosphorylation state of Rtg3 and its nucleocytoplasmic disposition needs further clarification, which will require identification of residues in Rtg3 that are subject to phosphorylation under different cellular conditions. At present it thus remains possible that retrograde and TOR signaling may represent independent routes by which Rtg1 and/or Rtg3 are regulated and that both pathways ultimately converge on the same nucleocytoplasmic transport step.

Inhibition of the TOR kinases by rapamycin affects the expression of a large number of genes, including those regulated by nitrogen and glucose-sensitive signaling pathways, glycolytic genes, genes expressed during the diauxic shift, and r-protein and rRNA genes (Beck and Hall, 1999; Cardenas et al., 1999; Hardwick et al., 1999; Powers and Walter, 1999; C. Kao, T. Powers, P. Walter, G. Crabtree, P. Brown, unpublished observations). By contrast, we find that only a small subset of these targets are expressed differentially in our nitrogen source experiments, in particular the *GLN3*- and *RTG*-dependent genes described above. These observations suggest that distinct branches of the TOR pathway may be regulated independently according to the precise nutritional state of the cell and, moreover, suggest that TOR activity may be modulated by multiple upstream nutritional signals. On the other hand, many of the glutamine-regulated genes identified in this study are affected to some extent by rapamycin treatment (T. Powers,

unpublished results). Thus, we believe that TOR signaling is likely to represent an important mechanism by which the availability of glutamine and/or glutamate is coupled to changes in gene expression in eukaryotic cells. One challenge now at hand is to understand how TOR activity is regulated under different nitrogen conditions. Such studies will undoubtedly prove invaluable toward deciphering the role that this pathway plays in promoting normal cell growth.

Acknowledgements

We are grateful to J. Derisi for his expertise and leadership and to P. Walter and other members of the Department of Biophysics and Biochemistry for their help in establishing DNA microarray technology at UC San Francisco. We thank H. Bennett for her expert instruction in preparing fluorescently-labeled cDNAs. We thank D. Botstein for strains and C. Kao for help with data analysis and for sharing her unpublished results. We thank K. Burtis for help with microarray development at UC Davis. We also thank the members of the Powers lab for their assistance during the course of this work as well as J. Derisi for valuable discussions. Finally, we thank S. Burgess, W. Heyer, M. Niwa, and M. Singer for comments on the manuscript. This work was supported by laboratory start up funds as well as a faculty research grant from U.C. Davis (to T. P.) and by grant GM 59034 from the National Institutes of Health (to E. K. O.). A. K. is an HHMI predoctoral fellow.

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Figure Legends

Figure 1. Examples of differences in gene expression during growth of yeast cells in the presence of different sources of assimilable nitrogen. Strain S288c was grown to mid-log phase in MD media containing the indicated nitrogen sources. Total mRNA was isolated and Northern blot analysis was performed, probing for the specified mRNAs. **(A)** Control transcripts showing no significant differences under the conditions tested. **(B)** Transcripts displaying similar levels of expression in MD-glutamine and MD-glutamate. **(C)** Transcripts displaying more complex patterns of expression.

Figure 2. Glutamine is both a global activator and repressor of gene expression. Scatter plots show pair-wise comparisons of gene expression profiles of S288c cells grown in the presence of glutamine, urea, or glutamine + urea. (A) MD-glutamine *versus* MD-urea. (B) MD-urea *verus* MD-glutamine + urea. (C) MD-glutamine *versus* MD-glutamine + urea. (D) control experiment comparing MD-glutamine with itself. For each plot, the x-axis depicts cDNA samples labeled with Cy5 dye and the y-axis depicts samples labeled with Cy3 dye.

Figure 3. Rtg1 and Rtg3 are required for expression of distinct metabolic genes in MD-urea. **(A)** Summary of metabolic genes (indicated in bold) subject to glutamine-mediated transcriptional repression (listed in Table II; note that *CIT1* is not listed in Table II as its MD-glutamine/MD-urea expression ratio of ~2.0 fell below the cut off value of 3.0 required for listing). Genes depicted were similarly repressed in MD-glutamine and MD-

glutamate, except for *GLN1* (see Figure 1). **(B)** Nitrogen source shift experiment. Wild type (S288c), *rtg1*Δ (PLY037), and *rtg3*Δ (PLY039) cells were grown in MD-glutamine until 0.5 OD_{600}/ml and were either harvested (lanes 1, 4, and 7) or transferred to MD-glutamine (lanes 2, 5, 8) or MD-urea (lanes 3, 6, 9) media for 30 minutes before harvesting. RNA was prepared and analyzed by Northern blotting and probed for the specified mRNAs.

Figure 4. Rtg1 and Rtg3 are localized within the nucleus under glutamine-limiting conditions. *rtg1*Δ (EY0733) or *rtg3*Δ (EY0735) cells were transformed with pRtg1-GFP or pRtg3-GFP, respectively, and were grown to 0.5 OD_{600}/ml in MD-glutamine or MD-urea and examined by fluorescence microscopy. Punctate nuclear fluorescence was observed for both Rtg1-GFP and Rtg3-GFP in MD-urea. The nuclear disposition of this GFP-based fluorescence was confirmed by its co-localization with DAPI-stained nuclear DNA (data not shown).

Figure 5. Rtg1 and Rtg3 are localized within the nucleus following rapamycin treatment in a *TOR1*-dependent manner. **(A)** *rtg1*Δ (EY0733) or *rtg3*Δ (EY0735) cells expressing Rtg1-GFP or Rtg3-GFP, respectively, were treated with drug vehicle alone (left panels) or with 1 μg/ml of rapamycin (right panels) for 5 minutes, followed by examination by fluorescence microscopy. Pronounced nuclear accumulation of both Rtg1-GFP and Rtg3-GFP was observed in cells treated with rapamycin. **(B)** The experiment in (A) was repeated using cells that carried the dominant rapamycin resistant *TOR1-1* allele.

Figure 6. Induction of *RTG*-dependent target genes by rapamycin. Wild type (S288c), *rtg1*Δ (PLY037), and *rtg3*Δ (PLY039) cells were grown in MD-glutamine to 0.5 OD_{600}/ml and were treated either with drug vehicle (lanes 1, 5, and 9) or with rapamycin for 15 (lanes 2, 6, 10), 30 (lanes 3, 6, 9), or 60 minutes (lanes 4, 8, 12). Cells were then harvested and RNA was prepared and analyzed by Northern blotting, probing for the specified mRNAs.

Figure 7. Regulated nucleocytoplasmic transport of Rtg1 and Rtg3 requires Rtg1, Rtg2, and Rtg3. **(A)** Rtg1-GFP and Rtg3-GFP were visualized in *rtg2*Δ (EY0734) cells in the absence (left panels) or presence (right panels) of rapamycin. In contrast to wild type cells, neither Rtg1-GFP nor Rtg3-GFP relocate to the nucleus following rapamycin treatment (compare with Figure 5A). **(B)** Rtg1-GFP was visualized in *rtg3_* (EY0735) cells (upper panel) and Rtg3-GFP was visualized in *rtg1*Δ (EY0733) cells (lower panel). Note that Rtg1-GFP cannot accumulate in the nucleus upon rapamycin treatment in *rtg3*Δ cells. In contrast, Rtg3-GFP is localized constitutively to the nucleus in *rtg1*Δ cells, even in the absence of rapamycin addition.

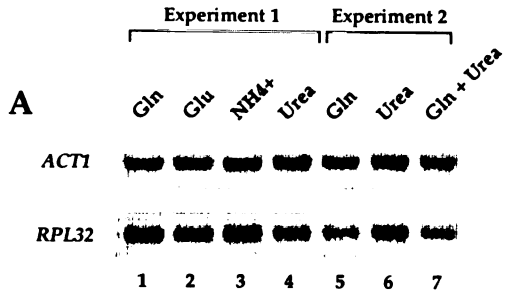
Figure 8. **(A)** Msn5 is required for export of Rtg1 and Rtg3 from the nucleus. **(A)** *msn5*Δ (EY0736) and *msn5*Δ *rtg2*Δ (EY0744) cells were transformed with pRtg1-GFP (upper panels) or pRtg3-GFP (lower panels) and grown to 0.5 OD_{600}/ml in SCD media lacking uracil and were examined by fluorescence microscopy. Rtg1-GFP and Rtg3-GFP were localized constitutively within the nucleus in *msn5*Δ cells but not in *msn5*Δ*rtg2*Δ cells. **(B)** *RTG*-dependent target genes remain responsive to TOR signaling

in *msn5*Δ cells. Wild type (K699) and *msn5*Δ (EY0736) cells were grown in YPD until 0.5 OD_{600}/ml and were treated either with drug vehicle (lanes 1 and 3) or with rapamycin (lanes 2 and 4) for 30 minutes (this time of incubation corresponded to the peak induction of *RTG*-dependent gene expression observed in the rapamycin time course in Figure 6). Cells were then harvested and RNA was prepared and analyzed by Northern blotting, probing for the specified mRNAs.

Figure 9. Rtg3 is a phosphoprotein and is differentially phosphorylated following rapamycin treatment. **(A)** Cells expressing Rtg1-HA_3 (PLY047), Rtg2-HA_3 (PLY089), and Rtg3-HA_3 (PLY050) were grown to 0.5 OD_{600}/ml in YPD and were treated either with drug vehicle alone or with rapamycin for 15 minutes. Extracts were prepared and Western blot analysis was performed using Anti-HA monoclonal antibodies to detect each protein. No change in the abundance or relative mobility of Rtg1 or Rtg2 could be detected following rapamycin treatment. In contrast, a portion of Rtg3 showed an increased mobility (arrowhead) following rapamycin treatment, compared to its mobility in the absence of rapamycin (asterick). **(B)** Wild type (K699) and *rtg2*Δ (EY0734) cells transformed with pRtg3-zz and were grown to 0.5 OD_{600}/ml in SCD media lacking uracil. Cells were then treated with drug vehicle or with rapamycin for 15 minutes. Extracts were prepared and Rtg3-zz was immunoprecipitated with IgG sepharose and either mock-treated or treated with phosphatase prior to Western blot analysis, as indicated. Increased mobility of a portion of Rtg3-zz following rapamcyin treatment is indicated (arrowhead). **(C)** Wild type (K699) cells carrying pRtg3-zz were grown to 0.5

OD_{600}/ml in MD-glutamine or MD-urea and processed as in (B). For each experiment in A-C, all samples were from the same gel. Identical results were obtained in three separate experiments.

Figure 10. Model for involvement of the TOR kinase in nitrogen-dependent regulation of the Rtg1 and Rtg3 transcription factors. See text for details.


Experiment 1
Experiment 2
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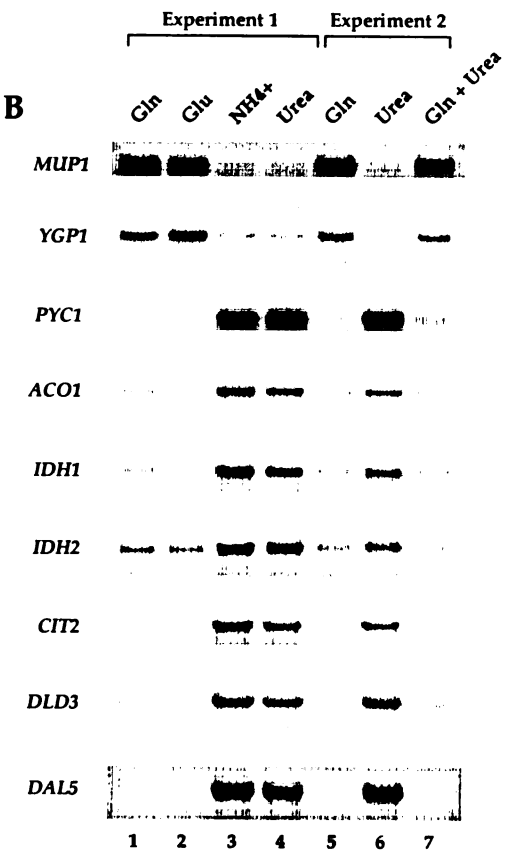
Gln
Glu
NH4+
Urea
Gln
Urea
Gln + Urea
ACT1
RPL32
1 2 3 4 5 6 7


Experiment 1
Experiment 2
B
Gln
Glu
NH4+
Urea
Gln
Urea
Gln + Urea
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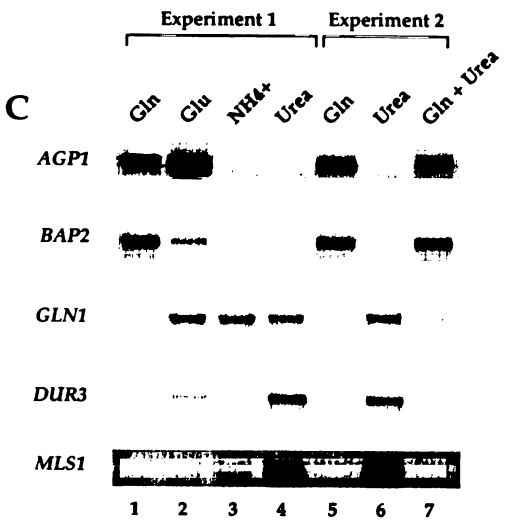
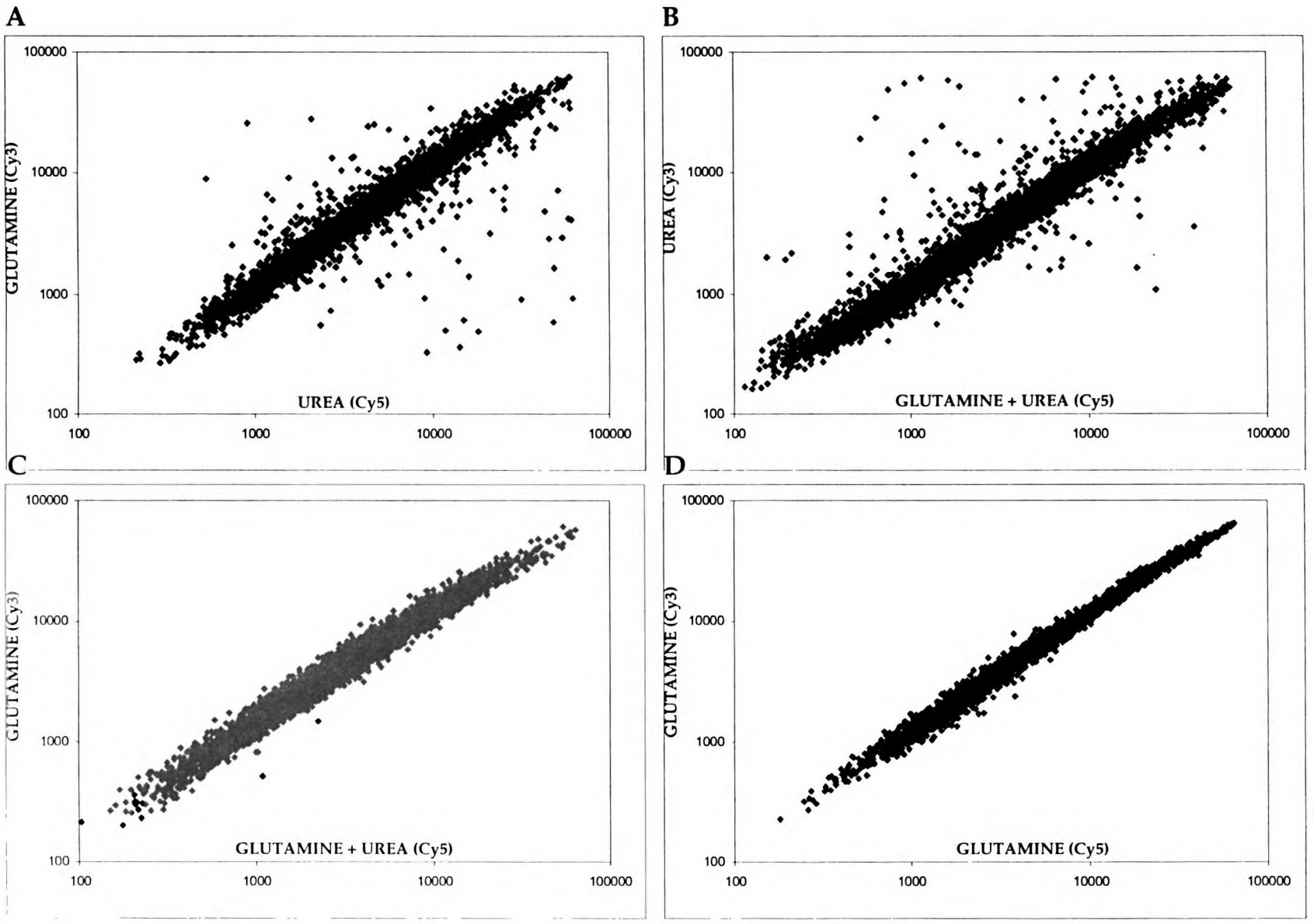
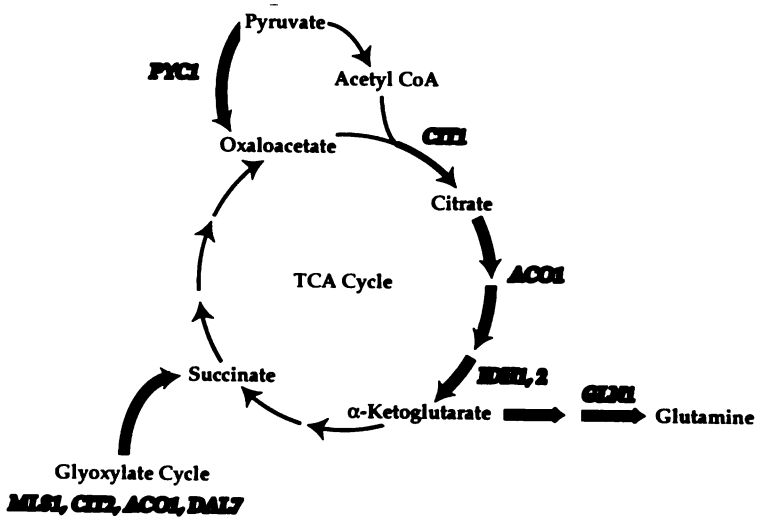
YGP1
PYC1
ACO1
IDH1
IDH2
CIT2
DLD3
DAL5
1 2 3 4 5 6 7

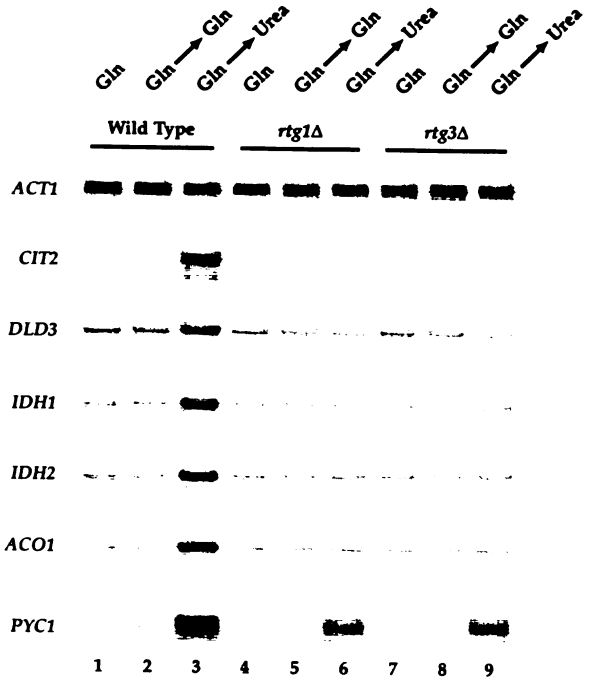

Experiment 1
Experiment 2
C
Gln
Glu
NH4+
Urea
Gln
Urea
Gln + Urea
AGP1
BAP2
GLN1
DUR3
MLS1
1 2 3 4 5 6 7

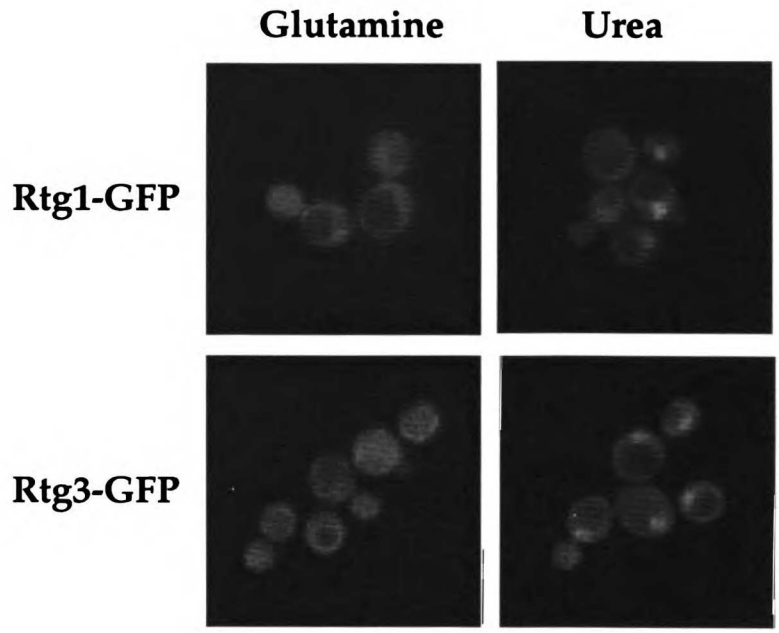

A
100000
10000
1000
100
GLUTAMINE (Cy3)
UREA (Cy5)
100
1000
10000
100000
B
100000
10000
1000
100
UREA (Cy3)
GLUTAMINE + UREA (Cy5)
100
1000
10000
100000
C
100000
10000
1000
100
GLUTAMINE (Cy3)
GLUTAMINE + UREA (Cy5)
100
1000
10000
100000
D
100000
10000
1000
100
GLUTAMINE (Cy3)
GLUTAMINE (Cy5)
100
1000
10000
100000

A

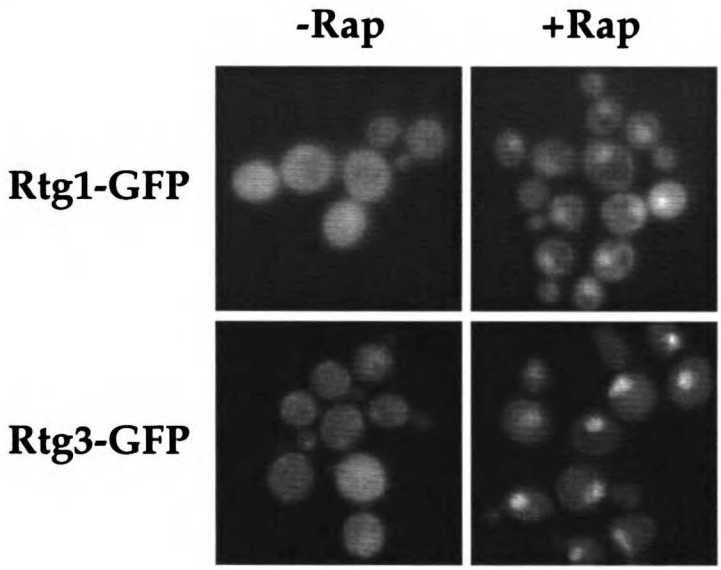

Pyruvate
PYC1
Acetyl CoA
CIT1
Oxaloacetate
Citrate
TCA Cycle
ACO1
IDH1, 2
Succinate
α-Ketoglutarate
GLN1
Glutamine
Glyoxylate Cycle
MLS1, CIT2, ACO1, DAL7

B

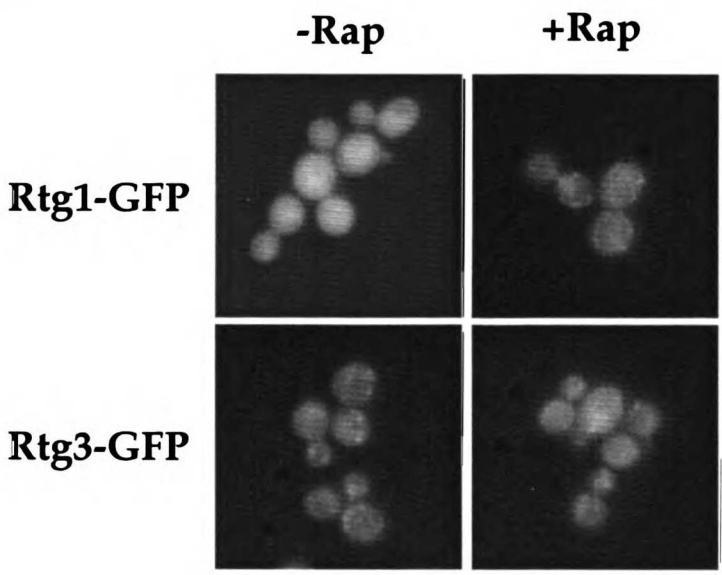

Gln
Gln → Gln
Gln → Urea
Wild Type
rtg1Δ
rtg3Δ
ACT1
CIT2
DLD3
IDH1
IDH2
ACO1
PYC1
1 2 3 4 5 6 7 8 9

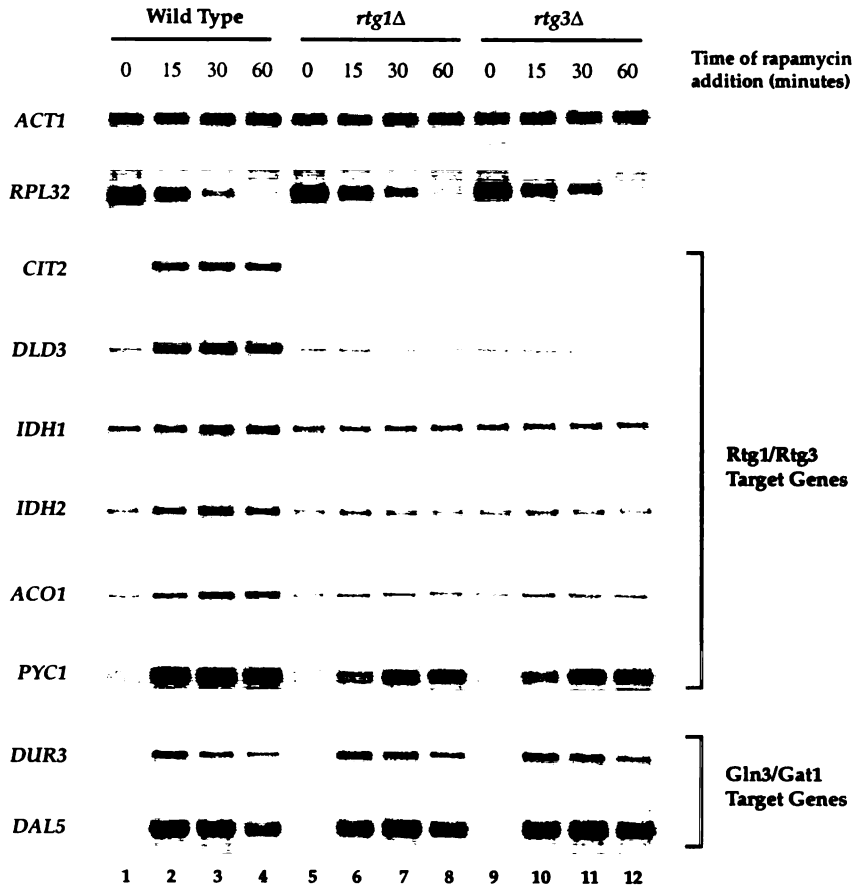

Glutamine
Urea
Rtg1-GFP
Rtg3-GFP

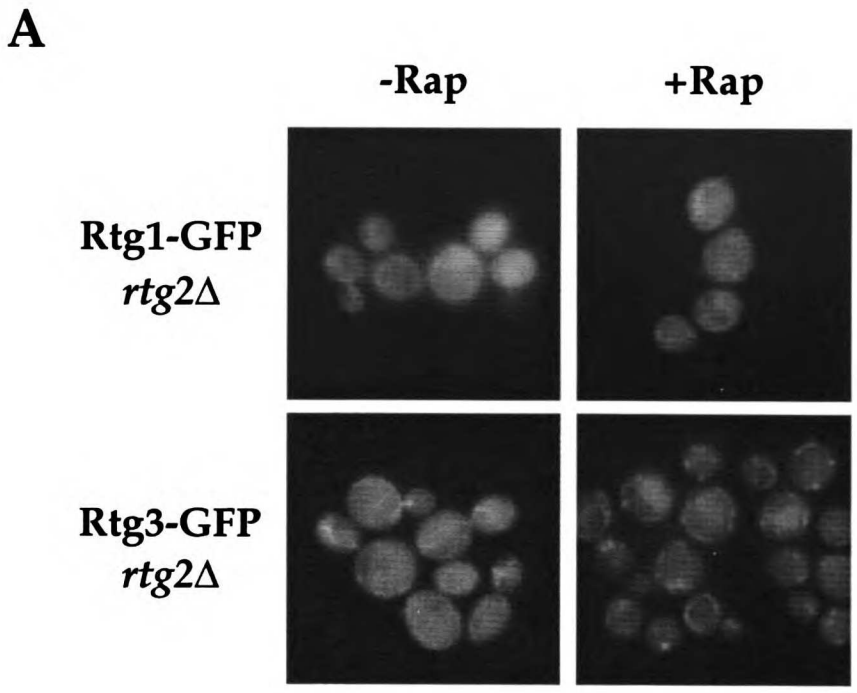
A



B




Wild Type
rtg1Δ
rtg3Δ
0 15 30 60 0 15 30 60 0 15 30 60
Time of rapamycin addition (minutes)
ACT1
RPL32
CIT2
DLD3
IDH1
IDH2
ACO1
PYC1
DUR3
DAL5
Rtg1/Rtg3 Target Genes
Gln3/Gat1 Target Genes
1 2 3 4 5 6 7 8 9 10 11 12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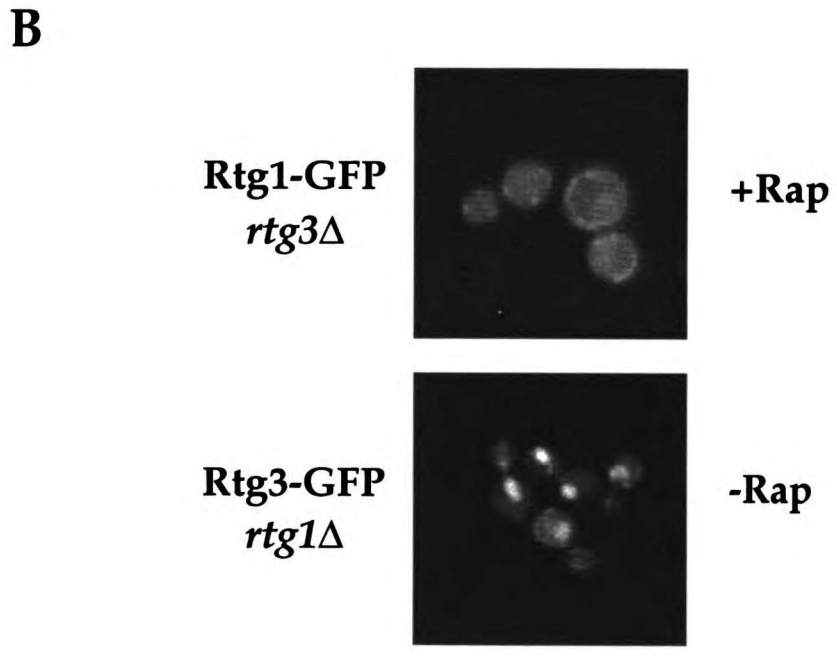
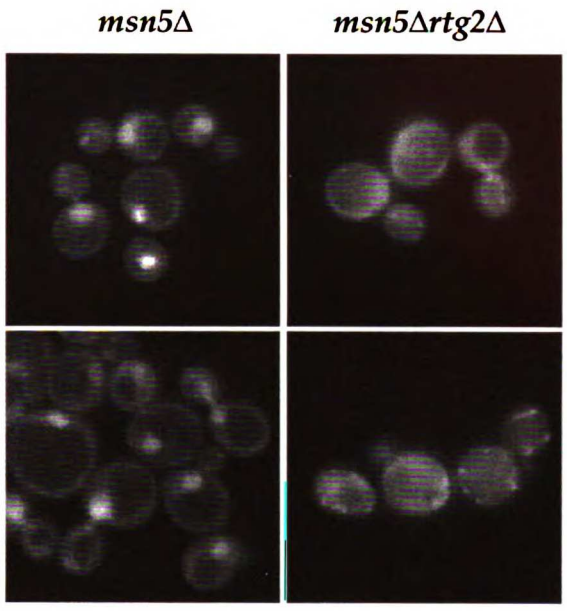
-Rap
+Rap
Rtg1-GFP
rtg2Δ
Rtg3-GFP
rtg2Δ


B
Rtg1-GFP
rtg3Δ
+Rap
Rtg3-GFP
rtg1Δ
-Rap

A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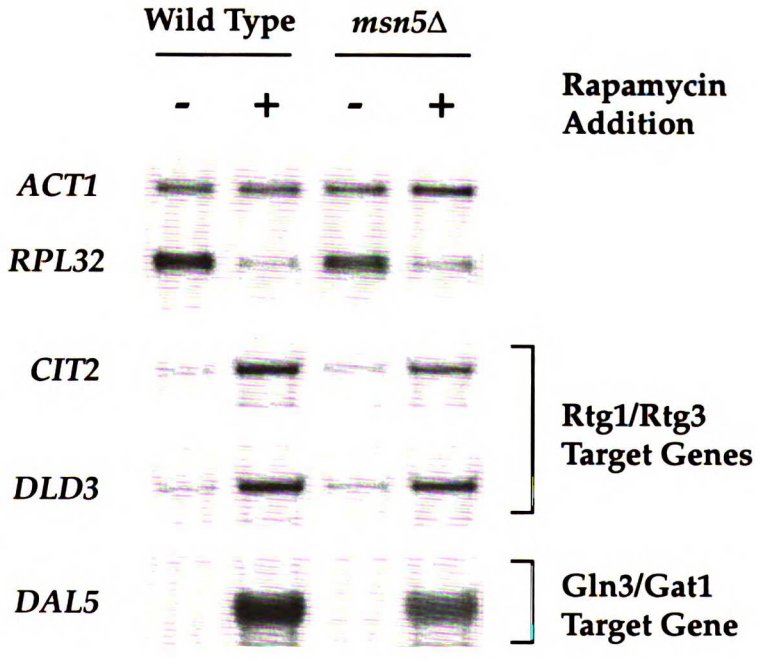
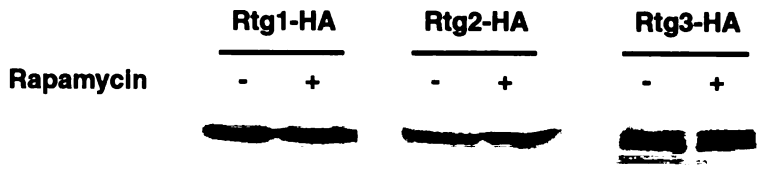
msn5Δrtg2Δ
Rtg1-GFP
Rtg3-GFP

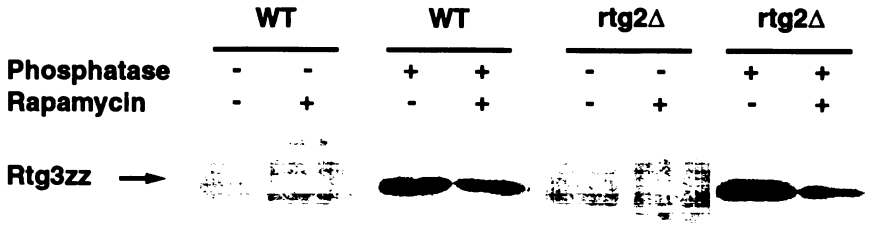
B


Wild Type
msn5Δ
- + - +
Rapamycin Addition
ACT1
RPL32
CIT2
DLD3
Rtg1/Rtg3 Target Genes
DAL5
Gln3/Gat1 Target Gene

A)

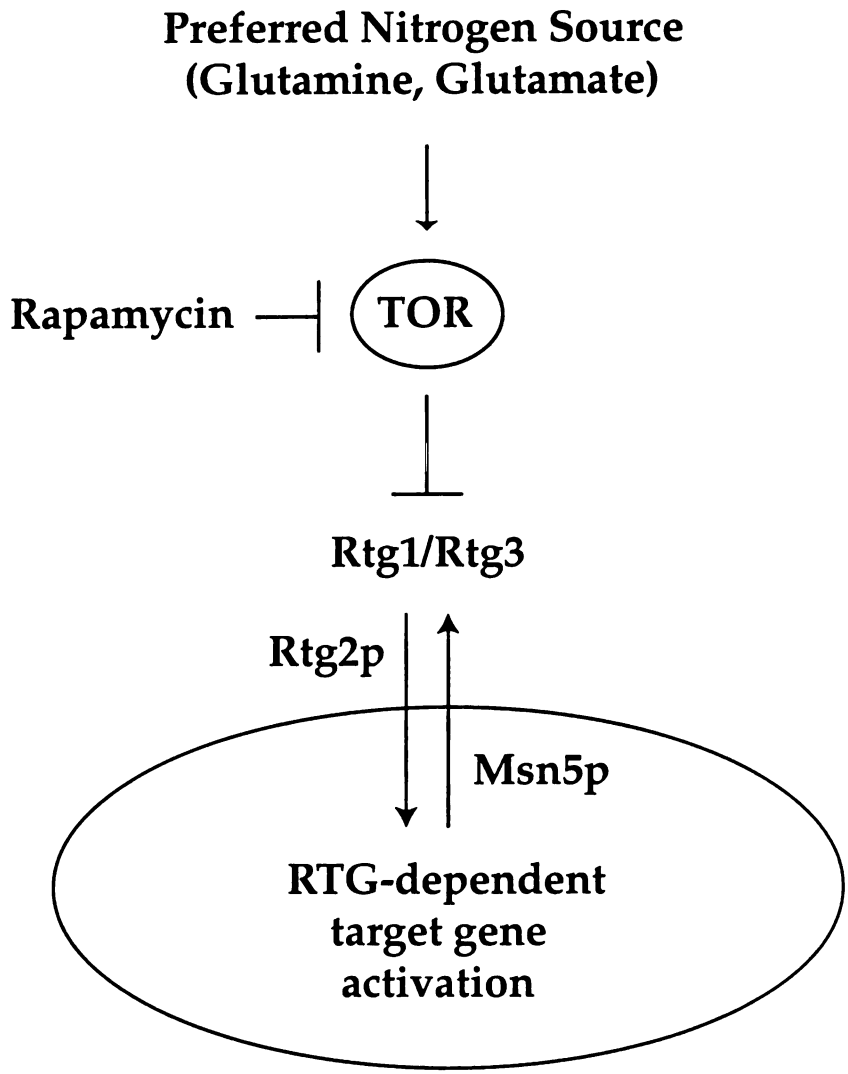

Rtg1-HA
Rtg2-HA
Rtg3-HA
Rapamycin
- + - + - +

B)


WT
WT
rtg2Δ
rtg2Δ
Phosphatase
Rapamycin
- - + + - - + +
- + - + - + - +
Rtg3zz

C)


U G U G
Ph'ase
- - + +
Rtg3zz


Preferred Nitrogen Source
(Glutamine, Glutamate)
Rapamycin
TOR
Rtg1/Rtg3
Rtg2p
Msn5p
RTG-dependent
target gene
activation

Conclusions

Our extensive knowledge of Pho4 phosphorylation, changes in activity and subcellular localization allowed us to uncover the mechanism by which phosphorylation of Pho4 regulates its activity. Interestingly, the five sites of phosphorylation on Pho4 have separable roles in regulating its localization and activity. Phosphorylation at two sites promotes its export, phosphorylation at a third site blocks its reimport, and phosphorylation at a fourth blocks its ability to activate transcription while in the nucleus. Thus, phosphorylation regulates Pho4's subcellular localization and its ability to activate transcription. These two mechanisms seem to play redundant roles as either one is sufficient to regulate Pho4 activity in response to changes in phosphate levels.

These results came as a surprise since we had expected that localization of Pho4 to the nucleus would result in the transcription of its target genes. The reasons why two overlapping mechanisms are used for regulating Pho4's activity are still unclear. One model is that separate regulation of nuclear localization and activity in the nucleus ensures a rapid and efficient response to changes in phosphate levels. Under phosphate-limiting conditions, the phosphate response is activated and a significant amount of cellular resources are devoted to the transcription, translation and secretion of phosphate scavenging, transport, and storage factors. As cells transition to high phosphate conditions they must rapidly turn off the energetically demanding phosphate response. In such conditions phosphorylation of SP6 would release Pho4 from its target promoters, phosphorylation of SP4 prevents the import of Pho4 once exported, and phosphorylation of SP2 and SP3 lead to the export of Pho4. This model is substantiated by our results that neither localization to the cytoplasm nor phosphorylation of SP6 were sufficient to fully

turn off the production of Pho5. Furthermore, work by Jeffrey et al (1) showed that there was a phosphorylation site preference by Pho80-Pho85 during in vitro phosphorylation reactions such that SP6 was phosphorylated first, SP4 second, and SP2 and SP3 last.

However, recent work in our lab has implicated the two mechanisms regulating Pho4 activity in differential gene expression in response to varying phosphate levels. Springer et al have shown that Pho4 under intermediate phosphate levels Pho4 is localized to the nucleus but *PHO5* is not expressed. Furthermore, under similar conditions the phosphate transporter Pho84, which is transcribed in a Pho4-dependent manner, is expressed (2). Miller et al had shown that in contrast to *PHO5*, *PHO84* is transcribed when Pho4 is nuclear and SP6 is phosphorylated (3). Thus, in intermediate phosphate levels Pho4 might be differentially phosphorylated on SP6 and not SP2 or SP3 leading to the transcription of *PHO84* and not *PHO5*. This indicates that under intermediate phosphate levels cells might only need to increase their phosphate uptake ability and not resort to scavenging of phosphate by phosphatases such as Pho5.

The glutamine response transcription factors, Rtg1 and Rtg3, parallel the behavior of Pho4 in many respects. We discovered that the localization of Rtg1-Rtg3 was regulated by glutamine levels or presence of rapamycin, that the phosphorylation of Rtg3 changed in the presence of rapamycin and that the export receptor Msn5 was involved in establishing the subcellular localization of these transcription factors. Interestingly, we also showed that localization of Rtg1-Rtg3 to the nucleus was not sufficient to induce their activity. Thus, similar to Pho4, Rtg1 and Rtg3 are influenced by multiple regulatory mechanisms including the regulation of their subcellular localization.

Despite the substantial increase in our knowledge of Rtg1 and Rtg3 many important issues remain unresolved. For instance, it is unclear if the changes in the phosphorylation state of Rtg3 result in a change in its activity. To answer this question, it is important to identify the phosphorylation sites on Rtg3 and investigate their role in regulating its activity. Furthermore, the role of Tor kinases in regulating the activity of Rtg1 and Rtg3 is unclear. The inhibition of Tor1 and Tor2 by rapamycin results in a change in the activity, localization and phosphorylation state of these transcription factors. However, it is unclear if Tor1 and Tor2 are directly involved in the cellular response to glutamine starvation. It is possible that Tor1 and Tor2 and glutamine starvation affect the same downstream component, which in turn acts on Rtg1 and Rtg3. It would be important to clarify the role of Rtg2 in this pathway. From our work it appears that Rtg2 is required at steps leading to the import of the Rtg1-Rtg3 complex to the nucleus. It is possible that Rtg2 regulates a component of the nuclear transport machinery which imports Rtg1 and Rtg3 into the nucleus. Lastly, it would be important to investigate the nature of the additional regulatory levels that control Rtg1 and Rtg3 activity in the nucleus.

One of our laboratory's goals in studying the *PHO* pathway in detail has been to establish platforms and models to study other similar systems. The work in this thesis demonstrates the usefulness of such an approach. Our experimental approach in studying the glutamine starvation response and the Rtg1-Rtg3 transcription factor complex was greatly aided by our knowledge of Pho4. Furthermore, our study of these two systems revealed a common theme in the regulation of transcription factor activity in signaling pathways. Both Rtg1-Rtg3 and Pho4 change their subcellular localization in response to the availability of the appropriate nutrients. However, their localization to the nucleus is

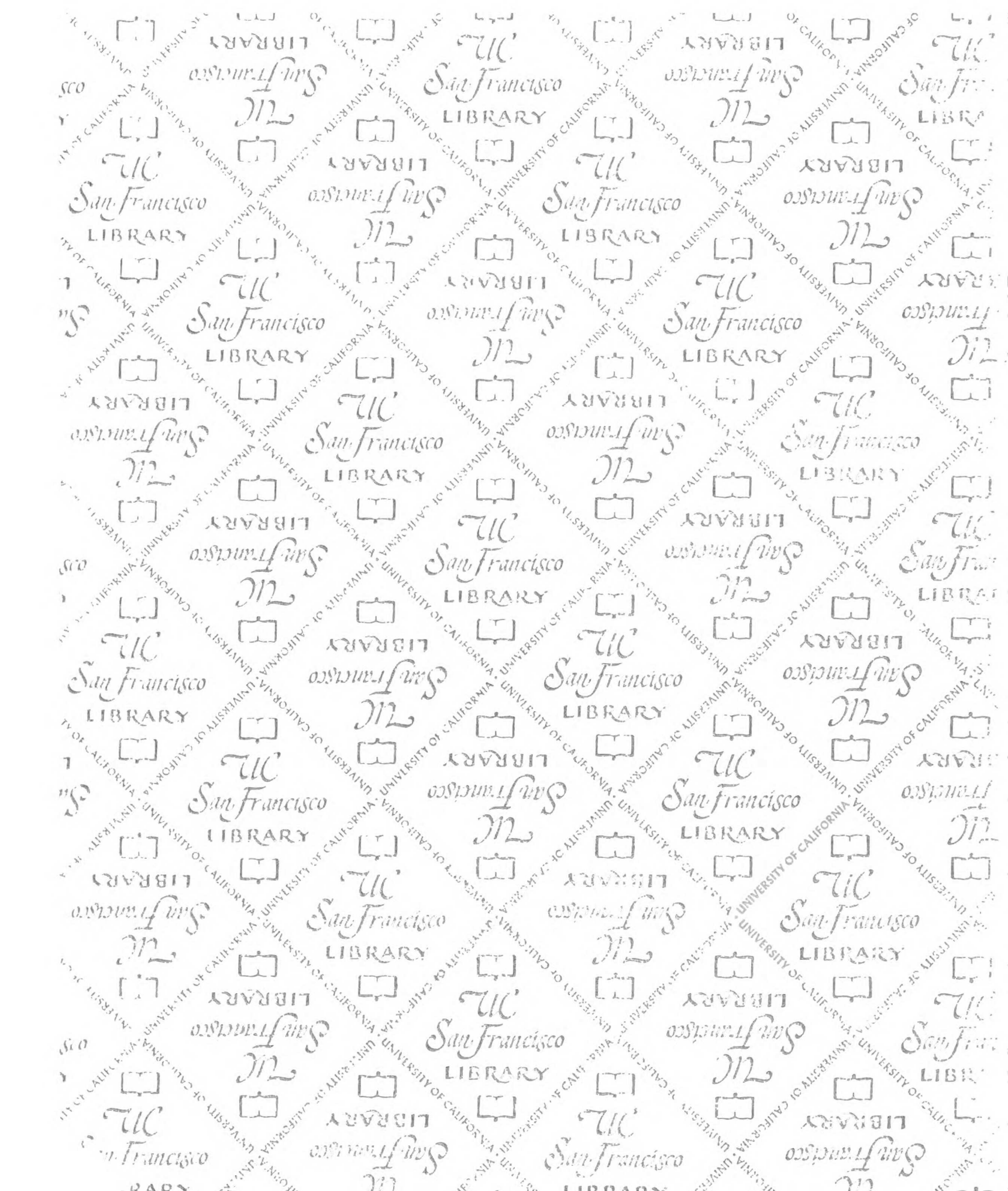
not sufficient to induce their activity indicating that multiple modes of regulation are used in controlling their activity. The next challenge is to clarify the biological importance of employing multiple regulatory modes in these nutrient signaling pathways.

References:

1. Jeffrey DA et al. *J Mol Biol.* 2001. 306(5):997-1010.

2. Springer M et al. Unpublished Results.

3. Miller N et al. Unpublished Results.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