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CIS-ACTING SEQUENCES INVOLVED IN TARGETING TRM1P-II TO THE INNER NUCLEAR MEMBRANE IN *SACCHAROMYCES CEREVISIAE*

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ABSTRACT

The mechanism of targeting a peripheral protein to the inner nuclear membrane (INM) is not understood. Sequences of Trm1p-II, a peripheral INM protein in *Saccharomyces cerevisiae*, were examined to define the *cis*-acting regions mediating targeting. Both a 9-amino acid nuclear localization sequence (NLS) targeting β-galactosidase to the nuclear interior as well as a 60-amino acid membrane targeting sequence directing β-galactosidase to the INM were uncovered. Trm1p-II may possess two NLSs; a classical basic NLS at amino acids 95-102 (NLS#1) and a putative NLS at amino acids 544-552 (NLS#2). NLS#1 is likely recognized by the classical importin, importin β, based on amino acid sequence similarities with known importin β substrates, while NLS#2 is recognized by the non-classical importers, Nmd5p and Kap120p. Several regions of Trm1p-II are necessary for INM targeting, including the N-terminus and two internal regions, as deleting these regions releases Trm1-II-GFP from the INM into the nucleoplasm. However, a single *cis*-acting sequence of Trm1p-II (amino acids 89-151) is sufficient to target passenger protein(s) to the INM. Competition experiments suggest Trm1p-II may be tethered to a second nuclear component, functioning in addition to the membrane partner, to retain Trm1p-II within the nucleus. Ice2p was identified in a genome-wide screen for mutants that mislocate Trm1-II-GFP, suggesting Ice2p may have a role in targeting of Trm1p-II to the INM; deletion of *ICE2* releases Trm1-II-GFP from the INM into the nucleoplasm. However, a β-galactosidase fusion protein containing amino acids 73-151 of Trm1p-II is maintained at the INM in *ice2* cells suggesting Ice2p may have a role in the distribution of Trm1p-II around the INM (possibly by regulating a

protein(s) involved in this process) rather than functioning directly by binding Trm1p-II at the membrane. As Trm1p-II is acetylated at its N-terminus, we propose a bipartite model for targeting to the INM that requires this protein modification to expose a *cis*-acting sequence within amino acids 89-151 for binding Trm1p-II to its INM-binding partner. We believe Trm1p-II is targeted to a specific site at the INM and subsequently spread from this site, accounting for the variations in nuclear localization observed with different Trm1p-II fusion proteins.

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AMP - ampicillin

bp - basepair

BSA - bovine serum albumin

dATP - deoxyadenosine triphosphate

dCTP - deoxycytosine triphosphate

dGTP - deoxyguanine triphosphate

dNTP - deoxynucleotides

dTTP - deoxythymine triphosphate

DAPI - 4', 6'-diamidino-2-phenylindole dihydrochloride

DNA - deoxyribonucleic acid

DTT - 1, 4-dithiothreitol

EDTA - ethelenediaminetetraacetic acid

ER - endoplasmic reticulum

FG - phenylalanine- glycine

FITC - fluorescein isothiocyanate

FRAP - fluorescence recovery after photobleaching

GDW - glass distilled water

GDP - guanosine diphosphate

GFP - green fluorescent protein

GTP - guanosine triphosphate

INM - inner nuclear membrane

KAN - kanamycin

kb - kilobase

kDa - kilodaltons

LAP - lamina-associated polypeptide

LB - Luria broth

LBR - lamin B receptor

MDa - megadaltons

ul - microliter

ml - milliliter

mt - mutant

nm - nanometer

NLS - nuclear localization sequences

NPC - nuclear pore complex

ONM - outer nuclear membrane

PBS - phosphate buffered saline

PCR - polymerase chain reaction

PEG - polyethylene glycol

PNK - polynucleotide kinase

PNS - perinuclear space

RanBP1 - Ran binding protein 1

RanGAP - Ran GTPase Activating Protein

RanGEF - Ran Guanine Nucleotide Exchange Factor

RNA - ribonucleic acid

rpm - revolutions per minute

SDS - sodium dodecyl sulfate

SV40 - simian virus 40

TBE - Tris, boric acid, EDTA

TE - Tris, EDTA

tRNA - transfer RNA

YEPD - yeast extract, peptone, dextrose

YT - yeast tryptone

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CHAPTER ONE

GENERAL INTRODUCTION

Eukaryotic cells are defined by the presence of membrane-bound organelles that provide structural and functional compartmentalization for the cell. This compartmentalization allows various regions of the cell to be specialized for diverse functions, resulting in cellular reactions proceeding more efficiently as enzymes are concentrated at their site of function. Although compartmentalization is advantageous to the cell, it does create an inherent trafficking problem, as cells now require mechanism(s) to target proteins from their site of synthesis to their site of action. All nuclear genome-encoded proteins are synthesized in the cytoplasm and must be subsequently targeted to the various regions of the cell in which they function. In eukaryotic cells, this may require crossing a membrane, such as that of the mitochondria or nucleus, to gain access to the interior of an organelle.

The nucleus is the site of the essential cellular processes of DNA replication, RNA transcription and processing, and tRNA biogenesis (Fig. 1). The nucleus is surrounded by a double lipid membrane, composed of the outer nuclear membrane (ONM) and the inner nuclear membrane (INM). The ONM is contiguous with the endoplasmic reticulum (ER); therefore these membranes possess many of the same proteins. Though many proteins are shared between the ER and ONM, there is also a subset of proteins that localize exclusively to the ONM. The KASH (**K**larsicht/**A**NC-1/**S**yne **h**omology) domain proteins localize to the ONM and are conserved in *Drosophila*

Fig. 1: Nuclear structure.

The nucleus is surrounded by a double lipid membrane, composed of the outer nuclear membrane (ONM) and the inner nuclear membrane (INM). The ONM is contiguous with the endoplasmic reticulum (ER). The nuclear membrane is studded with nuclear pore complexes (NPCs) that allow transport of macromolecules between the nucleus and cytoplasm. The nucleolus, the most prominent subnuclear structure, is also pictured.

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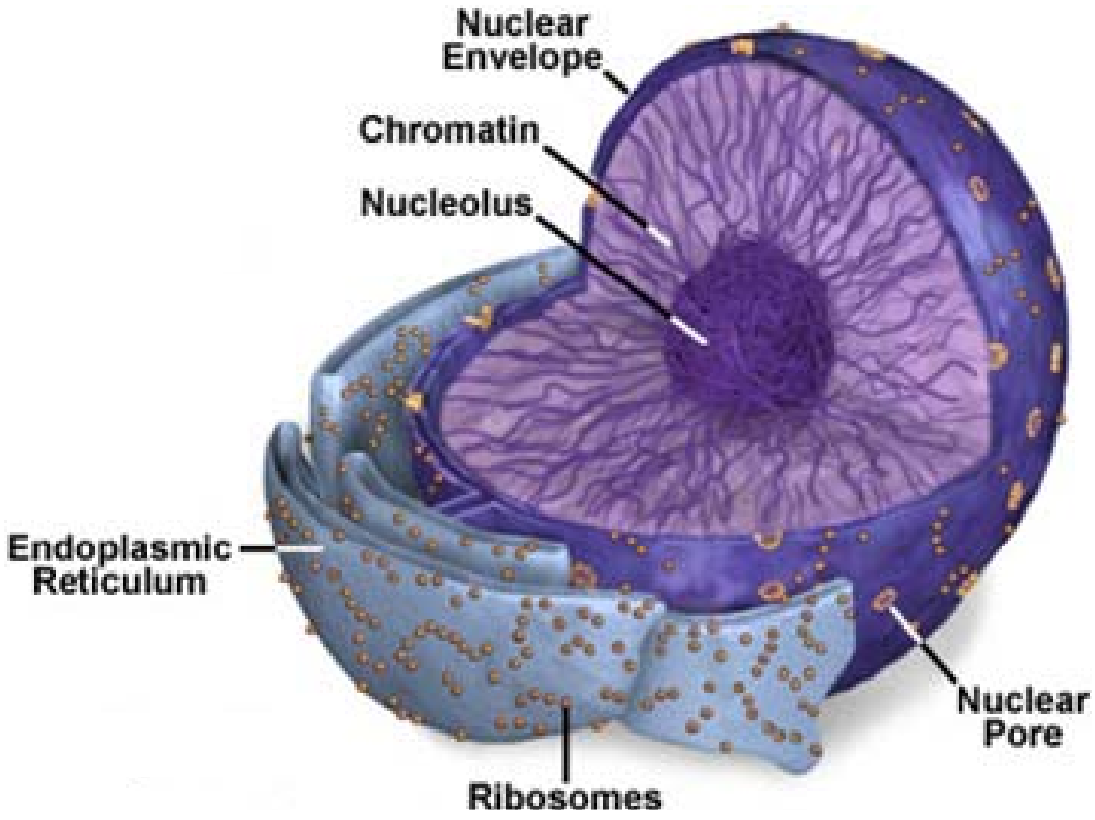

melanogaster, Caenorhabditis elegans, and humans (Reviewed in Starr and Fischer, 2005; Worman and Gunderson, 2006). The INM is distinct from the ONM and has a unique composition of proteins. The ONM and INM fuse at the pore membrane, the membrane region surrounding the nuclear pore complex (NPC).

Subnuclear structure of the nucleus

Many cellular processes occur simultaneously within the nucleus. This complex organelle is therefore divided into subdomains specialized to perform various functions. In contrast to the compartmentalization of the cell into organelles, the specialized regions of the nucleus are not physically separated by membranes. Little is known about the higher-order structure of the nucleus; most likely subnuclear organization results from interactions between various proteins and chromatin.

The most well-characterized subdomain of the nucleus is the nucleolus, an area enriched in splicing factors for rRNA transcription and processing, and the site for the assembly of ribosomes. Speckles, also called interchromatin granule clusters, are enriched in pre-mRNA splicing factors prior to their assembly into the spliceosome. The function of cajal bodies, also referred to as coiled bodies, remains unclear; however, these structures are enriched in molecules involved in RNA splicing, including small nuclear ribonucleoprotein complexes (snRNPs), heterogeneous nuclear ribonucleoprotein complexes (hnRNPs), small nuclear RNA (snRNA), fibrillarin, and coilin. Intermediate filaments called nuclear lamins interact to form a thin network lining the INM, termed the nuclear lamina, as well as an internal network dispersed throughout the nucleoplasm, called the nuclear matrix that provide a structural framework for the nucleus and a

scaffold for gene expression, respectively (Reviewed in Matera, 1999; Zimber *et al.,* 2004; Taddei *et al.,* 2004).

The INM is a highly complex region of the nucleus. Within the INM, there are structurally distinct regions that confer functional specialization; areas enriched in NPCs are associated with actively transcribed DNA while areas lacking NPCs are associated with silenced chromatin, such as the telomeres (Casolari *et al.,* 2004; Taddei *et al.,* 2004). Furthermore, the vertebrate INM contains unique integral proteins, such as the lamina-associated polypeptides (LAPs), the lamin B receptor (LBR), emerin, and MAN1, as well as peripherally associated proteins, including the nuclear lamins. There are several distinct isoforms of the vertebrate nuclear lamins. *LMNA* encodes lamins A and C which are expressed in terminally differentiated cells (Lin and Worman, 1993). Lamin A is a 664 amino acid protein while lamin C is a 572 amino acid protein. Lamins A and C share their first 566 amino acids; however, lamin C lacks approximately 100 amino acids at its C-terminus relative to lamin A. *LMNB1* and *LMNB2* encode lamins B1 and B2, respectively, expressed in all somatic cells. In addition, *LMNA* encodes lamin C2 while *LMNB2* encodes lamin B3, which are expressed exclusively in germ cells (Furukawa and Hotta, 1993; Furukawa *et al.,* 1994; Schütz *et al.,* 2005). The existence of various lamin isoforms seems to allow for tissue-specific expression. However, distinct function(s) of lamin B1 and B2 are unclear as these isoforms are both expressed in all somatic cells.

It is evident that INM proteins have a role in all aspects of nuclear structure and function, including assembly and maintenance of the organelle as well as regulation of gene expression. Evidence suggests nuclear proteins may also have a role in the global structure of the cell by providing a physical link between the nucleus and cytoskeleton

(Reviewed in Worman and Gundersen, 2006). The localization of integral ONM proteins containing conserved KASH domains, vertebrate Syne-1 and Syne-2 (also called Myne-1/Nesprin/NUANCE and Nesprin-2/Nesprin-2 Giant/nesp2G, respectively), *C. elegans* UNC-83 and ANC-1, and *D. melanogaster* Klarsicht is dependent on their interaction across the perinuclear space with integral INM proteins containing conserved SUN (**S**ad1/**UN**C-84 homology) domains, including Sun1/2 and UNC-84 (Padmakumar *et al.,* 2005; Crisp *et al.,* 2006; Starr and Han, 2002; McGee *et al.,* 2006). Nesprins, UNC-83, and ANC-1 bind to the cytoskeleton via actin in the cytoplasm, while Sun1/2 and UNC-84 bind to nuclear lamins in the nucleoplasm creating an intricate network connecting the plasma membrane to the nuclear membrane (Libotte *et al.,* 2005; Zhang *et al.,* 2005; Starr and Han, 2002). Though these proteins may have additional cellular functions, each contribute to the formation of this highly elaborate structural framework, which has been shown to be important for nuclear positioning and mechanical transduction (Fig. 2).

Nuclear pore complex

The NPC, a highly conserved structure shared by all eukaryotes, mediates transport between the cytoplasm and nucleus (Fig. 3). In *Saccharomyces cerevisiae* and vertebrates, the NPC is comprised of approximately 30 proteins called nucleoporins (nups), arranged in eight-fold symmetry to create an approximately 9 nm aqueous central channel through which macromolecules can move in both an active and passive manner (Rout *et al.,* 2000; Cronshaw *et al.,* 2002). In *S. cerevisiae*, molecules less than 47 kDa (Shulga *et al.,* 2000) are capable of passive diffusion through the central channel, while

Fig. 2: A model diagramming the connections between the nucleus and cytoskeleton.

KASH (**K**larsicht/**A**NC-1/**S**yne **h**omology)-domain containing proteins localize to the outer nuclear membrane (ONM), while SUN (**S**ad1/**UN**C-84 homology)-domain containing proteins localize to the inner nuclear membrane (INM). In the ONM, the KASH-domain proteins (Nesprin-2 Giant pictured) bind actin, a component of the cytoskeleton, which is proposed to contribute to nuclear positioning and migration. In the INM, the SUN-domain proteins (UNC-84 pictured) bind nuclear lamins to support and stabilize the nuclear membrane. Unknown complexes, which may include peripheral as well as integral proteins of the nuclear membrane, are represented by large purple ovals.

Figure obtained from: Libotte T, Zaim H, Abraham S, Padmakumar VC, Schneider M, Lu W, Munck M, Hutchison C, Wehnert M, Fahrenkrog B, Sauder U, Aebi U, Noegel AA. Karakesisoglou I. Lamin A/C-dependent localization of Nesprin-2, a giant scaffolder at the nuclear envelope. Mol Biol Cell. 2005; 16:3411-3424.
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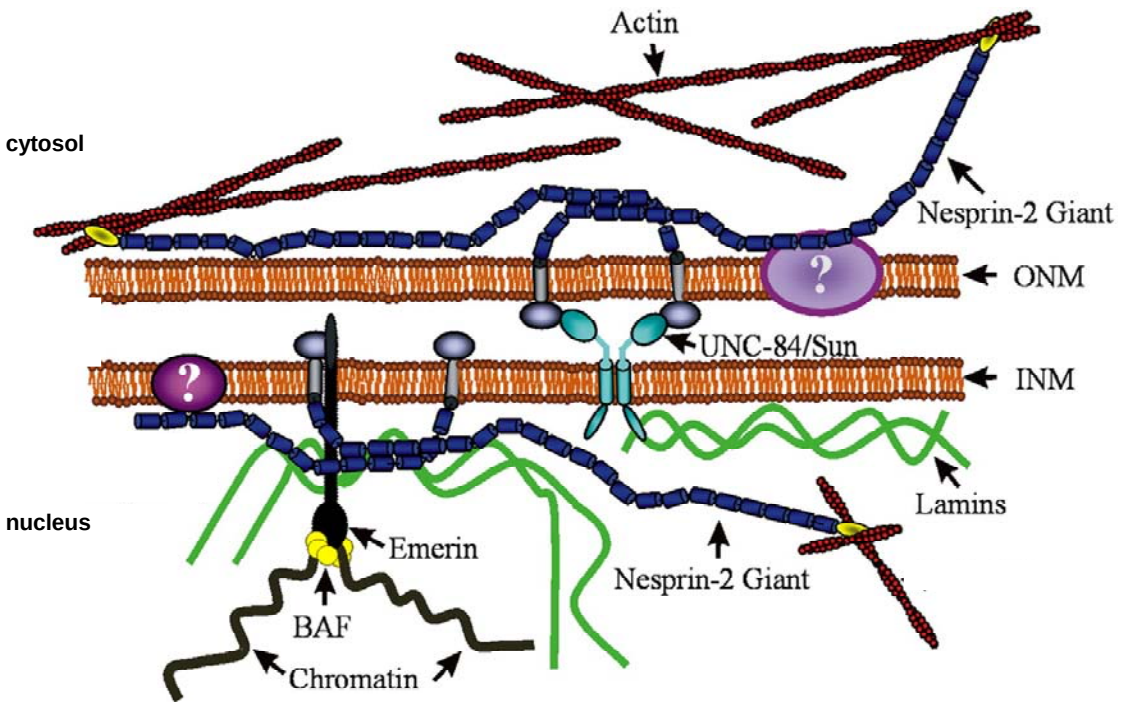

Fig. 3: Details of the nuclear pore complex within the nuclear membrane of eukaryotic cells.

The nuclear pore complex (NPC) is comprised of approximately 30 proteins called nucleoporins, arranged in eight-fold symmetry to create an approximately 9 nm aqueous central channel through which macromolecules can move in both an active and passive manner. The NPC extends on either side of the nuclear membrane, via individual filaments into the cytoplasm and a basket or cage, formed by filaments joined at their distal ends, into the nucleoplasm.

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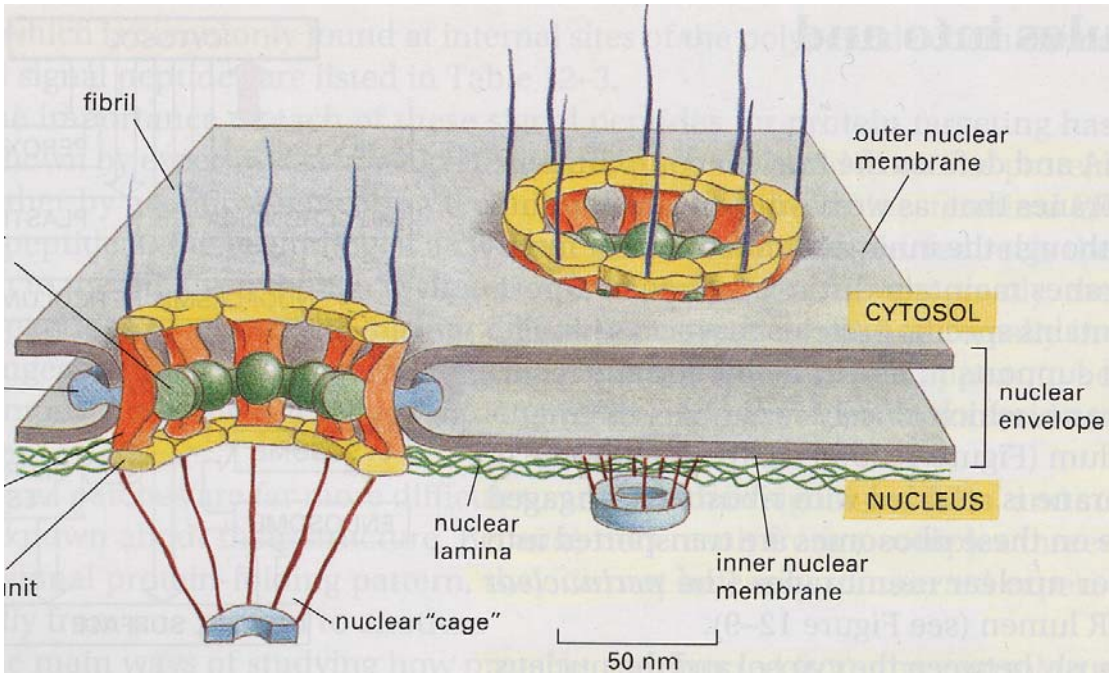


larger molecules must be actively transported by receptor proteins, called karyopherins that regulate nuclear-cytoplasmic traffic. In *S. cerevisiae*, the NPC is approximately 66 MDa, while in vertebrates it is approximately 125 MDa (Reichert *et al.,* 1990; Rout and Blobel, 1993). The composition of the NPC is relatively simple in comparison to the overall mass; however, this is likely due to the redundancy of the structure as most nucleoporins are present in multiple copies. The NPC extends on either side of the nuclear membrane, via individual filaments into the cytoplasm and a basket, formed by filaments joined at their distal ends, into the nucleoplasm; these structures are distinct and composed of different nucleoporins.

Nucleoporins are classified into three categories depending on their function within the NPC. In *S. cerevisiae*, 11 nucleoporins contain phenylalanine-glycine (FG) repeats that mediate their interaction with the various karyopherins and are thus classified as FG nucleoporins. A second class of nucleoporins act as a scaffold to organize the FG nucleoporins to optimize their interaction with the karyopherins during translocation through the central channel of the NPC. Finally, there is a group of nucleoporins that contain transmembrane domains that serve to anchor the NPC within the nuclear/pore membrane. In *S. cerevisiae*, Pom152p, is the only identified transmembrane nucleoporin, while in vertebrates, POM121 and gp210 are the known transmembrane anchoring nucleoporins (Wozniak *et al.,* 1994).

Most nucleoporins are distributed symmetrically throughout the NPC. However, in *S. cerevisiae*, four nucleoporins, Nup116, Nup100, Nup145N, and Gle1, are biased on a particular side of the NPC, though present on both. Other nucleoporins are restricted to a single face of the NPC; Nup42, Nup82, and Nup159 are strictly cytoplasmic, while

Nup1 and Nup60 are strictly nuclear (Rout *et al.,* 2000). The majority of nucleoporins are stationary within the NPC; however the vertebrate nucleoporins Nup50 and Nup98 have a dynamic nature. For example, Nup98 shuttles between the nucleoplasm, cytoplasm, and NPC (Griffis *et al.,* 2002; Daigle *et al.,* 2001; Zolotukhin and Felber, 1999; Lindsay *et al.,* 2002). The distribution of various nucleoporins may function in regulating the directionality of transport through the central channel of the NPC. For example, vertebrate Nup358 is exclusive to the cytoplasmic filaments and has high affinity for RanGTP, while vertebrate Nup153 is restricted to the nuclear basket and has low affinity for RanGTP (Reviewed in Vasu and Forbes, 2001). However, Walther *et al.* (2002) demonstrated that the cytoplasmic filaments, composed of RanBP2/Nup358, are dispensable in vertebrate NPCs, as their depletion did not inhibit nuclear import. Therefore, directionality of transport through the NPC is likely regulated by the distribution of multiple nucleoporins rather than that of individual nucleoporins.

Approximately one-third of the *S. cerevisiae* nucleoporins, likely those lining the central channel of the NPC, contain FG repeats (FxFG, GLFG, or FG) that bind directly to the karyopherins (Bednenko *et al.,* 2003; Rout *et al.,* 2000; Bayliss *et al.,* 2002). These hydrophobic repeats are usually separated by hydrophilic regions of 5–30 amino acids and, based on X-ray structure, appear to insert into hydrophobic pockets on the karyopherins during their translocation across the NPC (Bayliss *et al.,* 2000; Bayliss *et al.,* 2002). Both Zeitler and Weis (2004) and Strawn *et al.* (2004) found that deleting the FG domains from the five asymmetrically distributed nucleoporins, Nup42, Nup159, Nup1, Nup2, and Nup60, did not disrupt nuclear-cytoplasmic transport. However,

deleting the FG domain of Nup116 by itself or the GLFG domains of Nup116 and Nup100 in combination caused lethality indicating these regions are essential for nuclear-cytoplasmic transport. Furthermore, these studies indicated that there are several independent pathways for nuclear import as specific FG deletions inhibited import of some proteins whereas import of other proteins was unaffected (Strawn *et al.,* 2004; Zeitler and Weis, 2004).

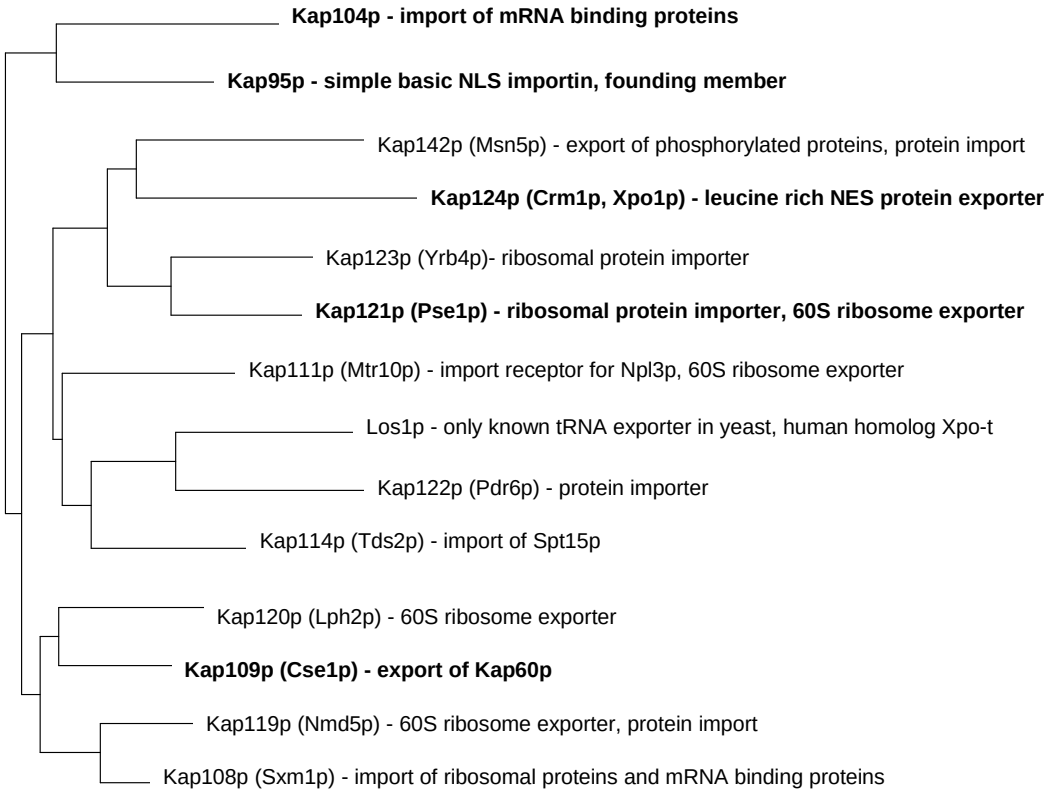
Karyopherins: Importin β family of transport receptors

In *S. cerevisiae*, there are 14 karyopherins, members of the importin β family of transport receptors, whereas in higher eukaryotes, there are approximately 20 members. In *S. cerevisiae*, the members of the importin β family are all related with regard to protein structure (Fig. 4). The various family members, ranging in size from 90–130 kDa, show little sequence similarity. However, all possess a Ran-binding domain at the N-terminus, multiple tandem anti-parallel amphipathic α-helices (HEAT repeats), and an acidic isoelectric point (Reviewed in Strom and Weis, 2001). The C-terminus of the karyopherin interacts directly with the cargo whereas additional regions mediate interaction with the FG domains of the nucleoporins. Importin β, the prototypic nuclear importer, has two distinct nucleoporin binding sites, at the N-terminus and C-terminus, respectively (Bayliss *et al.,* 2000; Bednenko *et al.,* 2003). Although the C-terminal nucleoporin binding site has a lower affinity for nucleoporins, it seems to contribute equally to nuclear import *in vivo*. Karyopherins bind directly to the nucleoporins via their FG repeat domains (Iovine *et al.,* 1995; Ryan and Wente, 2000) thus shuttling the cargo through the central channel of the NPC to either the nucleoplasm or cytoplasm. The

Fig. 4: Importin β family of transport receptors in *Saccharomyces cerevisiae*.

ClustalX-generated dendrogram of importin β family members in *S. cerevisiae (*Jeanmougin *et al.,* 1998). Trees built with Clustal are based solely on sequence similarity. Essential importin β family members are bolded.

Courtesy of David Stanford.



mechanism by which karyopherin-nucleoporin binding regulates transport is not clear, though several models have been proposed.

Of the 14 importin β family members in *S. cerevisiae*, 9 are unessential while 5, including importin β/Rsl1p/Kap95p, Kap104p, Pse1p/Kap121p, Cse1p, and Crm1p, are essential (reviewed in Gorlich and Kutay, 1999). The majority of karyopherins are dispensable individually, even though the import or export of their respective cargo may be necessary, implying most proteins likely possess multiple pathways for import/export. Importin β can bind cargo directly or act through an adaptor protein, importin α/Srp1p/Kap60p. This duality of importin β binding to its cargo increases the diversity of cargo recognized by importin β. Although importin β has only a single adaptor, importin α, in *S. cerevisiae*, at least six adaptors have been identified for importin β in vertebrates (Malik *et al.,* 1997). Co-crystallization of karyopherins bound to various cargo indicates karyopherins possess multiple cargo-binding sites; this suggests that distinct contacts are made between the karyopherin and cargo, depending on its identity, and proposes a mechanism for how a limited number of transport receptors can interact with such a large number of diverse substrates (Reviewed in Pemberton and Paschal, 2005).

The various importin β family members can have multiple roles in nuclear-cytoplasmic transport. Some karyopherins function exclusively in either import or export, while others regulate traffic in both directions. In *S. cerevisiae,* Kap104p, Kap114p, Pse1p/Kap121p, and Yrb4p/Kap123p mediate nuclear import, whereas Crm1p and Los1p have only been implicated in export. However, Msn5p/Kap142p, originally identified as an exporter of various proteins, including Pho4p, Mig1p, Far1p, and Ste5p, also functions to import a trimeric complex composed of Rpa1p, Rpa2p, and Rpa3p (RPA) involved in

DNA replication, repair, and recombination (Kaffman *et al.,* 1998; DeVit and Johnston, 1999; Blondel *et al.,* 1999; Mahanty *et al.,* 1999; Yoshida and Blobel, 2001). RPA also interacts with importin β in *msn5/kap142* cells, suggesting there are multiple pathways for import of the same protein *in vivo*.

Ran: regulator of nucleus-cytoplasmic exchange

Transport between the nucleus and cytoplasm through the NPC is bidirectional, with proteins imported into the nucleus and RNA exported to the cytoplasm, and usually requires the small GTPase, Ran. Ran is an approximately 25 kDa protein encoded by two genes, *GSP1* and *GSP2*, with the majority of protein produced by *GSP1* (Belhumeur *et al.,* 1993). The directionality of transport is regulated, in part, by the asymmetric distribution of Ran across the nuclear membrane; RanGTP concentration is high in the nucleus and low in the cytoplasm (Izaurralde *et al.,* 1997; Kalab *et al.,* 2002). High levels of RanGTP are maintained in the nucleus by the action of RanGEF (RCC1 in higher eukaryotes and Prp20p in *S. cerevisiae*), which is tethered to the chromatin and converts RanGDP to RanGTP (Aebi *et al.,* 1990; Ohtsubo *et al.,* 1991). RanGDP is concentrated in the cytoplasm by the action of RanGAP (Rna1p in *S. cerevisiae*), which hydrolyzes RanGTP to RanGDP (Becker *et al.,* 1995; Bischoff *et al.,* 1995; Hopper *et al.,* 1990). RanBP1 (Yrb1p in *S. cerevisiae*) also resides in the cytoplasm and stimulates RanGAP to hydrolyze RanGTP (Ouspenski *et al.,* 1995).

General models for nuclear import and export have been delineated. Import complexes contain a karyopherin bound, either directly or through an adaptor protein, to its respective cargo. Upon translocation to the nucleoplasm, RanGTP binds the

karyopherin-cargo complex weakening its affinity for the FG nucleoporins and causing its disassembly, thus releasing the cargo (Rexach and Blobel, 1995; Hieda *et al.,* 1999; Izaurralde and Adam, 1998). The importer-RanGTP complex is then recycled to the cytoplasm, where RanGTP is hydrolyzed to RanGDP, allowing the karyopherin to function in another round of nuclear import. Export requires formation of a tertiary complex in the nucleus containing the karyopherin, cargo, and RanGTP. Upon translocation to the cytoplasm, hydrolysis of RanGTP to RanGDP, activated by RanGAP, causes release of the karyopherin and cargo in the cytoplasm (Arts *et al.,* 1998; Fornerod *et al.,* 1997). RanGDP is then recycled to the nucleus and converted to RanGTP by the action of RanGEF (Fig. 5).

Protein targeting to the nucleus

The mechanisms of targeting to many organelles as well as the signals that mediate this targeting are defined. Proteins destined for the nucleus possess sequences on their surface known as nuclear localization sequences (NLS). These sequences are recognized by the karyopherin in the cytoplasm and subsequently target these proteins to the nucleus. Classical NLSs are characterized by enrichment in basic amino acids and can take several forms; a single cluster of basic amino acids or two clusters of basic amino acids separated by a spacer. The most well-studied NLS (P**KKKRK**V) is that of the SV40 large T antigen which contains a single stretch of basic amino acids (Kalderon *et al.,* 1984). In contrast, the NLS within nucleoplasmin exemplifies the classical bipartite NLS (**KR**PAAIKKAGQA**KKKK**) whereby two clusters of basic amino acids are required for function (Robbins *et al.,* 1991). The proposed mechanism for binding of the

Fig. 5: The RanGTPase cycle regulates nuclear-cytoplasmic transport.

(1) The importer (Imp)-cargo complex forms in the cytoplasm and is translocated through the central channel of the nuclear pore complex (NPC). Upon entry to the nucleus, RanGTP binds to the importer causing release of the cargo.

(2) The RanGTP-importer complex is recycled to the cytoplasm for use in another round of nuclear import.

(3) The RanGTP-exporter (Exp)-cargo complex forms in the nucleus and is translocated through the central channel of the NPC. In the cytoplasm, RanGTP is hydrolyzed to RanGDP by RanGAP causing release of the cargo.

(4) RanGDP and the exporter are then recycled to the nucleus where RanGDP is converted to RanGTP by RanGEF (not pictured).

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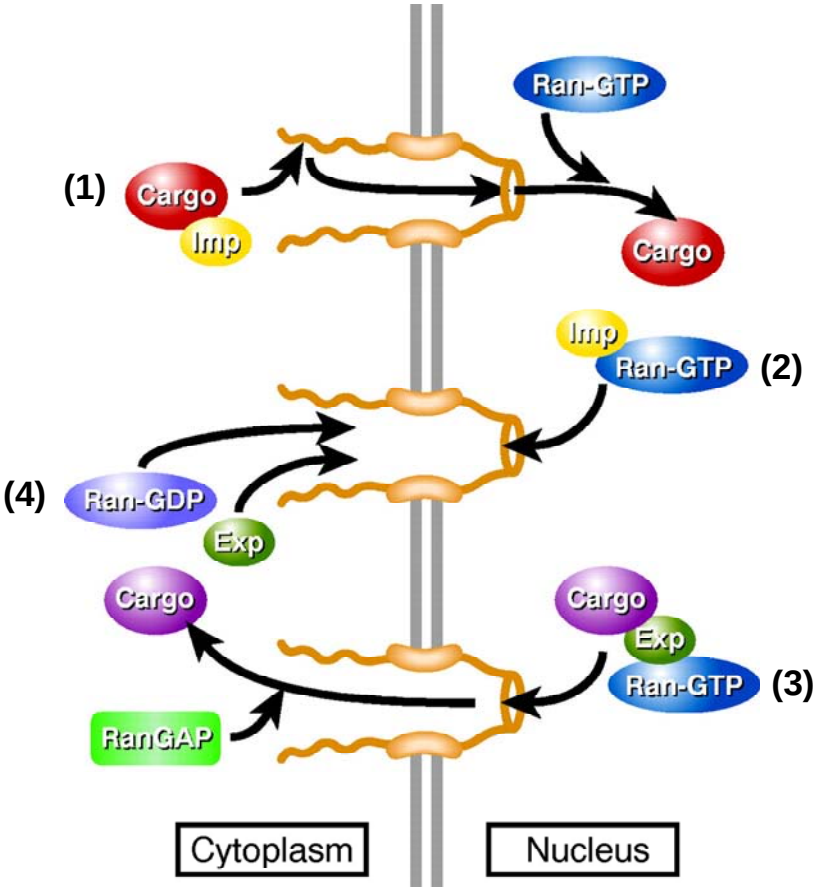

bipartite NLS by its karyopherin is that the two basic clusters are joined together by looping out of the spacer region. By this model, both the length and amino acid composition of the spacer can be varied without affecting the function of the NLS. This is supported by the observation that hypoxia-inducible factor 1 alpha (HIF1α) and hnRNP type I each have a functional bipartite NLS with a spacer of 32 and 30 amino acids, respectively, compared to nucleoplasmin which has a spacer of only 10 amino acids (Luo and Shibuya, 2001; Romanelli and Morandi, 2002; Robbins *et al.,* 1991). Additionally, mutations within the spacer of the nucleoplasmin NLS do not affect nuclear localization of a nucleoplasmin-pyruvate kinase fusion protein, indicating the amino acid composition of the spacer does not contribute to nuclear targeting (Makkerh *et al.,* 1996). Proteins containing either a monopartite or bipartite classical NLS are recognized and targeted to the nucleus by importin β. However, importin β does not bind directly to these signals, but rather requires the adaptor protein, importin α; thus, a heterotrimeric import complex must be formed.

Novel motifs that function as NLSs *in vivo* are continually being defined, such as that within Crz1p (IINGRKLKLKKSRRRSS) which requires both basic and hydrophobic amino acids for function (Polizotto and Cyert, 2001), the M9 domain of hnRNP A1, in which the 38 amino acid NLS is enriched in glycine rather than basic residues (Michael *et al.,* 1995; Pollard *et al.,* 1996), and the KNS signal within hnRNP K that does not require a karypopherin for import (Micheal *et al.,* 1997). As NLS motifs continue to be identified that do not fit the characteristics of a classical monopartite or bipartite NLS, it is becoming difficult to predict a functional NLS based solely on primary amino acid sequence. Additionally, multiple NLSs have been identified within a single protein (Luo

et al., 2004), suggesting that the variable NLSs may provide a means of regulation to alter the distribution of a protein under certain conditions. Post-translational modifications, such as phosphorylation, contribute to nuclear targeting as well; Mig1p and Pho4p are both targeted out of the nucleus by a phosphorylation-dependent mechanism (DeVit and Johnston, 1999; Kaffman *et al.,* 1998).

Protein targeting to the inner nuclear membrane

Most studies that examine targeting to the INM have focused on integral INM proteins, such as LAP1/2, LBR, emerin, and MAN1, rather than peripheral proteins. The current model for targeting integral proteins to the INM follows the "diffusion-retention" model (Smith and Blobel, 1993; Soullam and Worman, 1993, 1995). In this model, integral INM proteins are synthesized by ribosomes on the ER and insert co-translationally into the ER membrane. Saksena *et al.* (2004) found that the transmembrane region of LBR, through interactions with proteins in the ER translocon, directs LBR into a distinct pathway (targeting specifically to the INM) relative to proteins destined for other cellular membranes. As the ER is continuous with the ONM, LBR is able to diffuse to the ONM from the ER. Once in the ONM, LBR is able to access the INM via diffusion through the pore membrane (the membrane surrounding the NPC that represents the junction of the ONM and INM) and translocation of the nucleoplasmic domain through the lateral channels of the NPC. The lateral channels are approximately 10 nm in diameter and have been shown to exert an inherent size limitation, allowing only proteins less than 60 kDa to diffuse freely (Fig. 6; Hinshaw *et al.,* 1992; Akey and Radermacher, 1993); indeed, increasing the N-terminal nucleoplasmic domain of either

LBR (from approximately 22.5 kDa to approximately 70 kDa) or MAN1 (from approximately 50 kDa to approximately 100 kDA) prevented translocation of these proteins from the ONM to the INM (Soullam and Worman, 1995; Wu *et al.,* 2002). In addition, antibodies to gp210, an integral protein localized to the pore membrane in vertebrate NPCs, are sufficient to inhibit accumulation of an integral INM reporter protein at the INM, likely by blocking the lateral channels (Greber and Gerace, 1992; Ohba *et al.,* 2004). Most likely, integral INM proteins require a rearrangement of the NPC to allow movement through the lateral channel, explaining the requirement for ATP in the transport process (Ohba *et al.,* 2004). Once at the INM, integral INM proteins bind to stable nuclear components, such as the nuclear lamins, chromatin binding proteins, or chromatin itself (Fig. 7; Polioudaki *et al.,* 2001). Disrupting binding of integral INM proteins to lamins or chromatin binding proteins, such as HP1 or BAF, is sufficient to cause mislocalization of these proteins to the ER (Sullivan *et al.,* 1999). Though the experiments that were the basis for the "diffusion-retention" model were carried out for vertebrate integral INM proteins, similar results have subsequently been shown in *S. cerevisiae* for the integral INM proteins, Heh1p and Heh2p (King *et al.,* 2006), suggesting the mechanism for targeting of an integral protein to the INM may be a conserved process across eukaryotic organisms.

Ellenberg *et al.* (1997) provided the first direct evidence in support of the "diffusion-retention" model. Using fluorescence recovery after photobleaching (FRAP), they showed that LBR-GFP diffused rapidly in both the ER and ONM, while immobilized at the INM in live cells; similar diffusion profiles were subsequently

Fig. 6: Lateral channels of the nuclear pore complex.

Soluble nuclear proteins are transported through the central channel of the nuclear pore complex (NPC). In contrast, integral proteins that resident the inner nuclear membrane (INM) are thought to translocate through the lateral channels of the NPC.

Figure obtained from: Alberts B, Bray D, Lewis J, Raff M, Roberts K, and Watson JD. *Molecular Biology of the Cell Third Edition.* New York: Garland Publishing, 1994. Permission granted from publisher to reproduce figure.

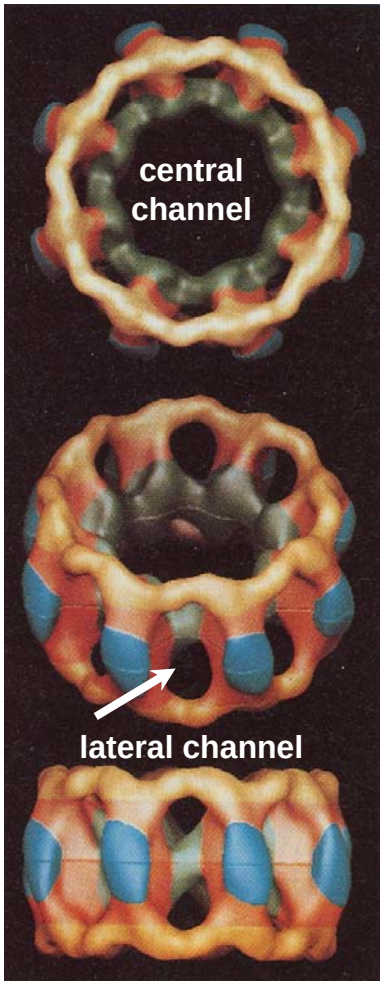

Fig. 7: The diffusion-retention model for targeting integral membrane proteins to the inner nuclear membrane.

Integral inner nuclear membrane (INM) proteins are translated in the cytoplasm and initially inserted into the endoplasmic reticulum (ER). These proteins possess a nuclear localization sequence (NLS; represented by gold circle) which is recognized by karyopherins which mediate their transport into the nucleus. Karyopherin α (triangle) recognizes the NLS and karyoperin β (pentagon) mediates interactions with the nuclear pore complex (NPC) to drive translocation across the nuclear membrane. Once in the nucleus, the integral INM protein binds to chromatin, chromatin-binding proteins, or nuclear lamins to be retained at the INM.

Figure obtained from: Kutay U, Muelhausser P. Taking a turn into the nucleus. Nature. 2006; 442:991-992.
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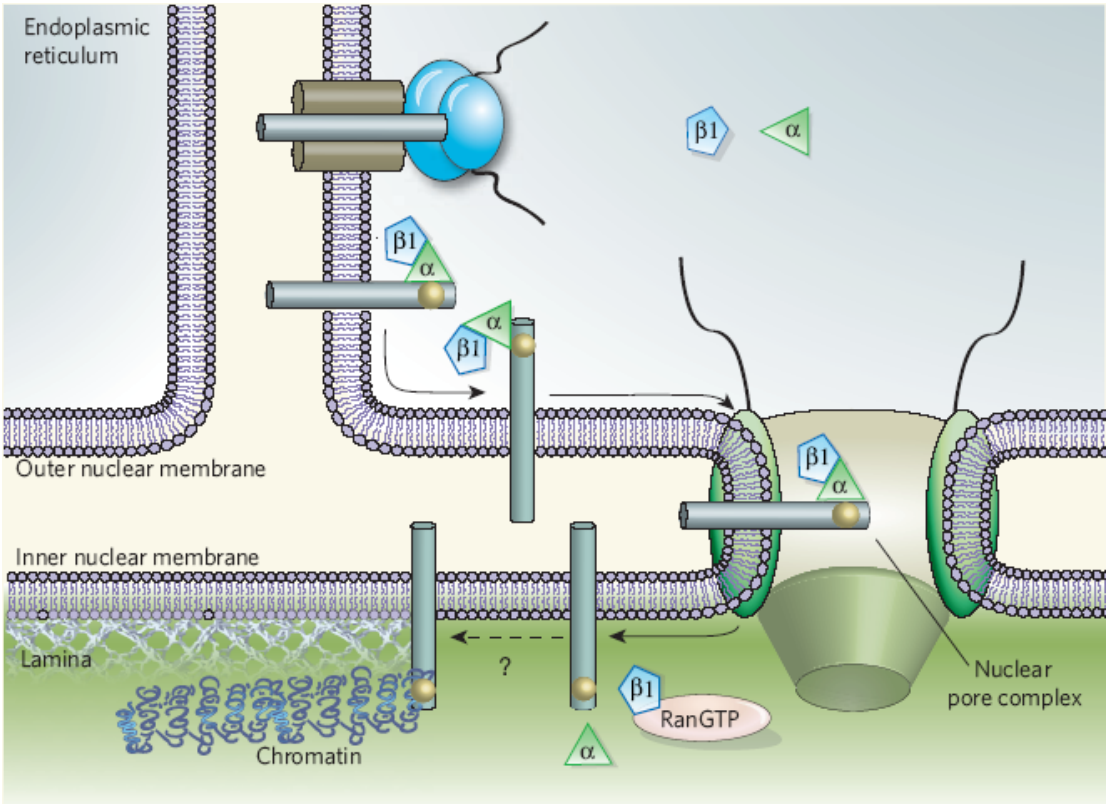

obtained for emerin and MAN1 (Ostlund *et al.,* 1999; Wu *et al.,* 2002). Additional experiments investigated the role of lamins in the diffusional mobility of integral INM proteins. Emerin and MAN1, which bind A-type lamins, were more mobile in lamin A-deficient fibroblasts compared to wild-type fibroblasts. In contrast, the diffusional mobility of LBR, which interacts with B-type lamins, was unaffected in lamin A-deficient fibroblasts (Ostlund *et al.,* 2006). These studies suggest that the nuclear lamins play an essential role in regulating localization and retention of integral INM proteins and may function as the primary nuclear tether to confer retention at the INM.

Mapping studies have also been conducted to define *cis*-acting sequences that confer INM targeting of integral nuclear proteins. LAP2β contains an N-terminal 76 amino acid sequence that binds to B-type lamins (B1 or B2) and is sufficient for targeting to the INM (Furukawa *et al.,* 1995; Furukawa *et al.,* 1998). LBR has two non-overlapping INM targeting sequences; the N-terminus and a distinct transmembrane domain are each sufficient to target LBR to the INM (Smith and Blobel, 1993; Soullam and Worman, 1993, 1995). A 6-amino acid region (DRLRHR) of glycoprotein B of human cytomegalovirus is sufficient for targeting to the INM, thus providing evidence that integral INM proteins have a distinct mechanism for targeting compared to soluble proteins of the nucleus (Meyer and Radsak, 2000). As integral proteins are targeted to the INM by a proposed mechanism does not require transport through the central channel of the NPC, the role of an NLS for targeting integral proteins to the INM remains unclear. LBR has two functional bipartite NLSs at its N-terminus (Soullam and Worman, 1993). In contrast, various nesprin isoforms possess multiple bipartite NLSs; however, these

sequences do not function *in vivo* and are not required for nuclear membrane targeting (Zhang *et al.,* 2001).

The mechanism for targeting soluble proteins to the nucleus (via active transport through the central channel of the NPC) as well as integral proteins to the INM ("diffusion-retention" model) is clear. However, a mechanism for targeting a soluble protein to a distinct subnuclear region, such as the INM, still remains unknown. Two possibilities exist for targeting a soluble protein to the INM. The soluble protein could bind to its membrane-binding partner, likely an integral INM protein, in the cytoplasm and diffuse to the INM through the lateral channels of the NPC via association with the integral INM protein. Alternatively, the soluble protein could be actively transported to the nucleus through the central channel of the NPC and subsequently targeted to the INM via an unknown mechanism. If the second proposed model is true, targeting to the INM would require both an NLS to gain access to the nuclear interior and a separate INM targeting motif. To date, no *cis*-acting INM targeting motif has been defined for a soluble nuclear protein.

In *S. cerevisiae*, approximately 60 proteins localize to the INM based on localization of the entire yeast proteome in live cells (Huh *et al.,* 2003). However, approximately 50% of these are either components of the NPC or karyopherins that function in nuclear-cytoplasmic transport. Several proteins that appear to be peripherally associated with the INM, including Trm1p, a tRNA modification enzyme, and Esc1p, which functions in chromatin silencing, are distributed uniformly along the INM (Rose *et al.,* 1995; Andrulis *et al.,* 2002). Our studies used Trm1p as a reporter to identify the necessary and sufficient signal(s) required to target a peripheral protein to the INM.

TRM1 background

TRM1 was identified through genetic complementation of a *trm1* mutation as the N^2,N^2-dimethylguanosine tRNA methyltransferase present in most eukaryotes (Ellis *et al.,* 1986). Trm1p catalyzes the addition of two methyl groups to G_{26} on both nuclear and mitochondrial tRNAs. *TRM1* is an unessential gene as its deletion does not cause a defect in tRNA function. However, *trm1* interacts genetically with *trm11*, another tRNA modification enzyme, suggesting that a single modification of tRNA may be dispensable, but loss of multiple modifications may alter tRNA structure and thus, inhibit its function (Purushothaman *et al.,* 2005).

Trm1p belongs to a family of proteins known as sorting isozymes wherein a single gene encodes an enzyme that functions in multiple cellular compartments (Gillman *et al.,* 1991). Trm1p is a class III sorting isozyme as it localizes to both the nucleus and mitochondria (Martin and Hopper, 1994). *TRM1* generates two classes of transcripts, each producing functional protein; however, approximately 90% of the transcripts initiate between position +14 to +36, while the remaining transcripts initiate at position –2 (Ellis *et al.,* 1987). The long form, Trm1p-I, begins translation at the first AUG (+1) producing a 570 amino acid protein. This protein possesses a 16-amino acid extension at the N-terminus that augments a mitochondrial targeting signal and targets Trm1p-I exclusively to the mitochondria (Ellis *et al.,* 1989). The short form, Trm1p-II, begins translation at the second AUG (+48) and produces a 554 amino acid protein which targets predominantly to the nucleus (in spite of possessing a mitochondrial targeting sequence [shared with Trm1p-I]). Within the nucleus, Trm1p-II localizes to the INM, appearing as ring-like structures in both live and fixed cells (Murthi and Hopper, 2005; Li *et al.,* 1989;

Rose *et al.,* 1992; 1995). The ONM and INM are indistinguishable by light microscopy in *S. cerevisiae*, and therefore, confirming the localization of Trm1p-II at the INM (relative to the ONM and/or ER) would require electron microscopy. However, we believe Trm1p-II does localize exclusively to the INM based on several observations: 1) the modification of G_{26} catalyzed by Trm1p occurs on pre-tRNAs prior to their nuclear export (Etcheverry *et al.,* 1979); and 2) altering the N-terminus of Trm1p-II, which likely inhibited N-terminal acetylation, resulted in accumulation of the protein in the nucleoplasm (rather than the cytoplasm) (Murthi and Hopper*,* 2005). Fractionation studies indicated Trm1p-II is a peripheral membrane protein rather than an integral protein as it is released from the membrane similar to a known peripheral NPC protein, Nup1p (Rose *et al.,* 1995).

Diseases resulting from mislocalization of nuclear proteins

Correct localization of proteins is critical as mislocalization of nuclear proteins causes alterations in gene expression in *S. cerevisiae* as well as various diseases in vertebrates. Most of these diseases are associated with mislocalization of a single nuclear protein. Collectively these disorders are termed “laminopathies” and include such diverse diseases as Emery-Dreifuss muscular dystrophy, Dunnigan-type familial partial lipodystrophy, Hutchinson-Gilford progeria, atypical Werner’s syndrome, Pelger-Huet anomaly, and Greenberg skeletal dysplasia (Cao and Hegele, 2000; Motsch *et al.,* 2005). The autosomal dominant form of Emery-Dreifuss muscular dystrophy results from mutations in the gene encoding lamins A/C (*LMNA*) while the X-linked form results from mutations in the gene encoding emerin (*EMD*), an integral INM protein. However, all the known mutations result in mislocalization of nuclear proteins. Autosomal dominant

Hutchinson-Gilford progeria is also caused by a single base substitution in the gene encoding lamins A/C and results in growth retardation, absence/loss of adipose tissue, alopecia, and premature atherosclerosis leading to early death (De Sandre-Giovannoli *et al.,* 2003).

How inappropriate targeting and maintenance of a single protein can result in such diverse phenotypes is unclear. Two hypotheses have been proposed to account for the cellular effects observed with the laminopathies (Holmer and Worman, 2001). The first contends that altering interactions between integral INM proteins and the lamins destabilizes the nuclear lamina, which functions as the structural framework of the nucleus, thereby making the nucleus susceptible to mechanical stress. By this hypothesis, cells that contract, such as skeletal and heart muscle, would be primarily affected. This is consistent with the pathology of many of these laminopathies, whereby most individuals die from heart-related complications. In contrast, the second theory contends that altering interactions between integral INM proteins and lamins disrupts the nuclear lamina, which functions as a scaffold for transcription factors. This ultimately affects gene expression by either activating or silencing genes inappropriately. However, to understand how mislocalization can result in the disease state, we must first uncover the mechanism for targeting proteins to the INM.

Aims of this study

The nucleus is a highly dynamic organelle, breaking down and reassembling with each round of mitosis in higher eukaryotic cells (reviewed in Güttinger *et al.,* 2009). Disassembly of the nucleus begins at the end of prophase and requires several cellular

events: fragmentation of the nuclear membrane into vesicles, dissociation of the NPCs, and depolymerization of the nuclear lamina. In addition, there is a parallel breakdown of the ER, which is contiguous with the ONM during mitosis; this organelles also fragments into vesicles in a process similar to that for the nuclear membrane. The NPCs dissociate into subunits as a result of phosphorylation of various nucleoporins. Disassembly of the nuclear lamina also results from phosphorylation (of the lamins), causing this structure to break down. The B-type lamins remain associated with the nuclear membrane vesicles, while lamins A and C are released as free dimers into the cytoplasm. Integral INM proteins are also phosphorylated during nuclear disassembly; this may be important for subsequent vesicle formation as well as dissociation of the nuclear membrane from both the chromosomes and the nuclear lamina. Throughout this process, the chromosomes condense and the nucleolus disappears (as transcription is unable to occur in the presence of condensed chromosomes).

Following telophase, the nuclear membrane begins to reassemble (reviewed in Güttinger *et al.,* 2009). The first step in reassembly of the nucleus involves binding of the nuclear membrane vesicles to the chromosomes, a process that likely involves both lamins and integral INM proteins. The vesicles fuse around the chromosomes and the NPCs begin to reassemble. The nuclear lamina begins to reform and chromosomes begin decondensing. The nuclear membrane vesicles, which are now bound to the chromosomes, begin to fuse, forming an intact nuclear membrane. At this point in the reassembly process, the nucleus excludes cytoplasmic molecules as well as many resident nuclear protein and molecules. However, the newly reformed nucleus begins to selectively import nuclear proteins from the cytoplasm via active transport through the

reformed NPCs. Finally, the nucleolus reassembles as the chromosomes decondense and transcription begins, completing mitosis.

In contrast to higher eukaryotic organisms, *S. cerevisiae* undergoes "closed mitosis" whereby the nucleus is maintained throughout mitosis. This provides a unique system for modeling and understanding complex eukaryotic cellular processes, such as nuclear targeting, structure, organization, and maintenance. Many of the essential cellular functions and molecules of higher eukaryotes are conserved within *S. cerevisiae,* particularly of our interest are those involved in nuclear targeting, including the nucleoporins, karyopherins, and Ran proteins. Therefore, discoveries concerning basic cell biology carried out in *S. cerevisiae* can provide insight into human cellular biology as well as aiding in the understanding of the pathogenesis of human disease.

Although targeting to the nucleoplasm is partially understood for soluble proteins, very little is known about subsequent targeting to distinct subnuclear regions. This thesis focused on defining a mechanism for targeting a peripheral nuclear protein, Trm1p-II, to the INM. *Cis*-acting sequences within Trm1p-II that mediate targeting, both to the nucleus and INM, are defined in Chapters 3 and 4, respectively. *Trans*-acting factors that function in targeting and retaining Trm1p-II at the INM are discussed in Chapter 5.

CHAPTER TWO

MATERIALS AND METHODS

S. cerevisiae strains

S. cerevisiae strain W303 *(MATα ade2-1 ura3-1 his3-1, 115 trp1-1 leu2-3, 112* obtained from S. Wente; Wente and Blobel, 1993*)* was used for the competition experiments and BY4741 (*MATa his3Δ leu2Δ met15Δ ura3Δ*; Open Biosystems, Huntsville, AL; Winzeler *et al.,* 1999) was used for all remaining experiments. *trm1, mak3,* and *ice2* are derivatives of BY4741 containing Kan^r replacements for individual genes*. kap114, kap120, kap123, los1, msn5, nmd5, pdr6,* and *sxm1* are derivatives of BY4741 containing Kan^r replacements for individual genes encoding various importin β family members (Open Biosystems, Huntsville, AL; Winzeler *et al*., 1999). *mtr10 (MATa his3Δ leu2Δ met15Δ ura3Δ mtr10::*Nat^r*)* was not included in the unessential deletion collection but was generated independently by gene replacement in the BY4741 background as described by Azad *et al*. (2001). *kap120nmd5* (*kap120*::Kan^r, *nmd5*::Nat^r) was generated by M. Whitney via mating and tetrad analysis (Nat^r; ClonNAT from Werner Bioagents).

One-step *S. cerevisiae* transformation

Procedure carried out as described in Chen *et al.* (1992). Yeast cultures were grown to stationary phase (providing a density of approximately 2.5 x 10^8 cells/ml culture). 250 ul of *S. cerevisiae* culture was pelleted in 1.5 ml microfuge tubes for each transformation. The supernatant was removed and 50 ng–1 ug DNA was added along

with 100 ul 1-step buffer (1 ml 1-step buffer: 100 ul 1 M DTT, 50 ul sssDNA, 200 ul 0.2 M LiAc, 660 ul 60% PEG 3350). Cells were resuspended in the buffer-DNA mixture, incubated at 42^{o}C for 30 min, plated on appropriate media (lacking appropriate amino acids to allow for selection of cells containing the plasmid), and grown for 2–3 days at 30^{o}C to isolate single colonies.

Isolation of genomic DNA from *S. cerevisiae*

S. cerevisiae cultures were streaked on solid media containing the appropriate selection. Individual colonies were inoculated with a toothpick into a 1.5 ml microfuge tube containing 200 ul zymolase 20-T solution (*Athrobacter luteus*, ICN Biomedicals Inc.). This mixture was incubated at 37^{o}C for 30 min. 200 ul phenol:chloroform and 20 ul 10% SDS were added, cultures were vortexed briefly, and centrifuged in a tabletop centrifuge for 10 min. The supernatant was transferred to a clean microfuge tube. 100% ethanol was added and this mixture was incubated at -70^{o}C for 20 min to precipitate the DNA. The mixture was centrifuged for 10 min in a tabletop centrifuge and the supernatant was removed. 70% ethanol was added to wash the pellet. After vortexing briefly, this mixture was centrifuged and the supernatant was removed to dry the pellet. The pellet was resuspended in 100 ul glass distilled water (GDW).

Indirect immunofluorescence

Indirect immunofluorescence was carried out as described by Pringle *et al*. (1991) with the modification previously described by Hopper *et al.* (1990). 10 ml *S. cerevisiae* cultures were grown to early– to mid–log phase (approximately 1 x 10^{7} cells/ml culture)

in a 30°C waterbath. 1.2 ml 37% formaldehyde (Fisher) was added to a 15 ml Falcon tube and the cultures were transferred to the tubes. These mixtures were vortexed briefly and centrifuged for 5 min at 3500 rpms at 4°C (Jouan tabletop centrifuge). Pellets were resuspended in 5 ml solution A (40 mM K_2HPO_4-KH_2PO_4, 500 uM $MgCl_2$) supplemented with 0.6 ml 37% formaldehyde and incubated at room temperature for various times, depending on the primary antibody to be used, in order to fix the cells (affinity-purified rabbit anti-β-galactosidase primary antibody = 45 min, Roche mouse anti-GFP primary antibody = 25 min). The cultures were centrifuged for 5 min at 3500 rpms at 4°C to pellet the cells. The cells were washed twice with solution A and resuspended in solution B (40 mM K_2HPO_4-KH_2PO_4, 500 uM $MgCl_2$, 1.2 M sorbitol). *S. cerevisiae* cell walls were digested by incubating in solution B supplemented with glusulase (PerkinElmer), β-mercaptoethanol (Sigma), and zymolase 20-T (ICN) for 30-60 min at 37°C, until visualization of a sample of a culture under the light microscope confirmed the cells walls had been digested. Cultures were washed twice with solution B and resuspended in various volumes of solution B depending on the density of the culture. Cells were adhered to glass slides using a 1:10 dilution in GDW of poly-L-lysine (Sigma). 10 ul of each cell culture was added to the individual wells of the slides. The supernatant was aspirated leaving the cells adhered to the glass slide. Solution F (0.73 mM KH_2PO_4 pH 7.4, 0.15 M NaCl, 0.015 M NaN_3, 0.1% BSA) was added to each well of the slide to inhibit non-specific binding of antibodies. Primary and secondary antibodies were diluted to their appropriate concentration with solution F. The cells were incubated with the primary antibody for 1 hr at room temperature and then washed 7 times with solution F. The cells were incubated with the secondary antibody for 1 hr at

room temperature in a moist Petri dish covered in foil and then washed 7 times with solution F. A 100 ng/ml solution of 4',6-diaminidino-2-phenyllindole dihydrochloride (DAPI) diluted in GDW was used to localize the nuclear and mitochondrial DNA within the cells. Mounting medium was added to the slides sufficiently to fill in all wells of the slides. Coverslips were applied and adhered to the slides using nail polish. Slides were stored at -20^{o}C. Primary antibodies were affinity-purified rabbit anti-β-galactosidase (Hopper *et al.,* 1990) used at 1:400 or 1:1000 dilutions, mouse anti-GFP (Roche) used at 1:250 or 1:500 dilutions, mouse anti-Nsp1p (Tolerico *et al*., 1999) used at 1:10,000 dilution, and rabbit anti-Kar2p (Scidmore *et al.,* 1993) used at 1:3500 dilution. Secondary antibodies were Cy3 goat anti-rabbit IgG, Cy3 goat anti-mouse IgG, FITC goat anti-rabbit IgG, and FITC goat anti-mouse IgG, each diluted 1:400.

Live cell imaging of *S. cerevisiae* cells using GFP

Individual colonies of *S. cerevisiae* expressing a GFP fusion protein were grown on solid media. Cells were transferred via a toothpick to a slide containing approximately 4 ul of 1X TBS for visualization of the GFP fusion protein in live cells. *In vivo* localization of various ADEPT deletions of Trm1-GFP was assessed using the FITC channel of a Nikon Microphot-GX microscope. This technique allowed for visualization of thousands of live cells from each individual colony (transformant). In addition, multiple (2–3) colonies (transformants) were tested to confirm the localization was consistent across the majority of cells in several independent colonies.

Microscopic imaging

Images were obtained using a Nikon Microphot-GX microscope equipped with a 60X objective and a SenSys CCD camera (Photometrics Ltd, Tucson, AZ). Multiple images were captured for each experiment. However, due to technical limitations, only 1–5 cells were often in focus in each image. This was particularly true when images of live cells were obtained. Therefore, representative images are shown for each confirmed result (confirmed by blinded review by another investigator within the lab) of the localization experiments reported in this thesis. Image processing was performed using QED software (Pittsburgh, PA) and Adobe Photoshop.

Western blot analysis

5 ml *S. cerevisiae* cultures were grown to log phase at 30°C, transferred to a 15 ml Falcon tube, pelleted at 1800x g for 5 min at 4°C, and resuspended in 1 ml cold GDW. These cultures were transferred to 1.5 ml microfuge tubes and pelleted at 1800x g for 5 min at 4°C. The supernatant was discarded and pellets were resuspended in 250 ul breaking buffer (50 mM Na_3PO_4 pH 7.2, 10 mM EDTA, 1 mM DTT, 0.1925 mg/ml PMSF, 4 ug/ml leupeptin, 1.6 ug/ml aprotinin). Glass beads were added and this mixture was vortexed for 5 min in the cold room. A hole was punctured in the bottom and top of the 1.5 ml microfuge tube, which was then inserted into a glass tube to isolate the cell extract by centrifuging at 10,000x g for 2 min at 4°C. This supernatant was then transferred to a new 1.5 ml microfuge tube, 30 ul 10% SDS was added and boiled for 7 min. Extracts were centrifuged at 10,000x g for 2 min in the cold room and protein extracts were transferred to new 1.5 ml microfuge tubes and stored at -80°C.

Concentration of the protein extracts was determined using the Peterson Protein Estimation Assay (Peterson, 1983). 15 ug of protein was added to each well of the 10% polyacrylamide gel. The primary antibodies were mouse anti-GFP (Roche) and rabbit anti-Trm1p (Li *et al.,* 1989), both used at 1:1000 and 1:5000 dilutions. The secondary antibodies were anti-mouse horseradish peroxidase conjugated antibody and anti-rabbit horseradish peroxidase conjugated antibody, both used at 1:7500 dilution. Blots were developed using the SuperSignal® West Femto Maximum Sensitivity Substrate (Pierce, Rockford, IL).

Preparation of chemically competent *E. coli* cells

A 5 ml culture of DH5α cells was grown overnight at 37°C in LB (1000 ml GDW, 5.0 g yeast extract, 8.0 g bacto tryptone, 5.0 g NaCl) with no antibiotic selection. This 5 ml culture was used to inoculate a 500 ml culture of LB with no antibiotic selection. The 500 ml culture was grown for approximately 4 hrs at 37°C with shaking until an OD of 0.500–0.700 was reached. The culture was split into two 250 ml centrifuge bottles and chilled on ice for approximately 20 min. These cultures were centrifuged at 5000 rpms for 15 min at 4°C (Jouan tabletop centrifuge) and the pellets were resuspended in 50 ml Cell Wash solution (10 mM Pipes pH 7.0, 60 mM $CaCl_2$, 15% glycerol). These cultures were centrifuged, washed with 20 ml Cell Wash solution, and combined into a 50 ml Falcon tube. These cultures were centrifuged again and resuspended in 4 ml Cell Wash solution. Cells were frozen in 200 ul aliquots and maintained at -80°C.

Chemical transformation of *E. coli* cells

Chemically competent DH5α *E. coli* cells were thawed on ice. 2.5 ul mini-prep DNA was added to 200 ul cells and incubated on ice for 30 min. Cells were heat-shocked at 42^{o}C for 90 sec and 250 ul LB was added. Cells were grown at 37^{o}C for 1 hr, plated on YT media containing 50 ug/ml ampicillin or 50 ug/ml kanamycin, depending on the antibiotic selection of the plasmid, and incubated at 37^{o}C overnight. The resulting single colonies were isolated for plasmid purification (Qiagen).

Preparation of electrocompetent *E. coli* cells

A 5 ml culture of DH5α was grown overnight at 37^{o}C in LB with no antibiotic selection. This 5 ml culture was used to inoculate a 500 ml culture of LB with no antibiotic selection. This culture was grown for approximately 4 hrs at 37^{o}C with shaking until an OD of 0.700–1.00 was reached. The culture was split into two 250 ml centrifuge bottles and chilled on ice for approximately 25 min. These cultures were centrifuged at 5000 rpms for 15 min at 4^{o}C (Jouan tabletop centrifuge) and the pellets were resuspended in 250 ml cold 1 mM Hepes pH 7.0. These cultures were centrifuged and washed with 125 ml 1 mM Hepes. These cultures were centrifuged, resuspended in 10 ml 10% glycerol and combined into a 50 ml Falcon tube. This culture was centrifuged again and cell pellets were resuspended in 2.5 ml cold 10% glycerol and frozen in 85 ul aliquots maintained at -80^{o}C.

Electroporation of *E.coli* cells

Electrocompetent DH5α *E. coli* cells were thawed on ice. 2.5 ul mini-prep DNA was added to 80 ul cells. Cells were transferred to chilled Gene Pulser cuvettes (Bio-Rad) and electroporated using the settings: 2.5 kvolts, 25 uF and 200 ohms on a Bio-Rad Gene Pulser System. 250 ul LB was added to the cells and the cultures were transferred to 1.5 ml microfuge tubes. These cultures were grown for 45 min at 37^{o}C, shaking horizontally. Cultures were plated on YT media containing 50 ug/ml ampicillin, incubated overnight at 37^{o}C, and the resulting single colonies were isolated for plasmid purification (Qiagen).

Isolation of plasmid from *E. coli*

Plasmid DNA was isolated from *E. coli* cells using the Qiagen mini-prep kit following manufacturer's instructions.

DNA manipulations

DNA restriction enzyme products and PCR products were resolved on agarose gels of appropriate percentage in 1X TBE (10X: 500 ml GDW, 108 g Sigma Tris base, 55 g boric acid, 7.44 g disodium EDTA). Gel slices containing the desired digestion fragment or PCR product were excised and the DNA was purified using the Qiagen gel purification kit according to manufacturer's instructions. Additionally, PCR products were purified directly using the Qiagen PCR purification kit according to manufacturer's instructions.

Dephosphorylation of DNA restriction enzyme fragments was carried out by incubating with shrimp alkaline phosphatase (MBI Fermentas) at 37^{o}C for approximately

1 hr, deactivating the enzyme at 65°C for 20 min, and purifying the DNA via the PCR purification protocol (Qiagen) before use in ligation.

Plasmid construction

Plasmid constructions were performed in *Escherichia coli* strain DH5α. *Pfu* Ultra High Fidelity DNA polymerase (Stratagene) was used to generate *TRM1* PCR fragments for cloning. Blunt end PCR products were ligated into either the TOPO Blunt vector (Invitrogen) or pGEM-T vector (Promega) following the manufacturer's instructions. Restriction enzyme fragments generated from TOPO or pGEM-T vectors were subcloned into the appropriate yeast vector in a 3:1 ratio of insert: vector using T4 DNA Ligase (Promega). *Bam*HI or *Bgl*II (Promega) *TRM1* fragments were cloned into the pFB1-7u and pFB1-67u vectors (Moreland *et al.,* 1987). *TRM1* deletion constructs lacking the endogenous promoter were cloned into the pEMBLyex4 vector and expressed under *GAL10*/*CYC1* regulation. *TRM1* deletion constructs with the endogenous promoter were cloned into either pRS416, a centromeric vector (Sikorski and Heiter, 1989), or pRS426, a 2μ high copy vector (Christianson *et al*., 1992). Constructs were verified by restriction enzyme digestion, PCR using GoTaq DNA polymerase (Promega) and DNA sequencing (performed by the Core Facility at the Pennsylvania State University College of Medicine). Sequences were compared to those within *Saccharomyces* Genome Database (SGD; yeastgenome.com) for *TRM1*.

PCR

50 or 100 ul PCR reactions were set up including 5–10X PCR buffer (GoTaq versus *Pfu* buffer, respectively), 1–2 ul dNTP mixture (5 mM mix: 10 ul 100 mM dATP, 10 ul 100 mM dTTP, 10 ul 100 mM dGTP, 10 ul 100 mM dCTP, 160 ul GDW; USB), 2 ul 20 uM oligonucleotides, 1 ul GoTaq or *Pfu* polymerase, 1 ul DNA (100 ng/ul), and GDW to bring total volume to 50 or 100 ul. Mineral oil was added to each reaction to prevent evaporation of the PCR mixture during thermocycling. Standard PCR conditions using the thermocycler were 94^{o}C for 3 min, 94^{o}C for 30 sec (denaturing), 50–65^{o}C for 30 sec (annealing), 72^{o}C for 2–4 min (extension), 25–35 cycles, 72^{o}C for 10 min, with only the annealing temperature and extension times modified based on the melting temperature of individual oligonucleotides and length of PCR product, respectively.

PCR from bacterial colonies

PCR was carried out as outlined above, however in place of the addition of DNA, a bacterial colony isolated from an LB+AMP plate was inoculated in the PCR mixture. A 0.5 ml LB+AMP culture was inoculated with the bacteria for plasmid purification if the candidate was verified correct by PCR.

Overlap extension/fusion PCR

To generate the various ADEPT deletion constructs, regions of *TRM1* were deleted using the protocol described by Ho *et al.* (1989) for overlap extension/fusion PCR. In this technique, the first round of PCR generated two *TRM1* DNA fragments (via separate PCR reactions) engineered to have overlapping complementary ends. These

individual PCR fragments were purified (Qiagen PCR purification kit) and combined in a second PCR reaction. During the second PCR reaction, the PCR fragments annealed via their complementary overlapping ends. This fusion molecule then serves as the template for future rounds of PCR by allowing the 3' overlap of each strand to serve as a primer for the 3' extension of the complementary strand. The fusion DNA product is subsequently amplified with continued PCR cycles generating a *TRM1* PCR fragment lacking various internal sequences (such as ADEPT#3 or ADEPT#4), depending on the oligonucleotides used in round 1.

T4 polynucleotide kinase reaction

Oligonucleotides were diluted to a final concentration of 10 ng/ul for use in the polynucleotide kinase reaction in which phosphates are added to the oligonucleotides. 2 ul 10X PNK buffer, 1 ul PNK, 10 ul 10ng/ul oligonucleotide, 2 ul 10 uM dATP, 0.5 ul 100X BSA and 4.5 ul GDW was incubated at 37^{o}C for approximately 1 hr 30 min. The reaction was stopped by incubating at 65^{o}C for approximately 25 min.

Annealing oligonucleotides

15 ul phosphorylated oligonucleotides, 4 ul 10X PNK buffer and 6 ul GDW were mixed together to anneal the oligonucleotides and make dsDNA. The mixture was boiled for approximately 5 min, then cooled on benchtop overnight.

DNA sequencing

DNA sequencing was carried out by the Core Facility at the Pennsylvania State University College of Medicine.

Oligonucleotides

Oligonucleotides were synthesized in the Pennsylvania State University College of Medicine Core Facility.

CHAPTER THREE

Trm1p-II CONTAINS A NOVEL PUTATIVE NUCLEAR LOCALIZATION SEQUENCE AT THE C-TERMINUS

ABSTRACT

Various *cis*-acting regions of Trm1p were examined for their role in targeting Trm1p-II to the nucleus. Fragments of Trm1p were fused to a non-nuclear protein, β-galactosidase, to determine whether they could function as an NLS. A 9-amino acid region present in the C-terminus of Trm1p-II (amino acids 544–552) was capable of targeting β-galactosidase to the nucleus, indicating this sequence meets the criteria for a putative NLS (Dingwall and Laskey, 1991). This putative NLS was distinct from the previously mapped NLS within amino acids 95–102 of Trm1p-II (Rose *et al.,* 1992) based on its amino acid composition. The known NLS (NLS#1 [amino acids 95–102]) closely resembled the classical NLS within the SV40 Large T Antigen (Kalderon *et al.,* 1984), while the putative NLS (NLS#2 [amino acids 544–552]) was similar to an NLS identified within Crz1p (Polizotto and Cyert, 2001). Although unconfirmed experimentally, NLS#1 is likely imported by importin β, through its adaptor importin α, based on common amino acid sequence characteristics with the SV40 NLS, known to be recognized by importin β. We show that the putative NLS (NLS#2) of Trm1p-II is regulated by at least two different importers, Nmd5p and Kap120p, as individual deletion of the genes encoding these importers decreases the nuclear pool of a Trm1p fusion protein (amino acids 492–570-β-galactosidase). Further, a *S. cerevisiae* strain lacking both *KAP120* and *NMD5* maintained a pool of Trm1 (492–570)-β-galactosidase in the

nucleus suggesting there is at least one more unidentified nuclear importer involved in this process.

INTRODUCTION

Proteins are synthesized in the cytoplasm and subsequently targeted to their site of function within the cell. Proteins that reside in the nucleus possess NLSs on their surface that are recognized by karyopherins which function to mediate transport of proteins and RNAs between the nucleus and cytoplasm. An NLS must exhibit distinct characteristics; it must be necessary for targeting the endogenous protein to the nucleus and sufficient to target a non-nuclear protein to the nucleus (Dingwall and Laskey, 1991).

The classical basic NLS, exemplified by the NLS within SV40 Large T antigen, is enriched in basic amino acids (Kalderon *et al.,* 1984). Basic amino acids are hydrophilic and therefore regions of a protein enriched in basic amino acids would likely be exposed on the surface. Exposure of an NLS on the surface of a protein allows for its recognition by importin β or the importin β adaptor, importin α. Similarly, the classical bipartite NLS, exemplified by nucleoplasmin, consists of two clusters of basic amino acids separated by a variable length spacer (Robbins *et al.,* 1991). The function of this NLS is not affected by either the amino acid composition or length of the spacer. Rather, it requires the looping out of the basic amino acid clusters to expose them on the surface of the protein.

Previous studies on Trm1p-II identified a classical NLS within amino acids 95–102. Rose *et al.* (1992) showed that altering the five basic residues in combination to glutamic acid inhibited the function of this NLS as Trm1p-II accumulated in the

cytoplasm with apparent exclusion of the nucleus. However, Christophe *et al.* (2000) advised that the presence of a classical NLS within a protein may mask the presence of a second NLS within the same protein. It was possible that a pool of Trm1p-II, indistinguishable against the significant cytoplasmic background, remained in the nucleus in the Trm1p NLS mutant, resulting from targeting to the nucleus by a secondary NLS. We reasoned Trm1p-II may possess additional sequence(s) that function, alone or in combination with the classical NLS, to target the protein to the nucleus. Many proteins have been shown to possess multiple NLSs (Luo *et al.,* 2004; Soullam and Worman, 1993); therefore, we sought to determine whether any regions of Trm1p-II could function independently as an NLS.

The ADEPT hypothesis was developed as a tool to predict regions of sorting isozymes that function in protein targeting within the cell (Stanford *et al.,* 2000). ADEPT represents **A**dditional **D**omains that mediate **E**ukaryotic **P**rotein **T**argeting. The basis for the ADEPT concept was catalytic regions of proteins are conserved between eukaryotic and prokaryotic organisms. However, eukaryotes possess organelles and thus require targeting information in addition to sequences regulating catalytic function. Therefore, extra sequences present in eukaryotes and absent in the prokaryotic counterparts may function in cellular localization. This type of protein sequence analysis provides a directed approach for elucidating regions of proteins involved in cellular targeting.

We utilized the ADEPT hypothesis to identify regions of Trm1p that may have a role in targeting the protein within the cell, both to the mitochondria and to the nucleus. Protein sequences alignments between eubacteria, archaebacteria, and eukaryotic forms of Trm1p revealed four regions present in the eukaryotic sequences and absent from the

bacterial counterparts (Fig. 8). ADEPT #1 (amino acids 1–48) coincided with sequences known to augment a shared mitochondrial targeting sequence within Trm1p-I and Trm1p-II (Ellis *et al.,* 1989), while ADEPT#2 (amino acids 91–136) contained the known NLS (amino acids 95–102) shared between Trm1p-I and Trm1p-II (Rose *et al.,* 1992). This provided strong support for the ADEPT hypothesis and suggested the remaining ADEPTs, ADEPT#3 (amino acids 323–375) and ADEPT#4 (amino acids 492–570), may also have a role in the distribution of Trm1p-II within the cell. Interestingly, the ADEPT alignments revealed that the human, mouse, and plant sequences of Trm1p possessed a C-terminal extension not present in the other eukaryotic organisms included in this analysis, including *S. cerevisiae*. The role of this C-terminal extension, either with regard to cellular targeting or catalytic function of Trm1p, has not been reported.

In the following experiments, the role of ADEPT#3 and/or ADEPT#4 in nuclear targeting was investigated by localization of fusion proteins containing these *cis*-acting sequences of Trm1p-II using indirect immunofluorescence.

METHODS

Trm1p ADEPTs in pFB1-7u

ADEPT#3, ADEPT#4, and ADEPT#3+ADEPT#4 were amplified by PCR and inserted at the unique *Bam*HI site within the pFB1-7u vector (Fig. 9). The sequences of the oligonucleotides used to amplify the various Trm1p ADEPT fragments are listed in Table 1, while the oligonucleotide pairs and DNA templates are described in Table 2. ADEPT#3 (amino acids 323–375) was amplified with oligonucleotides ADT3-1 and ADT3-3 to produce an approximately 170 bp fragment containing a *Bam*HI site at its 5’

Fig. 8: ADEPT alignments of Trm1p sequences from eubacteria, archaebacteria, and eukaryotic organisms.

Protein sequence alignments utilizing representative organisms from the three major kingdoms, eubacteria, archaebacteria, and eukarya are compared. Colored segments represent catalytic and structural domains conserved among the three kingdoms. Black solid lines are regions present in eukaryotes only. Organisms represented from kingdom eukarya from top to bottom: humans, mouse, *Caenorhabditis elegans,* plant*, Schizosaccharomyces pombe, Saccharomyces cerevisiae, Candida albicans.* Eubacteria and Archaebacteria are last two sequences, respectively. Roman numerals I–IV show the borders and extent of ADEPT#1, 2, 3, and 4, respectively.

Figure from: Stanford DR, Martin NC, Hopper AK. ADEPTs: information necessary for subcellular distribution of eukaryotic sorting isozymes resides in domains missing from eubacterial and archael counterparts. Nucl Acid Res. 2000; 28:383-392.
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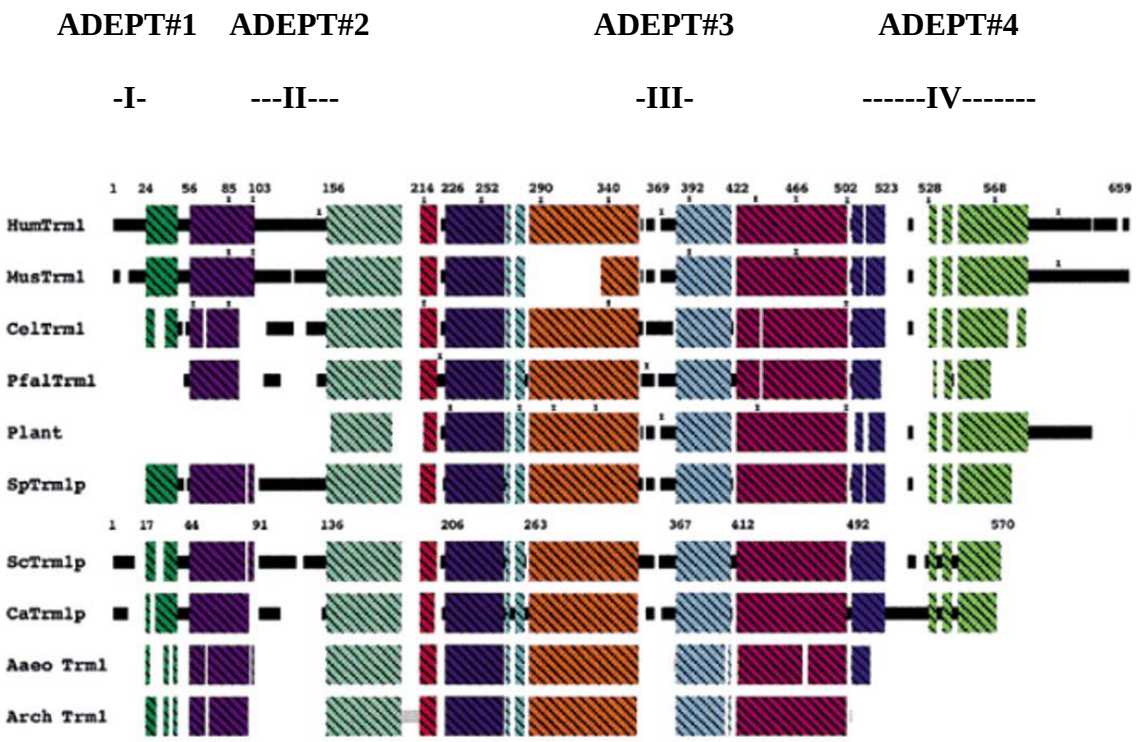


end and *Bgl*II-*Bam*HI sites at its 3' end for insertion in-frame with β-galactosidase. ADEPT#4 (amino acids 492–570) was amplified with oligonucleotides ADT4-3 and ADT4-4 to produce an approximately 250 bp fragment containing flanking *Bgl*II sites for insertion in-frame with β-galactosidase. ADEPT#3+ADEPT#4 was created by inserting *Bgl*II-ADEPT#4-*Bgl*II downstream of ADEPT#3 in pFB1-7u. pFB1-7u containing *Bam*HI-ADEPT#3-*Bgl*II-*Bam*HI was digested with *Bgl*II and *Bgl*II-ADEPT#4-*Bgl*II was inserted.

N-terminal ADEPT#4 of Trm1p (amino acids 492–530) was amplified with oligonucleotides KAS029 and ADT4-3 to produce an approximately 125 bp fragment flanked with *Bgl*II sites. C-terminal ADEPT#4 (amino acids 531–570) was amplified with oligonucleotides KAS030 and ADT4-4 to produce an approximately 130 bp fragment flanked with *Bgl*II sites. Each fragment was digested with *Bgl*II and inserted into the compatible *Bam*HI site of pFB1-7u.

Oligonucleotides (KAS041 and KAS042) corresponding to Trm1p amino acids 544–552, with 5' and 3' *Bam*HI sites were phosphorylated with polynucleotide kinase (PNK) and annealed to generate dsDNA. This fragment was inserted in the *Bam*HI site of pFB1-7u. The five basic amino acids at positions 544, 546, 547, 549, and 552 were mutated in combination to glutamic acid residues by phosphorylating oligonucleotides KAS043 and KAS044 with PNK and annealing to form dsDNA. Additionally, the four hydrophobic residues at positions 545, 548, 550, and 551 were changed in combination to glutamic acid residues by phosphorylating oligonucleotides KAS047 and KAS048 with PNK and annealing. The four hydrophobic residues at positions 545, 548, 550, and 551 were changed to alanine by phosphorylating oligonucleotides KAS055 and KAS056 with

PNK and annealing. The four hydrophobic residues at positions 545, 548, 550, and 551 were changed to asparagines by phosphorylating oligonucleotides KAS057 and KAS058 with PNK and annealing. The sequence of oligonucleotides KAS041–44, 47–48, and 55–58 are listed in Table 1.

The relevant constructs and their description are provided in Table 3. The plasmids containing the various Trm1p sequences were transformed into the *S. cerevisiae* strain, BY4741, using the one-step yeast transformation procedure described in Chapter 2, followed by localization of the resulting β-galactosidase fusion proteins by indirect immunofluorescence.

Indirect immunofluorescence

Indirect immunofluorescence was performed using the procedure of Pringle *et al*. (1990) described in Chapter 2, to localize the β-galactosidase fusion proteins. *S. cerevisiae* cells were fixed for approximately 45 min to optimize binding of the primary antibody, affinity-purified rabbit polyclonal anti-β-galactosidase antibody (Hopper *et al.*, 1990), used at 1:400 or 1:1000 dilution. The secondary antibody was Cy3-conjugated goat anti-rabbit IgG, used at 1:400 dilution.

RESULTS

Amino acids 544–552 of Trm1p-II can target a reporter protein to the nucleus

Experiments were performed to determine whether ADEPT#3, ADEPT#4, or ADEPT#3+ADEPT#4 play a role in the subcellular distribution of Trm1p-II. Each was fused in-frame to *E. coli* β-galactosidase and the location of the fusion protein was

Table 1: Sequence of the oligonucleotides used for amplification of *TRM1* fragments.

Name	Oligonucleotide sequence
ADT3-1	5-GGATCCAGTACTATGACTACTTACCAT-3
ADT3-3	5-GGATCCAGATCTCCCCTCGCAGAACTTACATTT-3
ADT4-3	5-AGATCTTGTGATGATGAGAAGAAAGAC-3
ADT4-4	5-AGATCTTGAAGTGTTGGGACGGGCTTT-3
KAS029	5-AGATCTCAGCTTTGAGTCATACTCTGA-3
KAS030	5-AGATCTTCGTTCGCACCAAATGAACAA-3
KAS041	5-GATCCAAACTAAGGAAGCTAAAAATTGTGAGAG-3
KAS042	5-GATCCTCTCACAATTTTTAGCTTCCTTAGTTTG-3
KAS043	5-GATCCGAACTAGAAGAACTAGAAATTGTGGAAG-3
KAS044	5-GATCCTTCCACAATTTCTAGTTCTTCTAGTTCG-3
KAS047	5-GATCCAAAGAAAGGAAGGAAAAAGAAGAAAGAG-3
KAS048	5-GATCCTCTTTCTTCTTTTTCCTTCCTTTCTTTG-3
KAS055	5-GATCCAAAGCAAGGAAGGCAAAAGCAGCAAGAG-3
KAS056	5-GATCCTCTTGCTGCTTTTGCCTTCCTTGCTTTG-3
KAS057	5-GATCCAAAAACAGGAAGAACAAAAACAACAGAG-3
KAS058	5-GATCCTCTGTTGTTTTTGTTCTTCCTGTTTTTG-3

Table 2: Oligonucleotide pairs and DNA templates used to amplify *TRM1* fragments.

PCR product	Oligonucleotides	Template	Template Source
ADEPT#3 (amino acids 323–375)	ADT3-1/ADT3-3	pGP19	G. Peng
ADEPT#4 (amino acids 492–570)	ADT4-3/ADT4-4	pGP19	G. Peng
N-terminal ADEPT#4 (amino acids 492–530)	KAS29/ADT4-3	pGP19	G. Peng
C-terminal ADEPT#4 (amino acids 531–570)	KAS30/ADT4-4	pGP19	G. Peng
ADEPT#4 (amino acids 544–552)	KAS41/42	pGP19	G. Peng
ADEPT#4 basic–acidic (amino acids 544–552)	KAS43/44	pGP19	G. Peng
ADEPT#4 hydrophobic–acidic (amino acids 544–552)	KAS47/48	pGP19	G. Peng
ADEPT#4 hydrophobic–alanine (amino acids 544–552)	KAS55/56	pGP19	G. Peng
ADEPT#4 hydrophobic–asparagine (amino acids 544–552)	KAS57/58	pGP19	G. Peng

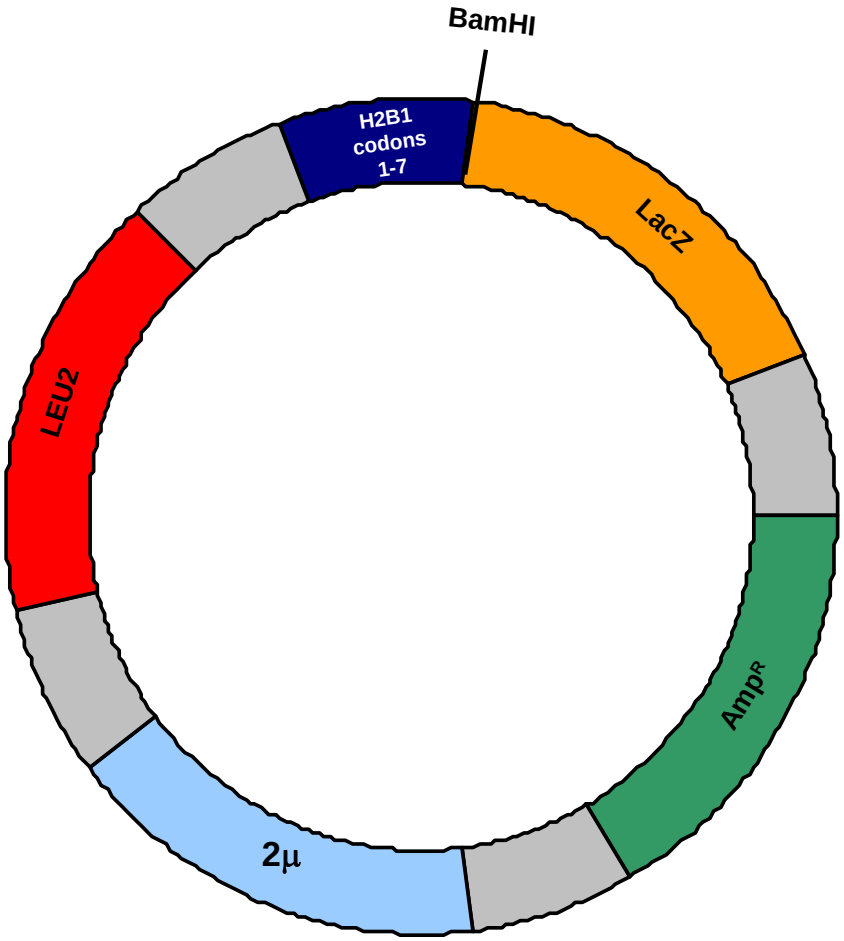
Table 3: Plasmids generated for the nuclear localization sequence experiments.

Plasmid	Encoded Fusion Protein
pFB1-7u	Histone H2B amino acids 1-14 fused to β-galactosidase
pFB1-7u+ADEPT#3	Trm1p amino acids 323-375 inserted in H2B-β-galactosidase
pFB1-7u+ADEPT#4	Trm1p amino acids 492-570 inserted in H2B-β-galactosidase
pFB1-7u+ADEPT#3+ ADEPT#4	Trm1p amino acids 323-375, 492-570 inserted in H2B-β-galactosidase
pFB1-7u+N-terminal ADEPT#4	Trm1p amino acids 492-530 inserted in H2B-β-galactosidase
pFB1-7u+C-terminal ADEPT#4	Trm1p amino acids 531-570 inserted in H2B-β-galactosidase
pFB1-7u+amino acids 544–552	Trm1p amino acids 544-552 inserted in H2B-β-galactosidase
pFB1-7u+amino acids 544–552 (basic–acidic)	Trm1p amino acids 544-552 (basic residues mutated to glutamic acid residues) inserted in H2B-β-galactosidase
pFB1-7u+amino acids 544–552 (hydrophobic–acidic)	Trm1p amino acids 544-552 (hydrophobic residues mutated to glutamic acid residues) inserted in H2B-β-galactosidase
pFB1-7u+amino acids 544–552 (hydrophobic–alanine)	Trm1p amino acids 544-552 (hydrophobic residues mutated to alanine residues) inserted in H2B-β-galactosidase
pFB1-7u+amino acids 544–552 (hydrophobic–asparagine)	Trm1p amino acids 544-552 (hydrophobic residues mutated to asparagines residues) inserted in H2B-β-galactosidase

Fig. 9: Schematic of the pFB1-7u vector.

TRM1 fragments were inserted into the *Bam*HI site of pFB1-7u to generate a Trm1-β-galactosidase fusion protein. *E. coli* β-galactosidase is a non-nuclear protein of approximately 116 kDa encoded by the *LacZ* gene. pFB1-7u, a plasmid containing the first 7 amino acids of histone H2B fused in frame to β-galactosidase produces a fusion protein that localizes predominantly in the cytoplasm

Figure adapted from: Moreland RB, Langevin GL, Singer RH, Garcea RL, Hereford LM. Amino acid sequences that determine the nuclear localization of yeast histone 2B. Mol Cell Biol. 1987; 7:4048-4057.

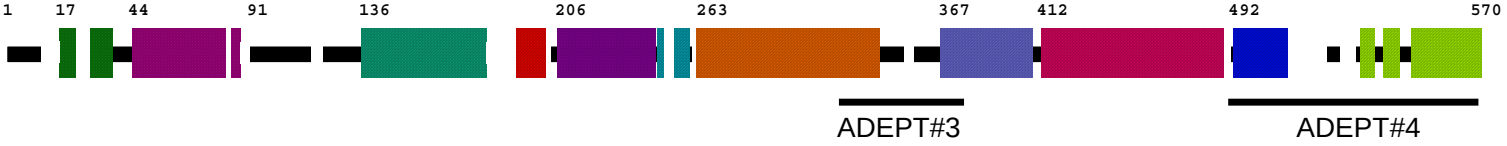


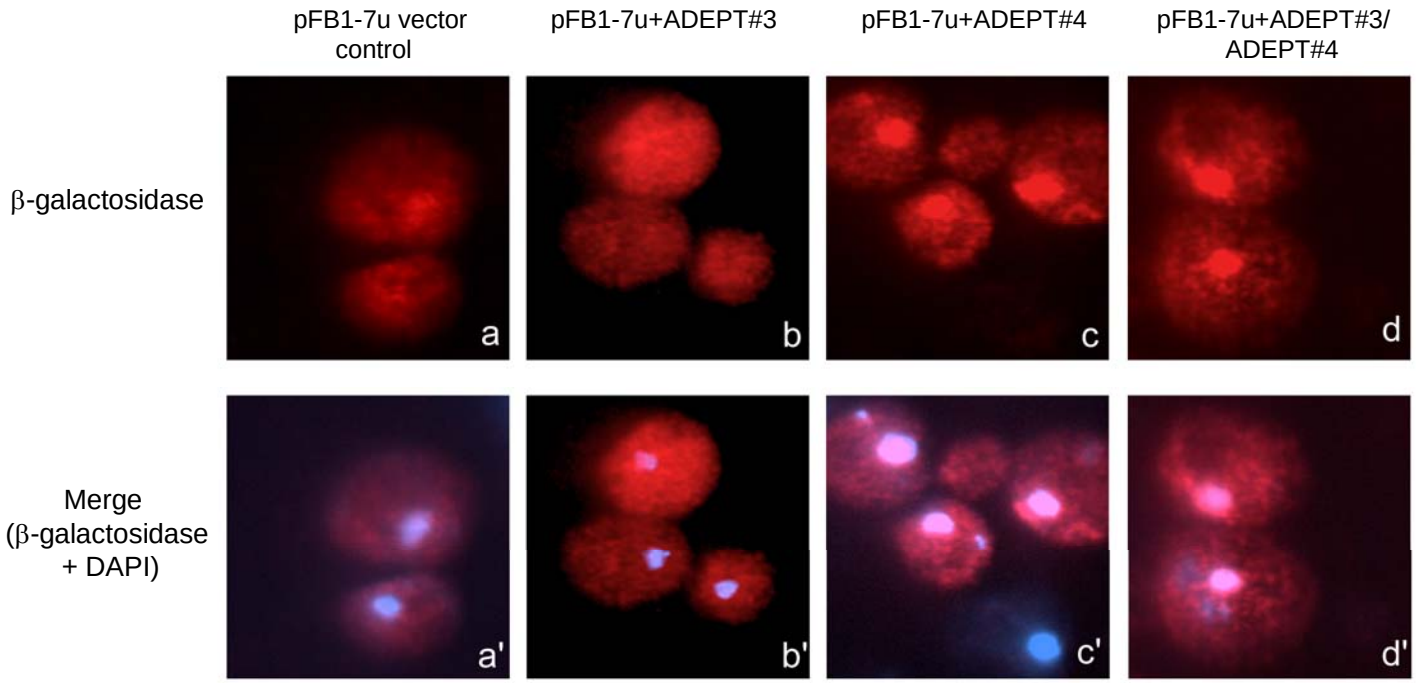
determined by indirect immunofluorescence. *E. coli* β-galactosidase is a non-nuclear protein of approximately 116 kDa encoded by the *LacZ* gene (Moreland *et al.,* 1987). pFB1-7u, a plasmid containing the first 7 amino acids of histone H2B fused in frame to β-galactosidase produces a fusion protein that localizes predominantly in the cytoplasm (Moreland *et al.,* 1987).

If a Trm1p fragment functions in targeting, either to the nucleus or INM, we anticipate a shift in the localization of the fusion protein from the cytoplasm to the nucleoplasm or INM, respectively. ADEPT#3 (amino acids 323–375)-β-galactosidase was diffusely localized in the cytoplasm (Fig. 10 b and b') similar to the pFB1-7u vector only control cells (Fig. 10 a and a'). In contrast, ADEPT#4 (amino acids 492–570)-β-galactosidase (Fig. 10 c and c') and ADEPT#3+ADEPT#4 (amino acids 323–375 + 492–570)-β-galactosidase (Fig. 10 d and d') accumulated in the nucleoplasm indicating the presence of a functional NLS within ADEPT#4 of Trm1p-II.

In order to identify the specific sequence functioning as the NLS within ADEPT#4 (amino acids 492–570), smaller ADEPT#4 fragments were amplified by PCR and inserted into pFB1-7u for localization of the fusion protein. N-terminal ADEPT#4 (amino acids 492–530)-β-galactosidase was localized diffusely in the cytoplasm (Fig. 11 c and c'), similar to pFB1-7u vector only control cells (Fig. 11 a and a'), indicating the NLS was not within this fragment of Trm1p-II. C-terminal ADEPT#4 (amino acids 531–570)-β-galactosidase accumulated in the nucleoplasm (Fig. 11 d and d'), even stronger than the entire ADEPT#4 fragment (amino acids 492–530) (Fig. 11 b and b'). Therefore, the region of Trm1p-II functioning as an NLS was within the C-terminus of ADEPT#4, amino acids 531–570. Though unclear why the C-terminal fragment of ADEPT#4 (amino

Fig. 10: Subcellular localization of pFB1-7u constructs containing various Trm1p ADEPT fragments transformed in the BY4741 strain. Trm1-β-galactosidase fusion proteins were localized by indirect immunofluorescence using affinity-purified rabbit polyclonal anti-β-galactosidase primary antibody (Hopper *et al.,* 1990) and Cy3-conjugated goat anti-rabbit IgG secondary antibody, each diluted 1:400. DAPI was used to detect DNA. (a) Cy3 image of pFB1-7u vector only control (a') Cy3 and DAPI merge of pFB1-7u vector only control (b) Cy3 image of pFB1-7u+ADEPT#3 (amino acids 323–375) (b') Cy3 and DAPI merge of pFB1-7u+ADEPT#3 (c) Cy3 image of pFB1-7u+ADEPT#4 (amino acids 492–570) (c') Cy3 and DAPI merge of pFB1-7u+ADEPT#4 (d) Cy3 image of pFB1-7u+ADEPT#3+ADEPT#4 (amino acids 323–375, 492–570) (d') Cy3 and DAPI merge of pFB1-7u+ADEPT#3+ADEPT#4.


1
17
44
91
136
206
263
367
412
492
570
ADEPT#3
ADEPT#4


pFB1-7u vector control
pFB1-7u+ADEPT#3
pFB1-7u+ADEPT#4
pFB1-7u+ADEPT#3/ ADEPT#4
β-galactosidase
Merge (β-galactosidase + DAPI)
a
b
c
d
a'
b'
c'
d'

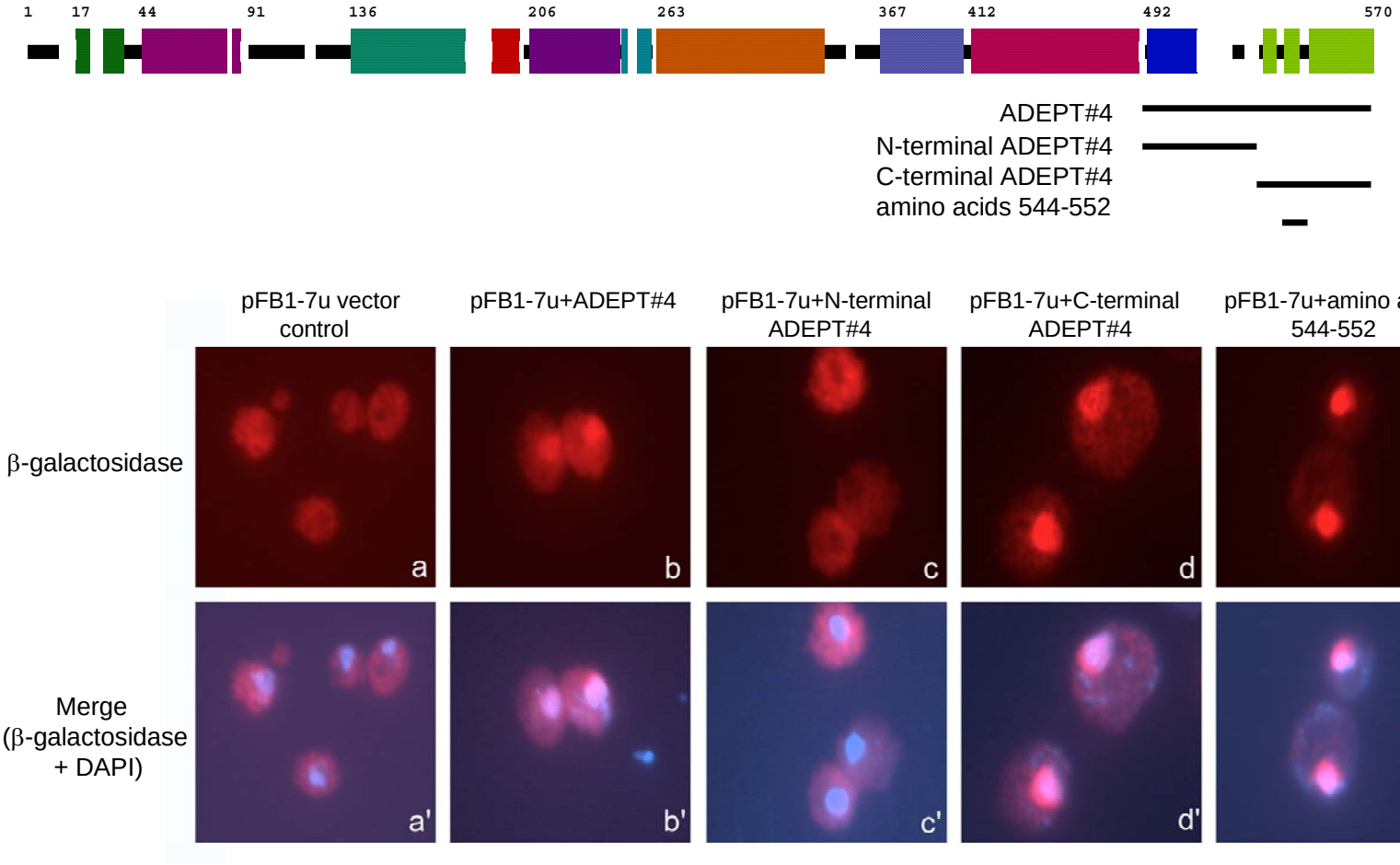
acids 492–530) appeared to target β-galactosidase more strongly to the nucleus relative to the entire ADEPT#4 region (amino acids 492–570), it may be due to differences in the exposure of the putative NLS within each fusion protein which may affect its ability to interact with the importin.

We examined the amino acid sequence of Trm1p-II within amino acid 531–570 and identified a stretch of nine residues, amino acid 544–552, enriched in basic amino acids that we hypothesized to be the putative NLS. Oligonucleotides corresponding to the nucleotide sequence of amino acids 544–552 were generated, annealed to form dsDNA, and inserted into pFB1-7u for localization of the resulting β-galactosidase fusion protein. This fusion protein containing Trm1p amino acids 544–552 accumulated in the nucleus (Fig. 11 e and e'), even stronger than the entire ADEPT#4 (amino acids 492–570) fusion protein (Fig. 11 b and b') or the C-terminal ADEPT#4 (amino acids 531–570) fusion protein (Fig. 11 d and d'). It is possible that the amino acids surrounding the putative NLS inhibit its function in the context of the endogenous protein (or even in the context of the ADEPT#4 region in the fusion proteins). As a result, when this sequence (amino acids 544–552) was liberated from the constraints of the endogenous protein (or ADEPT#4), it was able to more efficiently import β-galactosidase.

Both basic and hydrophobic amino acids are necessary for the function of the putative novel NLS within Trm1p-II

To determine which amino acids were necessary for the function of the putative NLS within amino acids 544–552, mutations were generated across the entire 9 amino acid region to determine their effect on the nuclear targeting of the β-galactosidase fusion

Fig. 11: Subcellular localization of pFB1-7u constructs containing various Trm1p ADEPT#4 fragments transformed in the BY4741 strain. Trm1-β-galactosidase fusion proteins were localized by indirect immunofluorescence using affinity-purified rabbit polyclonal anti-β-galactosidase primary antibody (Hopper *et al.,* 1990) and Cy3-conjugated goat anti-rabbit IgG secondary antibody, each diluted 1:400. DAPI was used to detect DNA. (a) Cy3 image of pFB1-7u vector only control (a') Cy3 and DAPI merge of pFB1-7u vector only control (b) Cy3 image of pFB1-7u+ADEPT#4 (amino acids 492–570) (b') Cy3 and DAPI merge of pFB1-7u+ADEPT#4 (c) Cy3 image of pFB1-7u+N-terminal-ADEPT#4 (amino acids 492–530) (c') Cy3 and DAPI merge of pFB1-7u+N-terminal-ADEPT#4 (d) Cy3 image of pFB1-7u+C-terminal-ADEPT#4 (amino acids 531–570) (d') Cy3 and DAPI merge of pFB1-7u+C-terminal-ADEPT#4 (e) Cy3 image of pFB1-7u+544–552 amino acids (e') Cy3 and DAPI merge of pFB1-7u+544–552 amino acids.


1
17
44
91
136
206
263
367
412
492
570
ADEPT#4
N-terminal ADEPT#4
C-terminal ADEPT#4
amino acids 544-552
pFB1-7u vector control
pFB1-7u+ADEPT#4
pFB1-7u+N-terminal ADEPT#4
pFB1-7u+C-terminal ADEPT#4
pFB1-7u+amino acids 544-552
β-galactosidase
Merge (β-galactosidase + DAPI)
a
b
c
d
e
a'
b'
c'
d'
e'

protein. If an amino acid was necessary for function of the NLS, then mutating that residue should shift the localization of the fusion protein from the nucleus to the cytoplasm. If an amino acid is not involved in the function of the NLS, then its mutation would not inhibit nuclear accumulation of the protein.

The requirement of basic amino acids for function of the classical basic NLS motif is clear. However, Polizotto and Cyert (2001) showed that the NLS within Crz1p required both basic and hydrophobic amino acids for function. There was a striking similarity in sequence between the NLS of Crz1p and the putative NLS of Trm1p (amino acids 544–552) (Table 4). Therefore, we predicted that the putative NLS within Trm1p may require both basic and hydrophobic amino acids for its function and sought to determine the contribution of the various amino acids within this NLS to its function. The mutations that were generated are summarized in Table 4.

Oligonucleotides were generated with altered nucleotide sequence(s) corresponding to amino acids 544–552 of Trm1p-II, annealed and inserted into pFB1-7u for localization of the resulting β-galactosidase fusion protein by indirect immunofluorescence. First, we changed the basic amino acids at positions 544, 546, 547, 549, and 552 to glutamic acid. When the β-galactosidase fusion protein containing the basic–acidic mutations was examined, the protein was restricted to the cytoplasm indicating this *cis*-acting region no longer functioned as an NLS (Fig. 12 b and b'). As we altered all five basic residues in combination, it was not possible to determine whether all five basic amino acids were required for function of the NLS or whether only individual residues were required. Future experiments individually mutating the basic residues

Table 4: Alteration of the putative nuclear localization sequence within ADEPT#4 of Trm1p-II (amino acids 544–552) to assay the necessary amino acids for function (identical and similar amino acids between Trm1p-II and Crz1p).

Protein	Amino Acid Sequence
Crz1p	Ile-Ile-Asn-Gly-Arg-Lys-Leu-Lys-Leu-Lys-Lys-Ser-Arg-Arg-Arg-Ser-Ser
Trm1p-II (putative NLS)	^{544}Lys-Leu-Arg-Lys-Leu-Lys-Ile-Val-Arg^{552}
Trm1p-II (basic–acidic)	^{544}**Glu**-Leu-**Glu**-**Glu**-Leu-**Glu**-Ile-Val-**Glu**^{552}
Trm1p-II (hydrophobic–acidic)	^{544}Lys-**Glu**-Arg-Lys-**Glu**-Lys-**Glu**-**Glu**-Arg^{552}
Trm1p-II (hydrophobic–alanine)	^{544}Lys-**Ala**-Arg-Lys-**Ala**-Lys-**Ala**-**Ala**-Arg^{552}
Trm1p-II (hydrophobic–asparagine)	^{544}Lys-**Asn**-Arg-Lys-**Asn**-Lys-**Asn**-**Asn**-Arg^{552}

within the amino acid 544–552 region of Trm1p could delineate their individual contribution. However, this result indicated a general requirement for basic amino acids within this putative NLS.

Polizotto and Cyert (2001) demonstrated the role of hydrophobic residues in the function of the NLS of Crz1p by removing the N-terminal isoleucines and showing that this sequence no longer functioned as an NLS. However, the hydrophobic residues of the putative NLS of Trm1p-II were interspersed throughout the 544–552 amino acid region. Therefore, we performed site-directed mutagenesis to alter individual hydrophobic residues within amino acids 544–552 to determine their effect on the function of this region as an NLS.

We generated oligonucleotides in which we altered the hydrophobic amino acids at positions 545, 548, 550, and 551 to glutamic acid. We chose to mutate the hydrophobic residues to acidic residues to be consistent with the mutations made previously for the basic residues within this region. The fusion protein containing the hydrophobic–acidic mutations accumulated in the cytoplasm (Fig. 12 c and c'), indicating the hydrophobic amino acids contribute to the function of the NLS. However, this mutation could have affected nuclear targeting in two ways: 1) the required hydrophobic residues were no longer present, or 2) the positive charge generated by the basic residues within this region was neutralized by the introduction of acidic residues and therefore the inhibition of nuclear accumulation was due to lack of positive charge rather than lack of hydrophobic residues.

To elucidate more clearly the role of the hydrophobic residues within amino acids 544–552 on the function of the putative NLS, additional mutations were generated. The

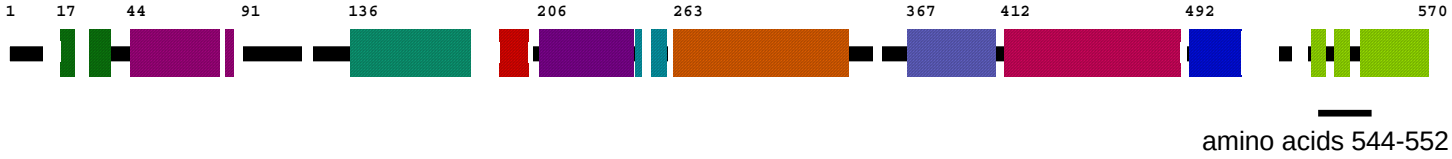
hydrophobic residues at positions 545, 548, 550, and 551 were changed in combination to alanine residues, as alanine is an uncharged amino acid and would not alter the overall positive charge within the amino acids 544–552 region. This fusion protein accumulated in the nucleus (Fig. 12 d and d'), similar to the fusion protein containing the wild-type NLS sequence (Fig. 12 a and a'). Therefore, mutating the hydrophobic residues to alanine did not inhibit the function of the NLS.

As alanine is slightly hydrophobic, it might be sufficient to compensate for the leucine and isoleucines within this region. Therefore, the hydrophobic residues were changed to asparagine to determine the effect of hydrophobicity on the function of the putative NLS. The previous mutations generated for the hydrophobic residues, hydrophobic–acidic and hydrophobic–alanine, would each have altered the structure of this Trm1p-II fragment since isoleucine and leucine differ significantly in size to both glutamic acid and alanine. In contrast, asparagine is similar in size to leucine and isoleucine. Therefore altering the hydrophobic residues at position 545, 548, 550, and 551 to asparagine would be conservative in size, though disrupting the hydrophobic nature of this region. The fusion protein wherein the hydrophobic residues were changed to asparagine was diffusely localized in the cytoplasm (Fig. 12 e and e'), suggesting the hydrophobicity of this region was important for nuclear targeting.

The putative NLS within Trm1p-II is recognized by the importers, Kap120p and Nmd5p

NLS#1 (amino acids 95–102; Rose *et al.,* 1992) within Trm1p-II resembles the classical NLS exemplified by that within the SV40 Large T Antigen and is most likely

Fig. 12: Subcellular localization of pFB1-7u constructs containing Trm1p amino acids 544–552 transformed in the BY4741 strain to determine which amino acids are necessary for function of the putative NLS within ADEPT#4. Trm1-β-galactosidase fusion proteins were localized by indirect immunofluorescence using affinity-purified rabbit polyclonal anti-β-galactosidase primary antibody (Hopper *et al.,* 1990) and Cy3-conjugated goat anti-rabbit IgG secondary antibody, each diluted 1:400. DAPI was used to detect DNA. (a) Cy3 image of pFB1-7u+544–552 amino acids (a') Cy3 and DAPI merge of pFB1-7u+544-552 amino acids (b) Cy3 image of pFB1-7u+544–552 amino acids (basic–acidic) (b') Cy3 and DAPI merge of pFB1-7u+544–552 amino acids (basic–acidic) (c) Cy3 image of pFB1-7u+544–552 amino acids (hydrophobic–acidic) (c') Cy3 and DAPI merge of pFB1-7u+544–552 amino acids (hydrophobic–acidic) (d) Cy3 image of pFB1-7u+544–552 amino acids (hydrophobic–alanine) (d') Cy3 and DAPI merge of pFB1-7u+544–552 amino acids (hydrophobic–alanine) (e) Cy3 image of pFB1-7u+544–552 amino acids (hydrophobic–asparagine) (e') Cy3 and DAPI merge of pFB1-7u+544–552 amino acids (hydrophobic–asparagine).


1
17
44
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136
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492
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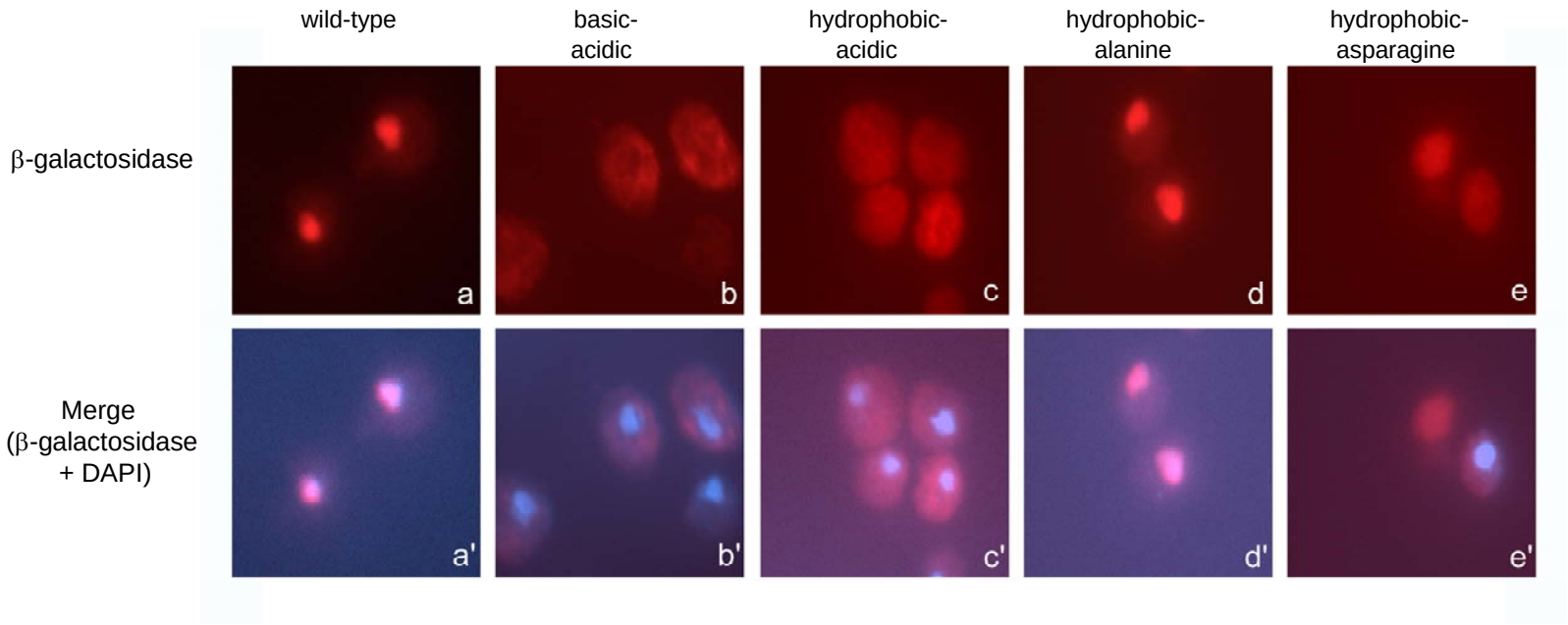
amino acids 544-552


wild-type
basic-acidic
hydrophobic-acidic
hydrophobic-alanine
hydrophobic-asparagine
β-galactosidase
Merge (β-galactosidase + DAPI)
a
b
c
d
e
a'
b'
c'
d'
e'

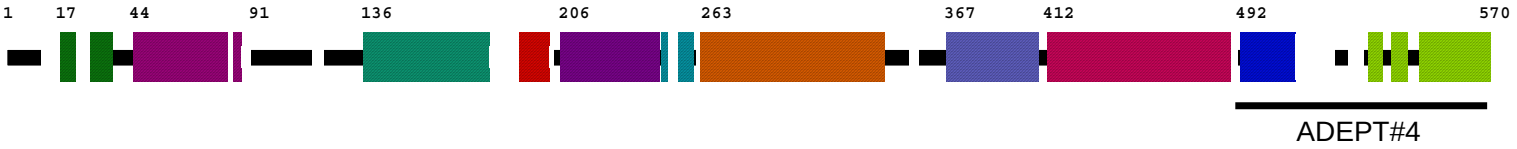
recognized by the karyopherin, importin β/Kap95p. To determine which of the importin β family member(s) mediated nuclear targeting of Trm1p-II via the putative NLS within ADEPT#4, the pFB1-7u plasmid containing ADEPT#4 (amino acids 492–570) was transformed into mutants containing individual deletions of each of the 9 unessential importin β genes, *kap114*, *kap120*, *kap123*, *los1, msn5*, *mtr10**, *nmd5*, *pdr6*, and *sxm1* (Winzeler *et al.,* 1999; *generated independently by M. Whitney). If an individual karyopherin was involved in the nuclear import of the β-galactosidase fusion protein, we predicted its deletion would cause a shift in localization (from the nucleus to the cytoplasm). As a control, pFB1-7u+ADEPT#2 (amino acids 73–151), containing the known NLS of Trm1p (NLS#1: amino acids 95–102), was transformed into these mutant strains. As NLS#1 is predicted to be recognized by importin β/Kap95p, an essential karyopherin not included in this screen, we did not anticipate observing any change in the localization of the ADEPT#2 (amino acids 73–151)-β-galactosidase fusion protein in any of the mutant strains being tested. The β-galactosidase fusion proteins were subsequently localized by indirect immunofluorescence in the mutant strains and compared to their localization in the wild-type parent strain, BY4741.

The ADEPT#4 (amino acids 492–570)-β-galactosidase fusion protein was examined in the individual karyopherin mutants to determine if its localization was altered in any of these strains. ADEPT#4 (amino acids 492–570)-β-galactosidase was maintained in the nucleus, similar to wild-type cells, in *kap114* (Fig. 13 b and b'), *kap123, los1, msn5, mtr10, pdr6,* and *sxm1* cells (data not shown). In both *kap120* and *nmd5* cells, the ADEPT#4 (amino acids 492–570)-β-galactosidase fusion protein was

diffused throughout the nucleus and cytoplasm with a marked reduction in the nuclear pool compared to wild-type cells (Fig. 13 c and c', d and d' compared to a and a'). As expected, the ADEPT#2 (amino acids 73–151)-β-galactosidase fusion protein accumulated in the nucleus in BY4741 cells and its localization was unaltered in any of the mutant karyopherin strains, including *kap120* and *nmd5* cells (data not shown). Though deletion of either *KAP120* or *NMD5* reduced the nuclear pool of the ADEPT#4 (amino acids 492–570)-β-galactosidase fusion protein, a nuclear pool remained. Therefore, a *kap120nmd5* double mutant was tested to determine whether there was an additive effect on localization when both karyopherin genes were deleted. However, there was no cumulative effect when *KAP120* was deleted in combination with *NMD5,* as a nuclear pool of the ADEPT#4 (amino acids 492–570)-β-galactosidase fusion protein remained, comparable to that present in the individual *kap120* or *nmd5* strains (Fig. 13 e and e'). This result suggested that there was at least one additional importin β family member mediating nuclear targeting of ADEPT#4 (amino acids 492–570)-β-galactosidase via the putative NLS. This is not unprecedented as many NLSs are recognized by several importin β family members; Pse1p/Kap121p and Kap123p both contribute to the import of the ribosomal protein, rpl25, and histones are imported by Kap114p, Pse1p/Kap121p, and Kap123p (Rout *et al.,* 1997; Mosammaparast *et al.,* 2001). Most likely, the putative NLS within ADEPT#4 is recognized by one of the five essential importin β family members not included in this screen.

We also examined the localization of the C-terminal ADEPT#4 (amino acids 531–570) and 9 amino acid (amino acids 544–552)-β-galactosidase fusion proteins in the

Fig. 13: Subcellular localization of pFB1-7u constructs containing Trm1p ADEPT#4 transformed in the unessential β-importin mutant strains. Indirect immunofluorescence using affinity-purified rabbit polyclonal anti-β-galactosidase primary antibody (Hopper *et al.,* 1990) and Cy3-conjugated goat anti-rabbit IgG secondary antibody, each diluted 1:400. DAPI was used to detect DNA. (a) Cy3 image of pFB1-7u+ADEPT#4/BY4741 (a') Cy3 and DAPI merge of pFB1-7u+ADEPT#4/BY4741 (b) Cy3 image of pFB1-7u+ADEPT#4/*kap114* (b') Cy3 and DAPI merge of pFB1-7u+ADEPT#4/*kap114* (c) Cy3 image of pFB1-7u+ADEPT#4/*kap120* (c') Cy3 and DAPI merge of pFB1-7u+ADEPT#4/*kap120* (d) Cy3 image of pFB1-7u+ADEPT#4/*nmd5* (d') Cy3 and DAPI merge of pFB1-7u+ADEPT#4/*nmd5* (e) Cy3 image of pFB1-7u+ADEPT#4/*kap120nmd5* (e') Cy3 and DAPI merge of pFB1-7u+ADEPT#4/*kap120nmd5*.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17
44
91
136
206
263
367
412
492
570
ADEPT#4

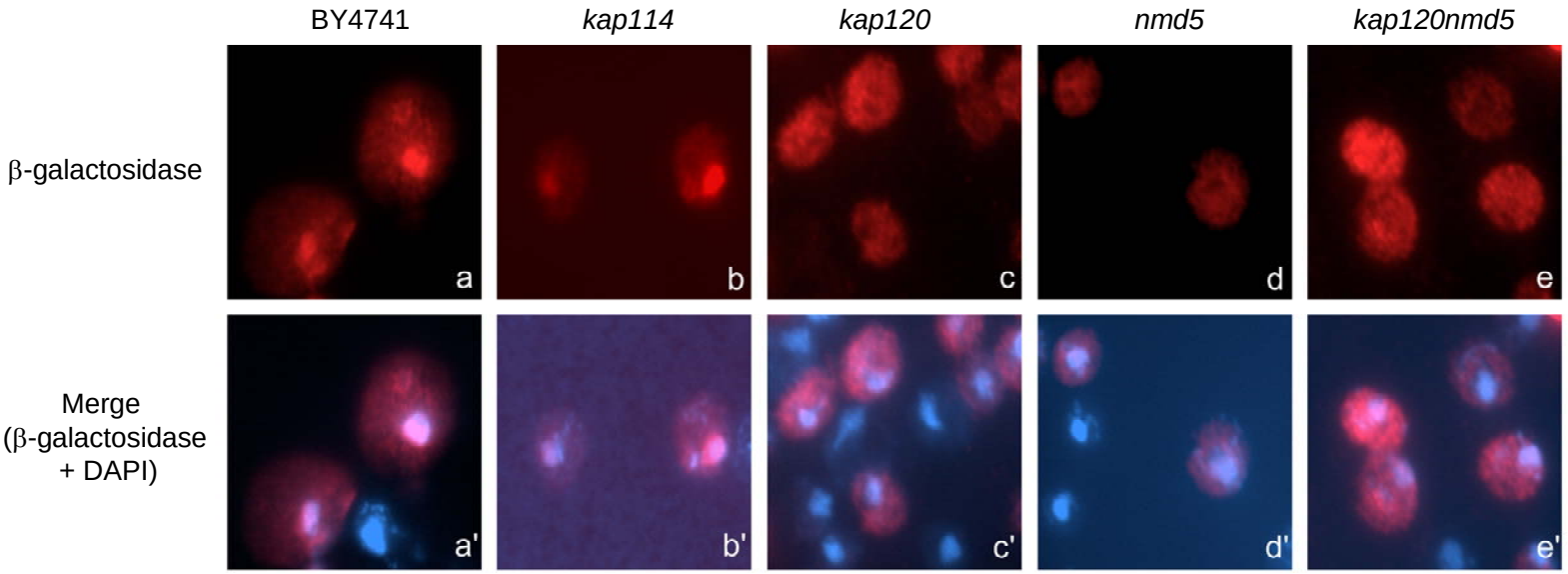
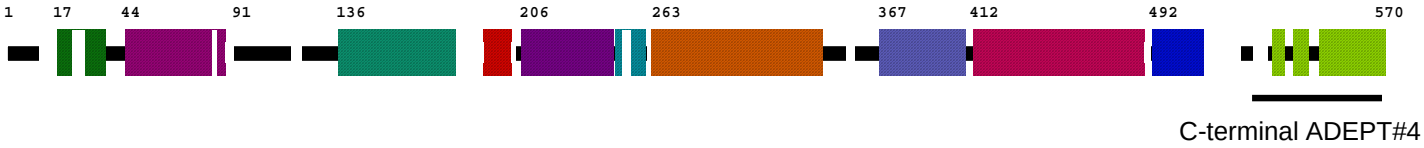

BY4741
kap114
kap120
nmd5
kap120nmd5
β-galactosidase
Merge
(β-galactosidase
+ DAPI)
a
b
c
d
e
a'
b'
c'
d'
e'

Fig. 14: Subcellular localization of pFB1-7u constructs containing Trm1p C-terminal ADEPT#4 transformed in the unessential β-importin mutant strains. Indirect immunofluorescence using affinity-purified rabbit polyclonal anti-β-galactosidase primary antibody (Hopper *et al.,* 1990) and Cy3-conjugated goat anti-rabbit IgG secondary antibody, each diluted 1:400. DAPI was used to detect DNA. (a) Cy3 image of pFB1-7u+C-terminal ADEPT#4/BY4741 (a') Cy3 and DAPI merge of pFB1-7u+C-terminal ADEPT#4/BY4741 (b) Cy3 image of pFB1-7u+C-terminal ADEPT#4/*kap114* (b') Cy3 and DAPI merge of pFB1-7u+C-terminal ADEPT#4/*kap114* (c) Cy3 image of pFB1-7u+C-terminal ADEPT#4/*kap120* (c') Cy3 and DAPI merge of pFB1-7u+C-terminal ADEPT#4/*kap120* (d) Cy3 image of pFB1-7u+C-terminal ADEPT#4/*nmd5* (d') Cy3 and DAPI merge of pFB1-7u+C-terminal ADEPT#4/*nmd5* (e) Cy3 image of pFB1-7u+C-terminal ADEPT#4/*kap120nmd5* (e') Cy3 and DAPI merge of pFB1-7u+C-terminal ADEPT#4/*kap120nmd5*.


1
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44
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136
206
263
367
412
492
570
C-terminal ADEPT#4

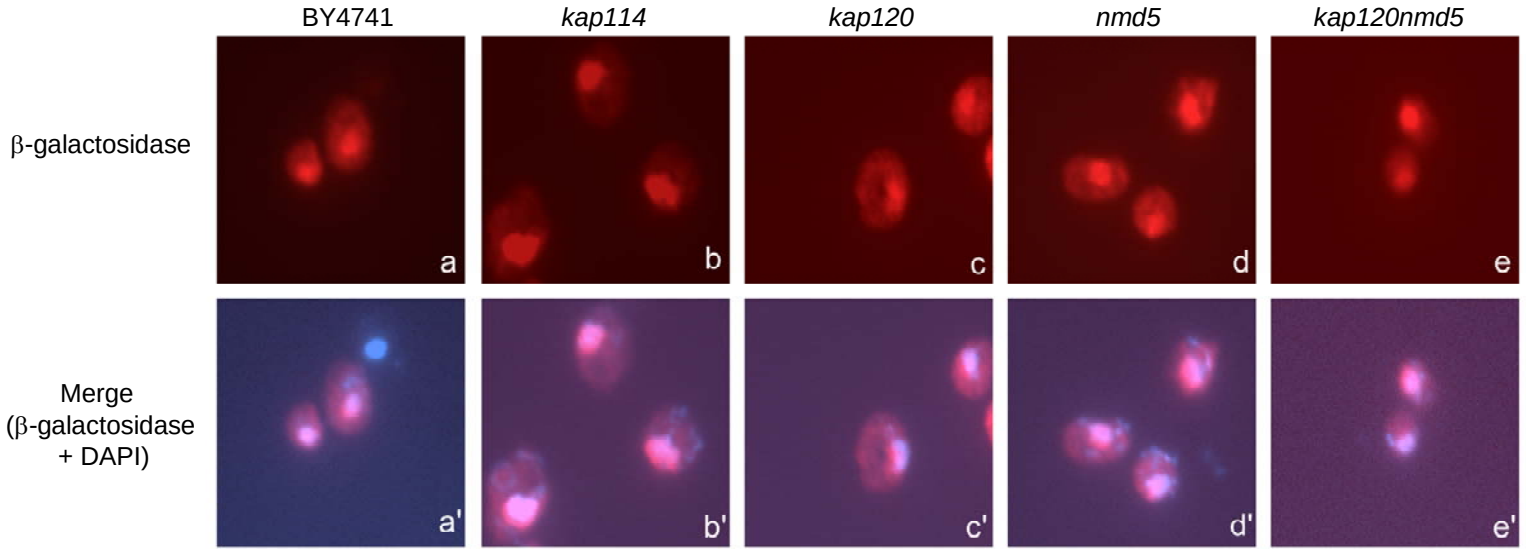
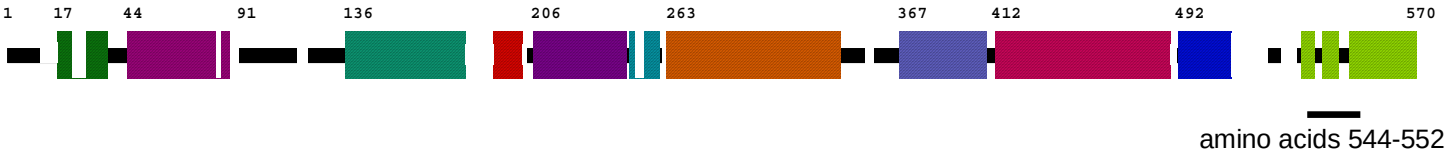

BY4741
kap114
kap120
nmd5
kap120nmd5
β-galactosidase
Merge
(β-galactosidase
+ DAPI)
a
b
c
d
e
a'
b'
c'
d'
e'

Fig. 15: Subcellular localization of pFB1-7u constructs containing Trm1p amino acids 544–552 amino acids transformed in the unessential β-importin mutant strains. Indirect immunofluorescence using affinity-purified rabbit polyclonal anti-β-galactosidase primary antibody (Hopper *et al.,* 1990) and Cy3-conjugated goat anti-rabbit IgG secondary antibody, each diluted 1:400. DAPI was used to detect DNA. (a) Cy3 image of pFB1-7u+544–552 amino acids/BY4741 (a') Cy3 and DAPI merge of pFB1-7u+544–552 amino acids/BY4741 (b) Cy3 image of pFB1-7u+544–552 amino acids/*kap114* (b') Cy3 and DAPI merge of pFB1-7u+544–552 amino acids/*kap114* (c) Cy3 image of pFB1-7u+544–552 amino acids/*kap120* (c') Cy3 and DAPI merge of pFB1-7u+544–552 amino acids/*kap120* (d) Cy3 image of pFB1-7u+544–552 amino acids/*nmd5* (d') Cy3 and DAPI merge of pFB1-7u+544–552 amino acids/*nmd5* (e) Cy3 image of pFB1-7u+544–552 amino acids/*kap120nmd5* (e') Cy3 and DAPI merge of pFB1-7u+544–552 amino acids/*kap120nmd5.*


1
17
44
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492
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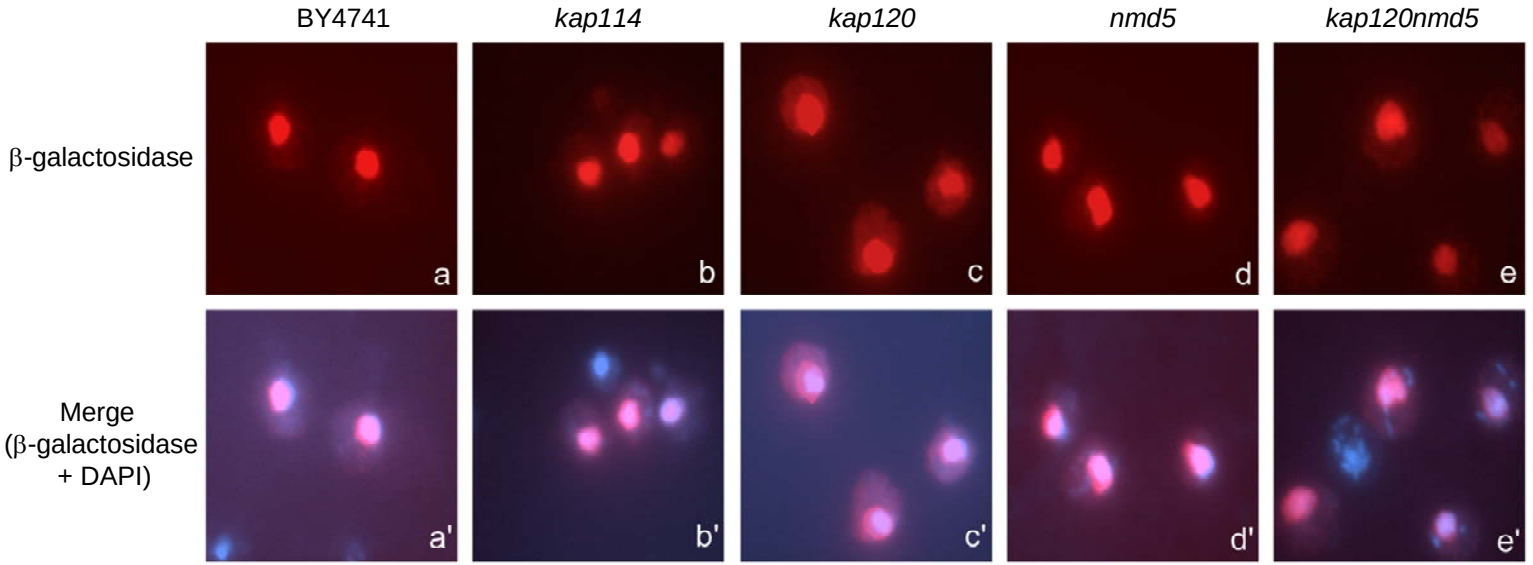
amino acids 544-552


BY4741
kap114
kap120
nmd5
kap120nmd5
β-galactosidase
Merge
(β-galactosidase
+ DAPI)
a
b
c
d
e
a'
b'
c'
d'
e'

unessential importin β mutants and compared their localization to: 1) that in wild-type cells and, 2) that of the entire ADEPT#4 (amino acids 492–570)-β-galactosidase fusion protein in the unessential importin β mutant strains. Surprisingly, there was no difference in the localization of either fusion protein in any of the mutant strains, including *kap120* and *nmd5* cells, relative to wild-type cells as the fusion proteins accumulated in the nucleus (Fig. 14 and 15). This result was in disagreement with the localization of the ADEPT#4 (amino acids 492–570) fusion protein wherein the nuclear pool was reduced in both *kap120* and *nmd5* cells. This suggested that either the structure within ADEPT#4 or the surrounding amino acids may have a role in the function of this NLS. The smaller fragments each lack 40 amino acids from the N-terminus of ADEPT#4, suggesting these amino acids may contribute to the function of the putative NLS. As the smaller fragments (amino acids 531–570 and amino acids 544–552) were liberated from the surrounding Trm1p-II amino acids, they could function in an independent manner than that of the entire ADEPT#4 fragment. A role for the surrounding amino acids in function of an NLS has been demonstrated previously for nucleoplasmin. Laskey *et al.* (1996) and Makkerh *et al.* (1996) demonstrated that the addition of neutral and acidic amino acids surrounding the second basic cluster of the bipartite NLS of nucleoplasmin promoted nuclear targeting. Perhaps the results we observe are similar in that the flanking amino acids have an effect on nuclear import of Trm1p-II. Future experiments altering the upstream amino acids within ADEPT#4 may provide insight to the contribution of these amino acids to the function of this putative NLS and provide an explanation for this discrepancy in localization.

All results from experiments described in this chapter are summarized in Table 5.

Table 5: Results from all experiments reported in Chapter 3.

Construct	**Independent:**		**Subcellular Localization**
	Cultures	**Transformations**	
pFB1-7u vector only/BY4741	1	2	Cytoplasmic
pFB1-7u+ADEPT#3/BY4741	1	2	Cytoplasmic
pFB1-7u+ADEPT#4/BY4741	1	2	Nuclear
pFB1-7u+ADEPT#3/ADEPT#4/ BY4741	1	2	Nuclear
pFB1-7u+N-terminal ADEPT#4/ BY4741	1	2	Cytoplasmic
pFB1-7u+C-terminal ADEPT#4/ BY4741	1	2	Nuclear
pFB1-7u+amino acids 544–552/ BY4741	1	2	Nuclear
pFB1-7u+amino acids 544–552/ BY4741 (basic–acidic)	1	2	Cytoplasmic
pFB1-7u+amino acids 544–552/ BY4741 (hydrophobic–acidic)	1	2	Cytoplasmic
pFB1-7u+amino acids 544–552/ BY4741 (hydrophobic–alanine)	1	2	Nuclear
pFB1-7u+amino acids 544–552/ BY4741 (hydrophobic–asparagine)	1	2	Cytoplasmic
pFB1-7u+ADEPT#4/*kap114*	1	2	Nuclear
pFB1-7u+ADEPT#4/*kap120*	1	2	Nuclear/Cytoplasmic
pFB1-7u+ADEPT#4/*nmd5*	1	2	Nuclear/Cytoplasmic
pFB1-7u+ADEPT#4/*kap120nmd5*	1	2	Nuclear/Cytoplasmic
pFB1-7u+C-terminal ADEPT#4/*kap114*	1	2	Nuclear
pFB1-7u+C-terminal ADEPT#4/*kap120*	1	2	Nuclear
pFB1-7u+C-terminal ADEPT#4/*nmd5*	1	2	Nuclear
pFB1-7u+C-terminal ADEPT#4/ *kap120nmd5*	1	2	Nuclear
pFB1-7u+amino acids 544–552/*kap114*	1	2	Nuclear
pFB1-7u+amino acids 544–552/*kap120*	1	2	Nuclear
pFB1-7u+amino acids 544–552/*nmd5*	1	2	Nuclear
pFB1-7u+amino acids 544–552/ *kap120nmd5*	1	2	Nuclear

DISCUSSION

The hypothesis that NLSs were of strictly two types, single or bipartite basic amino acid regions, has been overturned in recent years. Novel NLSs have been identified, suggesting there are likely still undefined motifs that function as NLSs *in vivo*. For Crz1p, a transcription factor that binds to calcineurin-dependent response elements, an NLS was identified that requires both basic and hydrophobic residues for function (Polizotto and Cyert, 2001). We defined a similar putative NLS within ADEPT#4 of Trm1p-II. The putative NLS within amino acids 544–552 was the second identified NLS within Trm1p-II. A classical basic NLS that shares sequence similarity with the prototypical NLS within SV40 Large T Antigen (Table 6) was identified previously at amino acids 95–102 (Rose *et al.,* 1992). The presence of more than one NLS within a protein is not unprecedented; for example, αCP2, belonging to a family of RNA-binding proteins including αCP1–4, contains two independent novel NLSs, each of which is functional *in vivo* (Chkheidze and Liebhaber, 2003). However, the identification of a second NLS within Trm1p-II raises many questions to the purpose of this seeming redundancy.

Several hypotheses can account for the presence of multiple NLSs within a protein: 1) allows for a secondary NLS to compensate for mutation(s) occurring in the primary NLS that ablate its function; 2) allows for regulation of import by the presence of NLSs of varying strengths, as has been suggested for 5-lipoxygenase (Luo *et al.,* 2004); or 3) allows for the different NLSs to function under different physiological conditions.

TRM1 is an unessential gene indicating this tRNA modification is not essential for tRNA function. The potential presence of multiple functional NLSs within Trm1p raises

Table 6: Alignments of known nuclear localization sequences with Trm1p-II known and putative sequences.

Protein	Amino Acid Sequence
SV40 Large T Antigen	Pro-Pro-**Lys-Lys-Lys-Arg**-Lys-Val
Trm1p-II NLS#1	^{95}Lys-Lys-Ser-**Lys-Lys-Lys-Arg**-Cys^{102}
Glucocorticoid Receptor	Arg-Lys-Thr-Lys-Lys-Lys-Ile-Lys-Gly
Nucleoplasmin	Lys-Arg-Pro-Ala-Ala-Thr-Lys-Lys-Ala-Gly-Gln-Ala-Lys-Lys-Lys-Lys
Crz1p	Ile-Ile-Asn-Gly-**Arg-Lys-Leu-Lys**-Leu-Lys-Lys-Ser-Arg-Arg-Arg-Ser-Ser
Trm1p-II NLS#2	^{544}Lys-Leu-**Arg-Lys-Leu-Lys**-Ile-Val-Arg^{552}

some questions about why an unessential protein would possess such an elaborate mechanism for nuclear targeting. Trm1p is capable of functioning at alternative locations and preventing its nuclear targeting does not result in any aberrant cellular phenotype. Further, as Trm1p-II with a mutated classical NLS was enzymatically functional, capable of modifying tRNAs in the cytoplasm (Rose *et al.,* 1992), there is evidently not a strict requirement for the modification catalyzed by Trm1p-II to occur in the nucleus, possibly in an ordered manner prior to tRNA export to the cytoplasm. It therefore seems counterintuitive that Trm1p-II would have evolved redundant nuclear import pathways as it can function as efficiently in either the nucleus or cytoplasm. This argues against the first hypothesis that the putative NLS acts as a backup to the classical NLS to ensure that Trm1p-II targets to the nucleus. Interestingly, it has been shown that inhibiting multiple modifications of tRNA, including that catalyzed by Trm1p, can cause growth defects (Purushothaman *et al.,* 2005). One explanation for the growth defects is that, although Trm1p-II activity is dispensable, Trm1p-II may perform an additional function, possibly in the nucleus, such as serving as a scaffold for tRNA to optimize other cellular interactions. It remains possible that there is some advantage to having Trm1p localize to the nucleus rather than the cytoplasm.

Previous studies have shown that altering NLS#1 (amino acids 95–102) of Trm1p-II restricts the majority of the protein to the cytoplasm, suggesting that NLS#1 is the predominant signal mediating nuclear import of Trm1p-II (Rose *et al.,* 1992). Although we did not demonstrate the role of the putative NLS in targeting endogenous Trm1p-II to the nucleus, we cannot rule out that this NLS contributes weakly to targeting. It is possible that a pool of Trm1p-II remained at the INM when the known NLS of

Trm1p-II was mutated, though our localization techniques were not sensitive enough to visualize its presence. Indirect immunofluorescence is a qualitative tool and does not allow for quantitative evaluation of the distribution of the protein. The known NLS within Trm1p-II is strong, allowing efficient import of the protein into the nucleus. It is likely that more sensitive techniques are required to definitively determine whether there is a nuclear pool of Trm1p-II remaining in the nucleus when the known NLS is mutated. It is possible that the putative NLS functions *in vivo*, but its role in targeting is overshadowed by the classical NLS. Therefore, it would be interesting to generate a double NLS mutant of Trm1p-II that lacks both the known NLS (amino acids 95–102) and the putative NLS (amino acids 544–552) and compare its localization using electron microscopy to: 1) that of individual NLS mutants of Trm1p-II, and 2) that of the endogenous protein.

It is also possible that the putative NLS of Trm1p-II functions under different conditions than those tested. It has been established within our lab that starving cells for amino acids, glucose, or phosphate cause tRNA to accumulate in the nucleus, possibly as a mechanism to regulate translation (Shaheen and Hopper, 2005; Shaheen *et al.*, 2007; Whitney *et al.*, 2007; Hurto *et al.*, 2007). Possibly, the tRNA modification enzymes are also regulated by a mechanism similar to that monitoring amino acids, glucose, and phosphate. In all experiments, the localization of the Trm1-β-galactosidase fusion proteins were examined at steady state, under optimal growth conditions. It is possible that the NLSs of Trm1p-II function differently when exposed to variations in growth conditions or cellular stress. It would therefore be interesting to examine the localization of endogenous Trm1p-II as well as Trm1p possessing mutations in the known NLS

and/or the putative NLS under different physiological conditions (starvation, stress, etc.) to determine if there are alterations in localization, thus addressing the third hypothesis.

Regardless of whether the putative NLS functions in the context of the endogenous protein, this amino acid sequence was sufficient to function as an NLS in the β-galactosidase fusion protein. This putative NLS appeared to be recognized and imported, in part, by Kap120p and Nmd5p, as individually deleting the genes for these karyopherins was sufficient to reduce the nuclear accumulation of the β-galactosidase fusion protein. Although both Kap120p and Nmd5p were implicated in the import of the putative NLS, deleting both genes in combination did not completely inhibit nuclear accumulation of the β-galactosidase fusion protein, indicating that there may be a third karyopherin which recognizes the putative NLS within ADEPT#4. Therefore, it would be interesting to examine the localization of Trm1-β-galactosidase fusion protein in the essential karyopherin mutants. Each of the essential importin genes has a temperature-sensitive mutation that causes the protein to be unstable at the non-permissive temperature. These experiments would require generation of a construct in which expression of the fusion protein can be induced after shifting the cells to the non-permissive temperature to inactivate the importin protein of interest. In the absence of regulated expression, the β-galactosidase fusion protein would be produced and imported into the nucleus at the permissive temperature and the effects of shifting to the non-permissive temperature would be indistinguishable against the nuclear pool accumulated at the permissive temperature.

Nmd5p/Kap119p has been previously identified as an importer of Crz1p (Polizotto and Cyert, 2001) and AlcR, an activator of the ethanol utilization pathway genes in *Aspergillus nidulans* (Nikolaev *et al.,* 2003). In *S. cerevisiae,* both Hog1 and Ssa4p redistribute in an Nmd5p-dependent fashion; Hog1p concentrates in the nucleus in response to osmotic stress (Ferrigno *et al.,* 1998) while Ssa4p, a member for the hsp70/hsc70 family of proteins, redistributes from the cytoplasm to the nucleus in response to cellular stress triggered by the addition of ethanol (Quan *et al.,* 2004). Therefore, there is sufficient evidence supporting a role for Nmd5p/Kap119p in nuclear import in response to stress conditions.

The role of Kap120p in importing protein to the nucleus is less definitive. Kap120p has a role in exporting Rpl11b, a protein of the 60S ribosomal subunit (Stage-Zimmermann *et al.,* 2000) and has a role in importing the MA protein, produced by proteolytic cleavage of the Gag polyprotein, of the Rous sarcoma virus (Butterfield-Gerson *et al.,* 2005). Interestingly, the role of Kap120p in importing Crz1p was not tested, as this karyopherin was not included in their screen (Polizotto and Cyert, 2001). As the putative NLS of Trm1p-II is similar in sequence to the NLS of Crz1p, it would be worthwhile to test the role of Kap120p in targeting Crz1p to the nucleus.

As we generated mutations of residues in combination in the putative NLS, rather than individually, we cannot determine individually which of the nine amino acids within this region are required for function. Therefore, it would be interesting to alter individual amino acids to determine a more definitive *cis*-acting sequence functioning as an NLS.

The ADEPT#4 (amino acids 492–570) fragment was mislocalized in the *kap120* and *nmd5* cells, in contrast to the smaller ADEPT#4 fragments (amino acids 531–570 and

544–552, respectively) whose localization was unaffected in these cells. One explanation to account for this discrepancy is that the upstream amino acids from 492–530, present in the larger ADEPT#4 fragment, contribute or regulate the function of the downstream NLS. It is also possible that there is a structural component for function of this NLS and the larger ADEPT#4 fragment is able to recapitulate the endogenous structure in the fusion protein, while the smaller fragments cannot. In this case, the sequences may each function as an NLS, but are recognized by distinct importers. We propose that the smaller ADEPT#4 fragments (amino acids 531–570 and 544–552, respectively) may function similar to the classical basic NLS. This hypothesis would predict that these smaller ADEPT#4 fragments would mislocalize in importin β/Kap95p mutant cells. Therefore, again it would be interesting to test the five essential importers. Finally, we cannot rule out the possibility that the plasmid backbone contributes to the function of the putative NLS within ADEPT#4. It is possible, though unlikely, that the junctions generated between the various *TRM1* fragments and the pFB1-7u backbone (Moreland *et al.,* 1987) contributed to the function of this region as an NLS, therefore accounting for the variations in localization between the different ADEPT#4 fragments. We suggest fusing these ADEPT#4 fragments to a different reporter, such as GFP, to verify the effects observed in the experiments described here.

We hypothesized two pathways for a peripheral INM protein to localize to the INM. The first model follows the pathways of integral INM proteins. In this case, Trm1p-II could bind its membrane counterpart, which is inserted into the ER membrane, and diffuse, by default, along the same pathway as the integral INM protein. This would require transversing the lateral channels of the NPC to gain access to the INM. The size

of Trm1p-II alone argues against this model, as the lateral channels of vertebrate NPCs can only accommodate proteins up to approximately 60 kDa. Both Trm1p-II, approximately 63 kDa, and Trm1p-II-GFP, approximately 89 kDa, locate to the INM (Ellis *et al.,* 1987; Murthi and Hopper, 2005). As Trm1p would be bound to an integral protein whose nucleoplasmic domain would translocate through the lateral channel, the entire domain moving through the lateral channel would exceed 63 kDa. Therefore, this model for targeting Trm1p-II to the nucleus is not likely. Additionally, no empirical evidence has been found for any peripheral nuclear protein targeting to the INM through the lateral channels of the NPC via binding to its membrane counterpart in the cytoplasm, either in *S. cerevisiae* or any other eukaryotic organism.

The second model involves a two-step targeting process; Trm1p-II is synthesized in the cytoplasm, imported through the central channel of the NPC to gain access to the nucleoplasm and subsequently targeted to the INM. In this scenario, Trm1p-II would require two signals, an NLS and an INM targeting sequence. Several lines of evidence support this model. The NLS within amino acids 95–102 of Trm1p-II resembles the NLS from SV40 Large T antigen imported actively through the central channel of the NPC by importin β/Kap95p (Kalderon *et al.,* 1984). When Rose *et al.* (1992) mutated the lysine residues within amino acids 95–102 (**KK**S**KKK**RC) in combination to glutamic acid residues (**EE**S**EEE**RC), the majority of Trm1p-II was no longer imported into the nucleus, but rather restricted to the cytoplasm. This is the predicted result if endogenous Trm1p-II is actively imported into the nucleus through the central channel of the NPC.

CHAPTER FOUR

A REGION OF Trm1p-II IS SUFFICIENT TO LOCATE TO THE INNER NUCLEAR MEMBRANE

ABSTRACT

Although targeting to the nucleus is regulated by signals on the surface of nuclear proteins, the mechanism of targeting to subnuclear regions, particularly the INM, remains unknown. To identify *cis*-acting sequences within Trm1p that contribute to its INM localization, we localized fused various fragments of Trm1p to β-galactosidase and assayed the localization of the fusion proteins. We identified a 60-amino acid region of Trm1p-II that is sufficient to target a non-nuclear protein, β-galactosidase, to the nucleus, specifically targeting to the INM. This 60-amino acid sequence of Trm1p-II represents the first identified domain of a peripheral nuclear membrane protein sufficient for targeting to the INM.

INTRODUCTION

We proposed that targeting a soluble protein to a specific subregion within the nucleus likely requires two signals: an NLS to direct the protein to the nuclear interior and a signal to target the protein specifically to a distinct subnuclear region, such as the nucleolus or INM. Sequences have been identified that regulate targeting to the nucleolus, such as those within Hsp70 and HIV tat proteins (Dang and Lee, 1989). Signals have also been identified that result in protein targeting to the INM; however, these characterized signals are for integral INM proteins (Smith and Blobel, 1993;

Soullam and Worman, 1993; 1995; Furukawa *et al.,* 1995, 1998; King *et al.,* 2006) that likely follow a distinct path to the INM relative to that of soluble proteins, such as Trm1p-II.

The best-characterized peripheral INM proteins are the vertebrate nuclear lamins that form the nuclear lamina, a filamentous network underlying the INM, which provides mechanical support for the nucleus. The mechanism of targeting of the nuclear lamins to the INM is not completely understood; all lamins are known to contain a functional NLS at their C-terminus and disrupting this NLS results in cytoplasmic accumulation (Loewinger and McKeon, 1988). The identification of a functional NLS within the lamins suggests these proteins enter the nucleus through the central channel of the NPC, similar to other soluble proteins that reside in the nucleus. However, how the nuclear lamins are subsequently targeted to the INM is not clear. Lamins bind many integral INM proteins, including LBR, various isoforms of the LAPs, emerin, and MAN1; it is possible that these interactions retain the nuclear lamins at the INM, where they polymerize forming a filamentous network. Obviously, much remains to be understood about the mechanism of subnuclear targeting, even for well-studied proteins, such as the nuclear lamins. Appropriate protein targeting of the nuclear lamins, as well as various integral INM proteins that interact with the lamins, is critical as many diseases have been identified resulting from mislocalization of these proteins.

Although there are no proteins in *S. cerevisiae* that share homologous sequence with the vertebrate nuclear lamins, there may be functional homologues. Identification of *cis*-acting sequences regulating INM localization of peripheral proteins in *S. cerevisiae*

may reveal additional proteins that possess similar targeting information leading to identification of putative lamin homologues.

We sought to identify regions of Trm1p-II that mediate targeting to the INM. Various *cis*-acting regions of *TRM1*, specifically the regions identified from the ADEPT alignments (Stanford *et al.,* 2000), were investigated for their role in targeting Trm1p-II to the INM. Deletion constructs were made for each of the ADEPTs of *TRM1* to determine the effect on the localization of full-length Trm1-II-GFP *in vivo*. There are several possible phenotypes for deletion of a *cis*-acting region of *TRM1*. If a region of *TRM1* is necessary for nuclear targeting, then its deletion would prevent Trm1p-II from accessing the nucleus and the protein would accumulate in the cytoplasm. If a region of Trm1p-II is necessary for INM targeting, then its deletion would release Trm1p-II from the INM into the nucleoplasm.

There are two general ways by which a *cis*-acting region of *TRM1* can be necessary for nuclear or INM targeting: 1) it may be required to interact with the import machinery to direct Trm1p-II to the nucleus or an INM binding partner to maintain Trm1p-II at the INM, or 2) removal of a region could alter the tertiary structure of the protein thereby preventing proper targeting. We must also consider the possibility that the protein may be unstable and/or the mislocalization observed could be an artifact of altering the endogenous structure of Trm1p-II.

Any sequence found to be necessary for targeting Trm1p-II to the INM must be further characterized to determine whether it is sufficient to mediate INM targeting. Ultimately, there may be several regions that function coordinately to target Trm1p-II to

the INM. We will also examine various combinations of Trm1p-II regions for their ability to target a nuclear reporter protein to the INM.

The aim of these studies was to identify a motif that mediates targeting specifically to the INM, as no sequence has yet been found for a peripheral nuclear protein. The discovery of a *cis*-acting region could then be used to search for other nuclear proteins that contain a similar motif and thus may share a similar mechanism for protein targeting.

METHODS

TRM1 ADEPT deletion constructs

The oligonucleotides used to generate the constructs and the oligonucleotide pairs and DNA templates are described in Tables 7 and 8, respectively. The following constructs, summarized in Table 9, were generated to study the role of each *TRM1* ADEPT in targeting to the INM. ADEPT#1 (amino acids 1-48) was deleted by amplifying *TRM1* from amino acid 49-570 using PCR. The endogenous translation start sites within Trm1p for Trm1p-I and Trm1p-II are located at amino acids 1 and 17, respectively. As Trm1p was amplified starting at amino acid 49, this fragment did not possess a translation start site. Therefore, the upstream oligonucleotide used for this PCR, KAS016, contained a *Sac*I site upstream of a consensus translational start sequence. Oligonucleotide KAS010 contained a *Sal*I site and annealed within the termination sequence of *TRM1* approximately 150 bps downstream of the translational STOP codon. PCR using a plasmid containing Trm1-GFP (pRS416-Trm1-GFP/pGP19; gift from G. Peng; Sikorski and Heiter, 1989) as the template and oligonucleotides KAS010 and

KAS016 produced an approximately 2466 bp fragment, containing Trm1p fused in-frame to GFP and flanked on the 5' end with *Sac*I and *Sal*I at the 3' end. This fragment was ligated into the TOPO blunt vector and transformed into *E. coli*. Candidates were digested with *Sac*I and *Sal*I and the Trm1-II-GFP fragment was inserted into the pEMBLex4 vector containing the *GAL10*/*CYC1* promoter as the *TRM1* promoter was absent.

ADEPT#2 (amino acids 73-151) was deleted from Trm1-II-GFP using overlap extension PCR (Ho *et al*., 1989) and pGP19 as the template. The upstream *TRM1* fragment was amplified with oligonucleotides TRM5B and KAS036. The downstream *TRM1* fragment was amplified with oligonucleotides KAS037 and TRM3B. These fragments were annealed via complementary overlapping sequences and amplified in a second round of PCR using oligonucleotides TRM5B and TRM3B. The final fragment contained *TRM1* with its endogenous promoter, amino acids 1-72, amino acids 95-102 (NLS#1), amino acids 152-570, GFP, and *TRM1* termination sequences approximately 150 bps downstream of the translational STOP codon. The restriction sites were *Xba*I at the 5' end and *Hind*III at the 3' end. This PCR fragment was inserted into the TOPO blunt vector and digested with *Xba*I and *Hind*III to isolate the Trm1-II-GFP fragment for ligation in both pRS416 and pRS426 (Sikorski and Heiter, 1989; Christianson *et al.,* 1992).

ADEPT#3 was deleted by overlap extension PCR. The upstream *TRM1* fragment was amplified with oligonucleotides KAS007 and KAS005. The downstream *TRM1* fragment was amplified with oligonucleotides KAS006 and KAS010. These PCR products were annealed via their complementary overlapping sequences and extended in

Table 7: Sequence of oligonucleotides used for amplification of *TRM1* ADEPT deletion fragments.

Name	Oligonucleotide sequence
KAS003	5-TTCTCGAGATTTCTGCATCACATACCAGATTGCATC-3
KAS004	5-ATGCAGAAATCTCGAGAAATGTCTAAAGGTGAAGAA-3
KAS005	5-AGTATCAACAGAAATTCTGCCCAGAGGTTGATT-3
KAS006	5-AGAATTTCTGTTGATACTAAATGTAAGTTCTGC-3
KAS007	5-AAGCTTGCCGCTTTGCTGTGGCACTTC-3
KAS009	5-CTCGAGACTGCCCTCCTGATTACTTTA-3
KAS010	5-GTCGACACTGCCCTCCTGATTACTTTA-3
KAS016	5-GAGCTCAAAATGTCTAAAGCAGAAATTCTTTTCCCT-3
KAS036	5-AAGAAGTCAAAAAAAAAGCGTTGTCATGAAATTCCCCATGT GAGGGAAGTTATT-3
KAS037	5-ACAACGCTTTTTTTTTGACTTCTTTAGATCTCTATTAAATTGT TGGATGGGATT-3
KAS038	5-TCTAGAATGTTGAAGGCTGCTATATCCA-3
KAS045	5-GAATCAACTCACGAAAGTGCGCTG-3
KAS046	5-CAGCGCACTTTCGTGAGTTGATTC-3
ADT4-1	5-ATGCAGAAATCATAAGAATTTTGATTAGTGTTAGAG-3
ADT4-2	5-TTCTTATGATTTCTGCATCACATACCAGATTGCATC-3
TRM3B	5-ACTTAAGCTTACTGCCCTCCTGATTAC-3

Table 8: Oligonucleotide pairs and DNA templates used to delete *TRM1* ADEPT fragments.

PCR product	Oligonucleotides	Template	Template Source
ADEPT#1Δ-GFP	KAS10/16	pGP19	G. Peng
ADEPT#4Δ-GFP	KAS3/7	pGP19	G. Peng
ADEPT#4Δ-GFP	KAS4/10	pGP19	G. Peng
ADEPT#4Δ-GFP	KAS7/10	KAS3/7, KAS4/10 PCR products	De Novo
ADEPT#3Δ-GFP	KAS5/7	pGP19	G. Peng
ADEPT#3Δ-GFP	KAS6/10	pGP19	G. Peng
ADEPT#3Δ-GFP	KAS7/10	KAS5/7, KAS6/10 PCR products	De Novo
ADEPT#2Δ-GFP	KAS7/37	pGP19	G. Peng
ADEPT#2Δ-GFP	KAS9/36	pGP19	G. Peng
ADEPT#2Δ-GFP	KAS7/9	KAS7/37, KAS9/36 PCR products	De Novo
Trm1mtNLS#1-GFP	KAS45/TRM3B	pGP19	G. Peng
Trm1mtNLS#1-GFP	KAS46/38	pYAR5	Rose *et al.,* 1992
Trm1mtNLS#1-GFP	KAS38/TRM3B	KAS45/TRM3B, KAS46/38 PCR products	De Novo

Table 9: Plasmids generated for the *TRM1* ADEPT deletion experiments and the fusion proteins encoded.

Plasmid	Encoded Fusion Protein
Trm1ΔADEPT#1-GFP	constitutive promoter (*CYC1*) and galactose-inducible promoter (*GAL10*) + TRM1 (amino acids 49-570) + GFP
Trm1ΔADEPT#2-GFP	*TRM1* promoter + *TRM1* (amino acids 1-72, 95-102, 152-570) + GFP
Trm1ΔADEPT#3-GFP	*TRM1* promoter + *TRM1* (amino acids 1-346, 368-570) + GFP
Trm1ΔADEPT#4-GFP	*TRM1* promoter + *TRM1* (amino acids 1-491) + GFP
Trm1mtNLS#1-GFP	constitutive promoter (*CYC1*) and galactose-inducible promoter (*GAL10*) + *TRM1* (amino acids 18-570) + GFP

a second round of PCR with oligonucleotides KAS007 and KAS010. The final *TRM1* fragment contained the endogenous *TRM1* promoter, amino acids 1-345, 368-570, GFP, and *TRM1* termination sequences approximately 150 bps downstream of the translational STOP codon flanked by *Hind*III at the 5' end and *Sal*I at the 3' end. This fragment was inserted into the TOPO blunt vector for digestion with *Hind*III and *Sal*I to isolate the Trm1-II-GFP fragment for ligation with pRS416 and pRS426 (Sikorski and Heiter, 1989; Christianson *et al.,* 1992).

ADEPT#4 was deleted by overlap extension PCR. The upstream *TRM1* fragment was amplified using oligonucleotides KAS007 and KAS003. The downstream *TRM1* fragment was amplified with oligonucleotides KAS004 and KAS010. These PCR fragments were annealed via their complementary overlapping sequences and extended in a second round of PCR using oligonucleotides KAS007 and KAS010. This fragment, containing the *TRM1* promoter, amino acids 1-491, GFP, and *TRM1* termination sequences approximately 150 bps downstream of the translational STOP codon, was ligated into the TOPO blunt vector and digested with *Hind*III and *Sal*I to isolate the Trm1-II-GFP fragment. This fragment was inserted into pRS416 and pRS426 (Sikorski and Heiter, 1989; Christianson *et al.,* 1992).

All plasmids were subsequently transformed into wild-type *S. cerevisiae* strain, BY4741, using the one-step yeast transformation protocol described in Chapter 2.

TRM1 ADEPTs in pFB1-67u

The following constructs were generated to identify *TRM1* ADEPT regions sufficient to target a reporter protein to the INM. The oligonucleotides used to generate

the constructs and the oligonucleotide pairs and DNA templates are described in Tables 10 and 11, respectively. A summary of the amino acids contained in each construct is presented in Table 12. A schematic of the pFB1-67u vector is presented in Fig. 16. ADEPT#1 was amplified with oligonucleotides KAS023 and KAS024 and flanked with *Bgl*II sites for insertion into pFB1-67u+ADEPT#3. *Bam*HI-ADEPT#3-*Bgl*II-*Bam*HI was digested with *Bgl*II and ADEPT#1 was inserted downstream of ADEPT#3 in pFB1-67u. ADEPT#2 (amino acids 73-151) was amplified by KAS021 and KAS022, producing an approximately 250 bp PCR fragment with flanking *Bgl*II sites. Amino acids 73-108 were amplified by oligonucleotides KAS025 and KAS026, producing an approximately 120 bp PCR fragment with flanking *Bam*HI sites. Amino acids 89-151 were amplified by oligonucleotides KAS027 and KAS028, producing an approximately 200 bp PCR fragment with flanking *Bam*HI sites. All ADEPT#2 fragments were cloned into the unique *Bam*HI site in pFB1-7u (schematic shown in Chapter 3), since these ADEPT fragments of Trm1p possess an NLS.

All plasmids were transformed into the *S. cerevisiae* strain, BY4741, using the one-step yeast transformation protocol described in Chapter 2, followed by localization of the fusion proteins by indirect immunofluorescence.

Indirect Immunofluorescence

Indirect immunofluorescence was performed using the procedure of Pringle *et al*. (1990) described in Chapter 2, to localize the β-galactosidase fusion proteins. Cells were fixed for approximately 45 min to optimize binding of the primary antibody, affinity-

Table 10: Sequence of oligonucleotides used for amplification of *TRM1* ADEPT fragments.

Name	Oligonucleotide sequence
ADT3-1	5-GGATCCAGTACTATGACTACTTACCAT-3
ADT3-3	5-GGATCCAGATCTCCCCTCGCAGAACTTACATTT-3
ADT4-3	5-AGATCTTGTGATGATGAGAAGAAAGAC-3
ADT4-4	5-AGATCTTGAAGTGTTGGGACGGGCTTT-3
KAS023	5-AGATCTATGTTGAAGGCTGCTATATC-3
KAS024	5-AGATCTTCCTTCCTTGACTATATTGA-3
KAS021	5-AGATCTAGTGTTACATGCATCAAGGCG-3
KAS022	5-AGATCTAGCATACCTAATGGCTCTTAA-3
KAS025	5-CGCGGATCCAGTGTTACATGCATCAAG-3
KAS026	5-CGCGGATCCATCATCGTTAGTTTCCGC-3
KAS027	5-CGCGGATCCGGCCAAAAGAGAAATAAT-3
KAS028	5-CGCGGATCCAGCATACCTAATGGCTCT-3

Table 11: Oligonucleotide pairs and DNA templates used to amplify *TRM1* ADEPT fragments for identifying *cis*-acting sequences sufficient for inner nuclear membrane targeting.

PCR product	Oligonucleotides	Template	Template Source
ADEPT#3	ADT3-1/3-3	pGP19	G. Peng
ADEPT#4	ADT4-3/4-4	pGP19	G. Peng
ADEPT#1	KAS23/24	pGP19	G. Peng
ADEPT#2	KAS21/22	pGP19	G. Peng
ADEPT#2mtNLS	KAS21/22	pYAR5	Rose *et al*., 1992
N-terminal ADEPT#2 (amino acids 73-108)	KAS25/26	pGP19	G. Peng
C-terminal ADEPT#2 (amino acids 89-151)	KAS27/28	pGP19	G. Peng

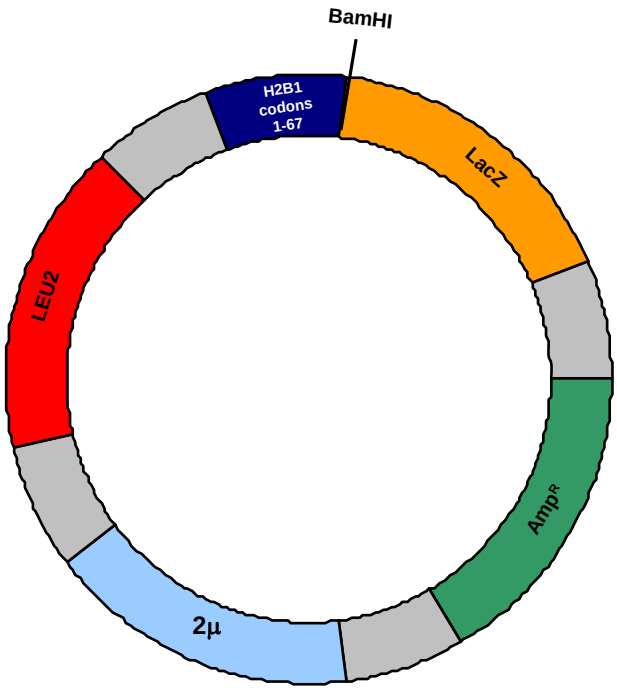
Table 12: Plasmids generated for identifying *cis*-acting sequences sufficient for inner nuclear membrane targeting and the fusion proteins encoded.

Plasmid	Encoded Fusion Protein
pFB1-67u	Histone H2B amino acids 1-67 fused to β-galactosidase
pFB1-67u+ ADEPT#3	Trm1p amino acids 323-375 inserted in Histone H2B amino acids 1- 67 fused to β-galactosidase
pFB1-67u+ ADEPT#4	Trm1p amino acids 492-570 inserted in Histone H2B amino acids 1- 67 fused to β-galactosidase
pFB1-67u+ ADEPT#3/ADEPT#4	Trm1p amino acids 323-375/492-570 inserted in Histone H2B amino acids 1-67 fused to β-galactosidase
pFB1-67u+ ADEPT#1/ADEPT#3	Trm1p amino acids 1-48/323-375 inserted in Histone H2B amino acids 1-67 fused to β-galactosidase
pFB1-7u+ ADEPT#2	Trm1p amino acids 73-151 inserted in Histone H2B amino acids 1-14 fused to β-galactosidase
pFB1-7u+ ADEPT#2 (mtNLS#1)	Trm1p amino acids 73-151 inserted in Histone H2B amino acids 1-14 fused to β-galactosidase
pFB1-7u+ amino acids 73-108	Trm1p amino acids 73-108 inserted in Histone H2B amino acids 1-14 fused to β-galactosidase
pFB1-7u+ amino acids 89-151	Trm1p amino acids 89-151 inserted in Histone H2B amino acids 1-14 fused to β-galactosidase

Fig. 16: Schematic of the pFB1-67u vector.

pFB1-67u contains 67 amino acids of histone H2B, including its strong nuclear localization sequence (NLS), fused in frame with *E. coli LacZ* resulting in a β-galactosidase fusion protein that accumulates strongly in the nucleus.

Figure adapted from: Moreland RB, Langevin GL, Singer RH, Garcea RL, Hereford LM. Amino acid sequences that determine the nuclear localization of yeast histone 2B. Mol Cell Biol. 1987; 7:4048-4057.



purified rabbit polyclonal anti-β-galactosidase antibody (Hopper *et al.,* 1990), used at 1:400 or 1:1000 dilution. The secondary antibody was Cy3-conjugated goat anti-rabbit IgG diluted 1:400.

RESULTS

ADEPT#1 and ADEPT#3 are both necessary for INM targeting of Trm1-II-GFP

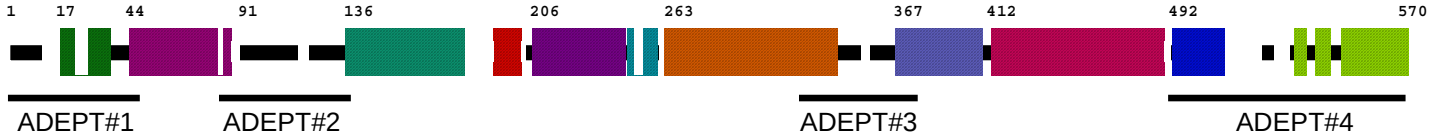
To identify regions necessary for INM localization of Trm1p-II, we deleted the various ADEPT sequences implicated in targeting from Trm1-II-GFP and assessed the location of the resulting mutant proteins. Wild-type Trm1-II-GFP localizes to the INM in both live and fixed cells (Fig. 17 a and b; and Murthi and Hopper, 2005). We anticipated finding regions involved in targeting to the nucleus, which upon their deletion would result in mislocalization of the Trm1-II-GFP protein from the nucleus to the cytoplasm. In addition, we anticipated identifying regions of Trm1p involved in INM targeting, which when deleted, would result in mislocalization of the Trm1-II-GFP protein from the INM into the nucleoplasm.

Removing the first 48 amino acids of Trm1p caused the release of Trm1-II-GFP from the INM into the nucleoplasm indicating that ADEPT#1 was necessary for maintaining Trm1p at the INM (Fig. 17 c and d). Deletion of ADEPT#2 caused accumulation of Trm1-II-GFP in the cytoplasm, even though the classical NLS within ADEPT#2 and the putative NLS within ADEPT#4 were conserved (Fig. 17 g and h). Perhaps deletion of the amino acids surrounding NLS#1 (amino acids 95-102) altered the structure of the protein such that NLS#1 within ADEPT#2 was no longer exposed on the surface of the protein, thereby preventing its interaction with its karyopherin and

preventing import to the nucleus. Alternatively, the Trm1-II-GFP deletion protein may be unstable; unfortunately, western analysis has not been carried out to evaluate this mutant protein. This result also suggested that the putative NLS within ADEPT#4 (amino acids 544-552) likely does not function, at all or efficiently under these conditions, in targeting Trm1p-II to the nucleus. Trm1-II-GFP with a mutant NLS#1 also accumulated in the cytoplasm (Fig. 17 e and f), similar to the localization observed previously by Rose *et al.* (1992) by indirect immunofluorescence using a anti-Trm1p antibody. Trm1ΔADEPT#2-GFP also mislocalized to the cytoplasm, again supporting the hypothesis that the putative NLS within ADEPT#4 does not function under these conditions. Deletion of ADEPT#3 caused release of Trm1-II-GFP from the INM into the nucleoplasm (Fig. 17 i and j) suggesting that this region was also necessary for maintaining Trm1p at the INM. Deletion of ADEPT#4 had no effect on the localization of Trm1-II-GFP as this fusion protein was maintained at the INM (Fig. 17 k and l). Since removal of ADEPT#4 did not impair nuclear import of Trm1-II-GFP at a detectable level, this again suggests that the putative NLS within ADEPT#4 may not function in the endogenous protein or under the conditions tested here. However, these experiments showed that deleting either ADEPT#1 or ADEPT#3 from Trm1-II-GFP caused the fusion protein to accumulate in the nucleoplasm, indicating these regions are necessary for INM localization of Trm1p.

Western analysis of the various ADEPT deletion proteins showed a significant band above the 83 kDa marker, which corresponds to the expected size of these GFP fusion proteins (Fig. 18). Wild-type Trm1-II-GFP, both ADEPT#3Δ-GFP fusion proteins,

Fig 17: Subcellular localization of various Trm1-II-GFP fusion proteins containing the deletion of individual ADEPTs and transformed in the BY4741 strain. Trm1-II-GFP fusion proteins were localized by autofluorescence in live cells. (a, b) wild-type Trm1-II-GFP (c, d) Trm1ΔADEPT#1-GFP (e, f) Trm1mtNLS#1-GFP (g, h) Trm1ΔADEPT#2-GFP (i, j) Trm1ΔADEPT#3-GFP (k, l) Trm1ΔADEPT#4-GFP.


1
17
44
91
136
206
263
367
412
492
570
ADEPT#1
ADEPT#2
ADEPT#3
ADEPT#4

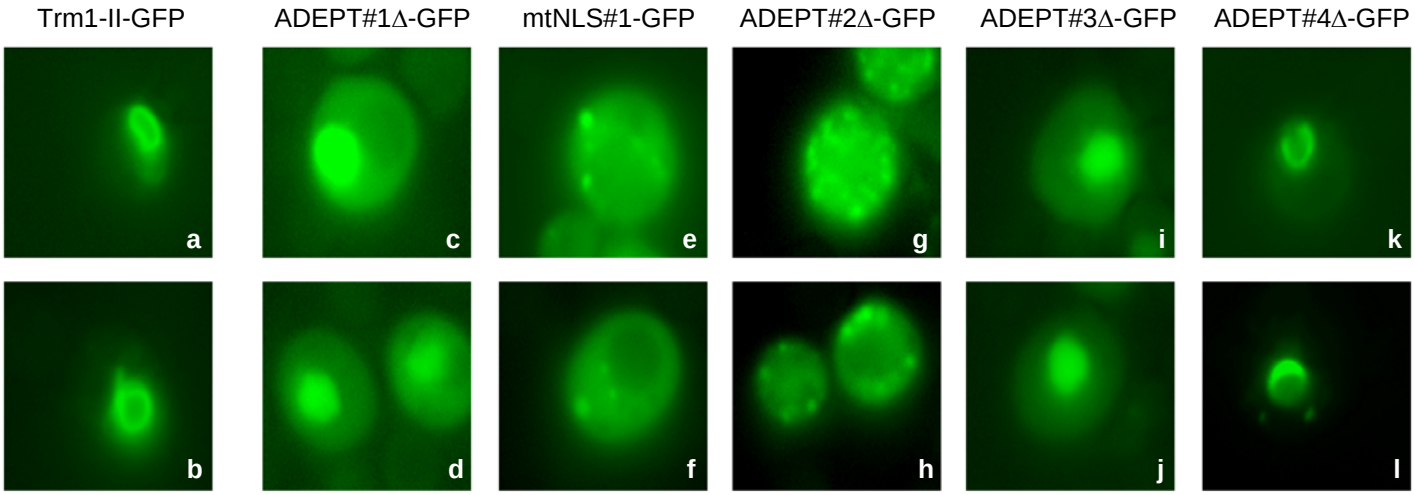

Trm1-II-GFP
ADEPT#1Δ-GFP
mtNLS#1-GFP
ADEPT#2Δ-GFP
ADEPT#3Δ-GFP
ADEPT#4Δ-GFP
a
b
c
d
e
f
g
h
i
j
k
l

Fig.18: Western analysis of Trm1-II-GFP fusion proteins containing the deletion of individual ADEPTs.

10% polyacrylamide gel containing 15 ug protein/well.

1- pRS426

2- Trm1-GFP (pRS426)

3-Trm1ΔADEPT#3-GFP (pRS426)

4-Trm1p (pRS426)

5-Trm1ΔADEPT#4-GFP (pRS426)

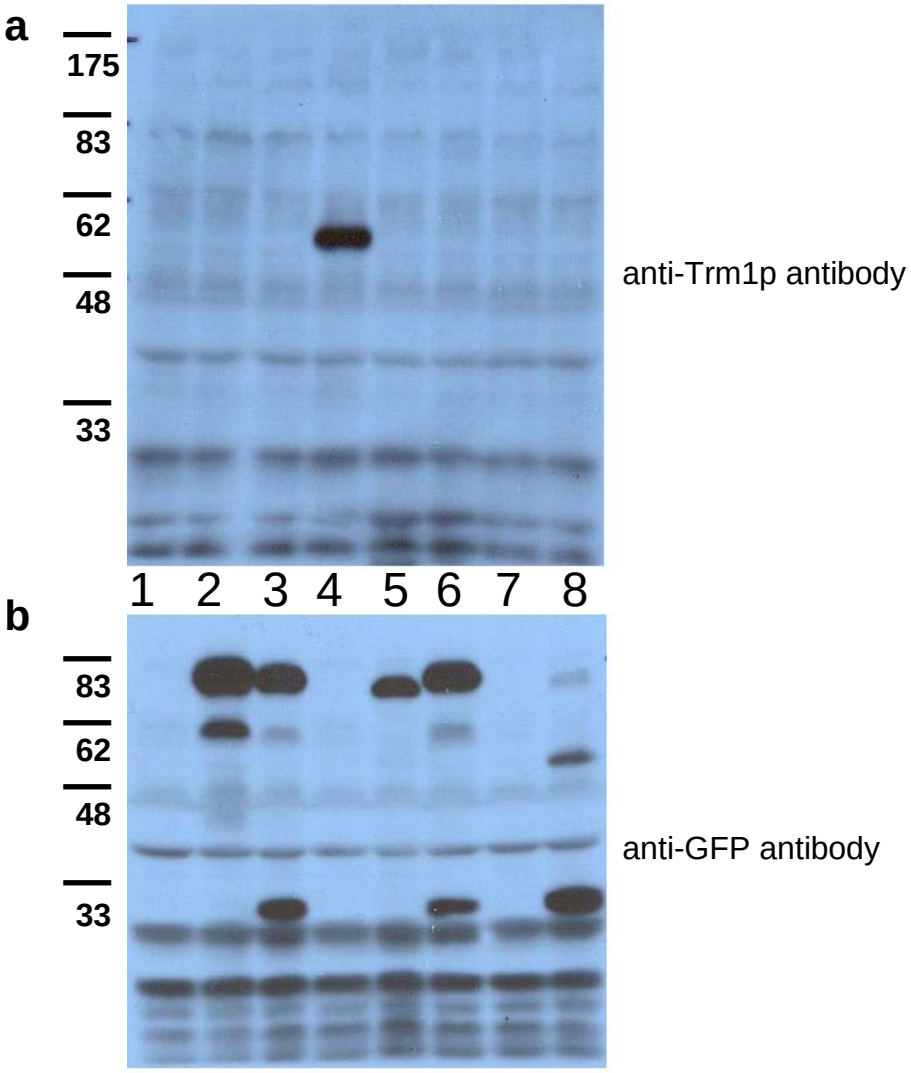
6- Trm1ΔADEPT#3-GFP (pRS426)

7- pEMBLyex4

8-Trm1ΔADEPT#1-GFP (pEMBLyex4)

a: anti-Trm1p antibody/BY4741 cells

b: anti-GFP antibody/BY4741 cells

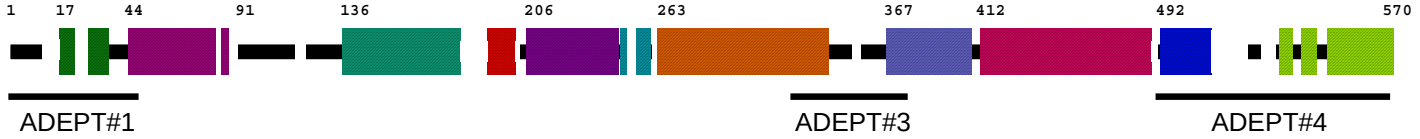

a
175
83
62
48
33
anti-Trm1p antibody
1 2 3 4 5 6 7 8
b
83
62
48
33
anti-GFP antibody

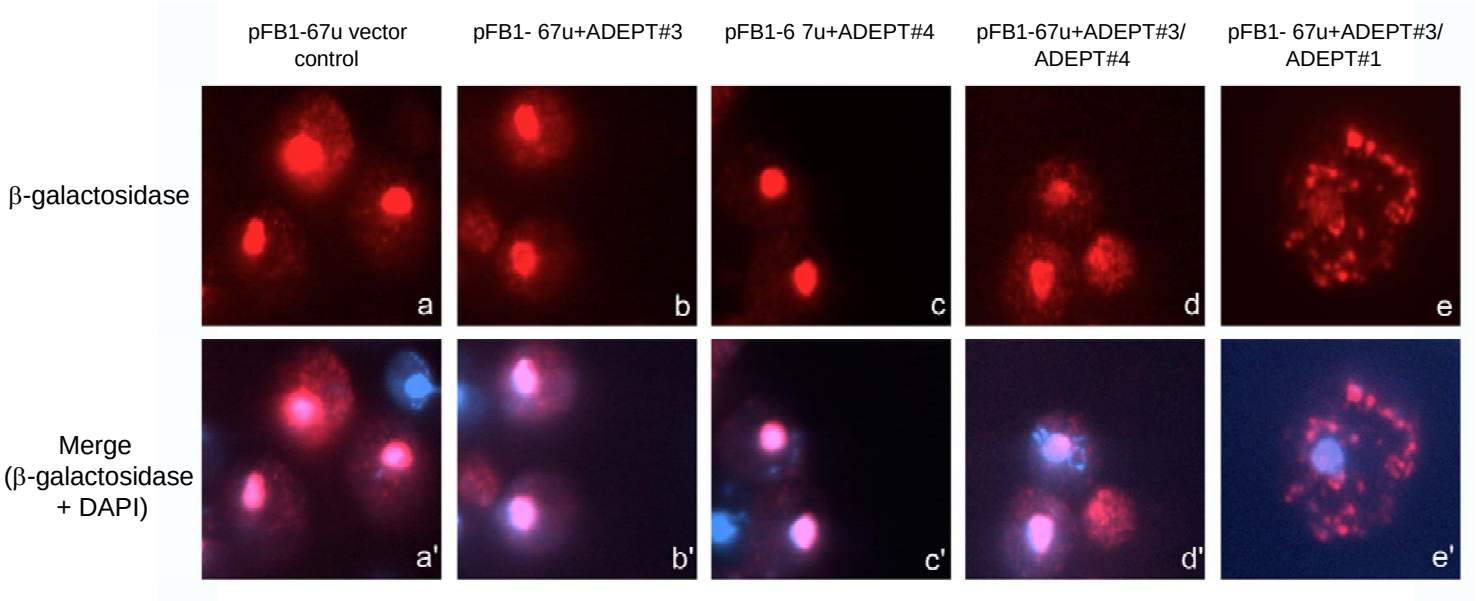
and the ADEPT#4Δ-GFP fusion protein showed bands of approximately equal size. For the ADEPT#1Δ-GFP protein, a portion of the pool ran at the expected size, while the majority of the protein ran slightly above the 48 kDa and slightly below the 33 kDa markers, suggesting this protein was unstable and degraded *in vivo* (Fig. 18).

ADEPT#1, ADEPT#3, and ADEPT#1+ADEPT#3 are not sufficient to target a β-galactosidase fusion protein to the INM

To identify which *cis*-acting regions of *TRM1* are sufficient for INM targeting, each of the ADEPTs was inserted in pFB1-67u for localization of the Trm1-β-galactosidase fusion protein by indirect immunofluorescence. pFB1-67u contains 67 amino acids of histone H2B, including its strong NLS, fused in frame with *E. coli LacZ* resulting in a β-galactosidase fusion protein that accumulates strongly in the nucleus (Moreland *et al.*, 1987). As this plasmid produces a protein that normally localizes to the nucleoplasm, we were looking for a fragment of *TRM1* capable of shifting the localization of the resulting fusion protein from the nucleoplasm to the INM. Plasmids expressing ADEPT#3 (amino acids 323-375)-β-galactosidase, ADEPT#4 (amino acids 492-570)-β-galactosidase and ADEPT#3+ADEPT#4 (amino acids 323-375, 492-570)-β-galactosidase were all transformed into BY4741 and indirect immunofluorescence using an affinity-purified rabbit polyclonal anti-β-galactosidase antibody (Hopper *et al.,* 1990) revealed all three fusion proteins were localized in the nucleoplasm (Fig. 19 b and b', c and c', d and d', respectively) indicating none of these *cis*-acting regions, on their own or in combination, was sufficient to target a nuclear protein to the INM.

Fig. 19: Subcellular localization of pFB1-67u constructs containing various Trm1p ADEPT fragments transformed in the BY4741 strain. Trm1-β-galactosidase fusion proteins were localized by indirect immunofluorescence using affinity-purified rabbit polyclonal anti-β-galactosidase primary antibody (Hopper *et al.,* 1990) and Cy3-conjugated goat anti-rabbit IgG secondary antibody, each diluted 1:400. DAPI was used to detect DNA. (a) Cy3 image of pFB1-67u vector only control (a') Cy3 and DAPI merge of pFB1-67u vector only control (b) Cy3 image of pFB1-67u+ADEPT#3 (amino acids 323-375) (b') Cy3 and DAPI merge of pFB1-67u+ADEPT#3 (c) Cy3 image of pFB1-67u+ADEPT#4 (amino acids 492-570) (c') Cy3 and DAPI merge of pFB1-67u+ ADEPT#4 (d) Cy3 image of pFB1-67u+ADEPT#3+ADEPT#4 (amino acids 323-375, 492-570) (d') Cy3 and DAPI merge of pFB1-67u+ADEPT#3+ADEPT#4 (e) Cy3 image of pFB1-67u+ ADEPT#3+ADEPT#1 (amino acids 323-375, 1-48) (e') Cy3 and DAPI merge of pFB1-67u+ADEPT#3+ADEPT#1.


1
17
44
91
136
206
263
367
412
492
570
ADEPT#1
ADEPT#3
ADEPT#4


pFB1-67u vector control
pFB1- 67u+ADEPT#3
pFB1-6 7u+ADEPT#4
pFB1-67u+ADEPT#3/ ADEPT#4
pFB1- 67u+ADEPT#3/ ADEPT#1
β-galactosidase
Merge (β-galactosidase + DAPI)
a
b
c
d
e
a'
b'
c'
d'
e'

Since ADEPT#1 (amino acids 1-48) and ADEPT#3 (amino acids 347-365) were each necessary for INM targeting of Trm1-II-GFP, as shown by the deletion experiments (Fig. 17 c, d and i, j, respectively), these regions were inserted in pFB1-67u in combination to determine the localization of a fusion protein containing both *cis*-acting regions necessary for targeting to the INM. Surprisingly, this fusion protein was present both in the nucleoplasm, as a result of the strong NLS within pFB1-67u, and in the cytoplasm (Fig. 19 e and e'). Within the cytoplasm, this fusion protein was localized in discrete patches, suggesting binding to membranes, possibly that of the mitochondria, perhaps as a result of the mitochondrial targeting signal within ADEPT#1 of Trm1p. This result was surprising because mitochondrial targeting sequences are generally located at the N-terminus of a protein and displacing to an internal region often prohibits efficient targeting to this organelle. However, there are exceptions, as Pfanner *et al.* (1987) showed sequences internal to a protein could function as efficiently for mitochondrial targeting as those restricted to the N-terminus. Although the mitochondrial targeting signal of Trm1p-I is located at the N-terminus in the endogenous protein, the possible targeting of the ADEPT#3+ADEPT#1-β-galactosidase fusion protein, with an internal mitochondrial targeting signal, to the mitochondria suggests that this sequence need not be restricted to the N-terminus for function. However, further experiments would need to be carried out to confirm this fusion protein localizes to the mitochondria. Colocalization studies using an antibody against a resident ER protein, such as Kar2p, may help address the localization of the ADEPT#3+ADEPT#1-β-galactosidase fusion protein.

ADEPT#2 is sufficient to target β-galactosidase to the INM

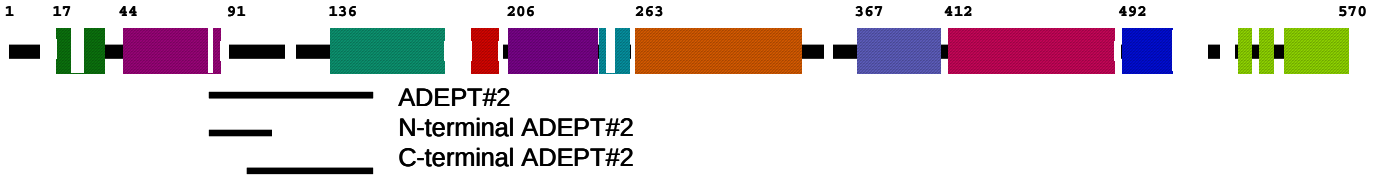
In contrast to the results for ADEPT#3, ADEPT#4, ADEPT#3+ADEPT#4, and ADEPT#3+ADEPT#1, ADEPT#2 (amino acids 73-151) was capable of targeting β-galactosidase to the INM (Fig. 20 b and b'). Although this fusion protein targeted to the INM, there were distinct differences in its localization compared to: 1) full-length Trm1-II-GFP viewed by autofluorescence, 2) Trm1-GFP viewed by indirect immunofluorescence using an anti-GFP antibody, and 3) endogenous Trm1p viewed by indirect immunofluorescence using an anti-Trm1p antibody (Rose *et al.,* 1992). Trm1-II-GFP located continuously at the INM forming ring-like structures in the majority of cells (Fig. 17 a and b). In contrast, the amino acids 73-151-β-galactosidase fusion protein was often not distributed entirely around the INM, but rather limited to approximately 1/3 or 1/2 of the INM, forming a crescent or half-moon structure in the majority of cells (Fig. 20 b and b'). Perhaps the Trm1 (amino acids 73-151)-β-galactosidase fusion protein lacked additional information required for spreading the protein throughout the entire INM. It would be interesting to elucidate what *cis*- and/or *trans*-acting factors are involved in the spreading of Trm1p around the entire INM. Further, it would be interesting to determine if Trm1p-II targets to a specific site at the INM and subsequently spreads from there as well as elucidating what may be preventing the spreading of the fusion proteins. Initial studies to define the initial targeting location within the INM for Trm1p utilizing the Trm1 (amino acids 73-151)-β-galactosidase fusion protein have not provided definitive results.

To further delineate the amino acids responsible for INM targeting, the initial amino acids region of Trm1p (73-151) was subdivided into amino acids 73-108 and

amino acids 89-151. The fragments overlapped at the NLS within ADEPT#2 (amino acids 95-102) to ensure that the fusion proteins targeted to the nucleus efficiently (and via the same import pathway). The Trm1 (amino acids 73-108)-β-galactosidase fusion protein located in the nucleoplasm (Fig. 20 c and c') while the Trm1 (amino acids 89-151)-β-galactosidase fusion protein was located at the INM (Fig. 20 d and d'). To determine whether the NLS (amino acids 95-102) within the region was involved in INM targeting, the 73-151 amino acid region of Trm1p was amplified from pYAR5, a vector which possesses *TRM1* containing a mutant NLS#1 (Rose *et al.,* 1992). In pYAR5, the five basic amino acids within the Trm1p NLS (amino acids 95-102) were changed to acidic amino acids; this was shown to result in mislocalization of the protein from the INM to the cytoplasm. The ADEPT#2 sequence (amino acids 73-151) with a nonfunctional NLS was subsequently inserted into pFB1-67u, which provided an ectopic NLS from histone H2B (Moreland *et al.,* 1987), and generated a β-galactosidase fusion protein containing the putative INM targeting sequence of Trm1p-II. This fusion protein located at the INM (Fig. 21 b and b') indicating that the endogenous NLS (amino acids 95-102) within Trm1p-II was dispensable for INM targeting and that it is likely the surrounding amino acids (amino acids 103-151) that function to direct the fusion protein to the INM.

In a genome-wide affinity purification study, Trm1p was reported to self-associate (Krogan *et al.,* 2004). It is possible then that the Trm1 (amino acids 73-151)-β-galactosidase fusion protein may target to the INM via its association with endogenous Trm1p-II present in wild-type cells. Therefore, we verified the localization of the Trm1 (amino acids 73-151)-β-galactosidase and Trm1 (amino acids 89-151)-β-galactosidase

Fig. 20: Subcellular localization of pFB1-7u constructs containing Trm1p ADEPT#2 fragments transformed in the BY4741 strain. Trm1-β-galactosidase fusion proteins were localized by indirect immunofluorescence using affinity-purified rabbit polyclonal anti-β-galactosidase primary antibody (Hopper *et al.,* 1990) and Cy3-conjugated goat anti-rabbit IgG secondary antibody, each diluted 1:400. DAPI was used to detect DNA. (a) Cy3 image of pFB1-7u vector only control (a') Cy3 and DAPI merge of pFB1-7u vector only control (b) Cy3 image of pFB1-7u+ADEPT#2 (amino acids 73-151) (b') Cy3 and DAPI merge of pFB1-7u+ADEPT#2 (c) Cy3 image of pFB1-7u+N-terminal ADEPT#2 (amino acids 73-108) (c') Cy3 and DAPI merge of pFB1-7u+N-terminal ADEPT#2 (d) Cy3 image of pFB1-7u+C-terminal ADEPT#2 (amino acids 89-151) (d') Cy3 and DAPI merge of pFB1-7u+C-terminal ADEPT#2.


1
17
44
91
136
206
263
367
412
492
570
ADEPT#2
N-terminal ADEPT#2
C-terminal ADEPT#2

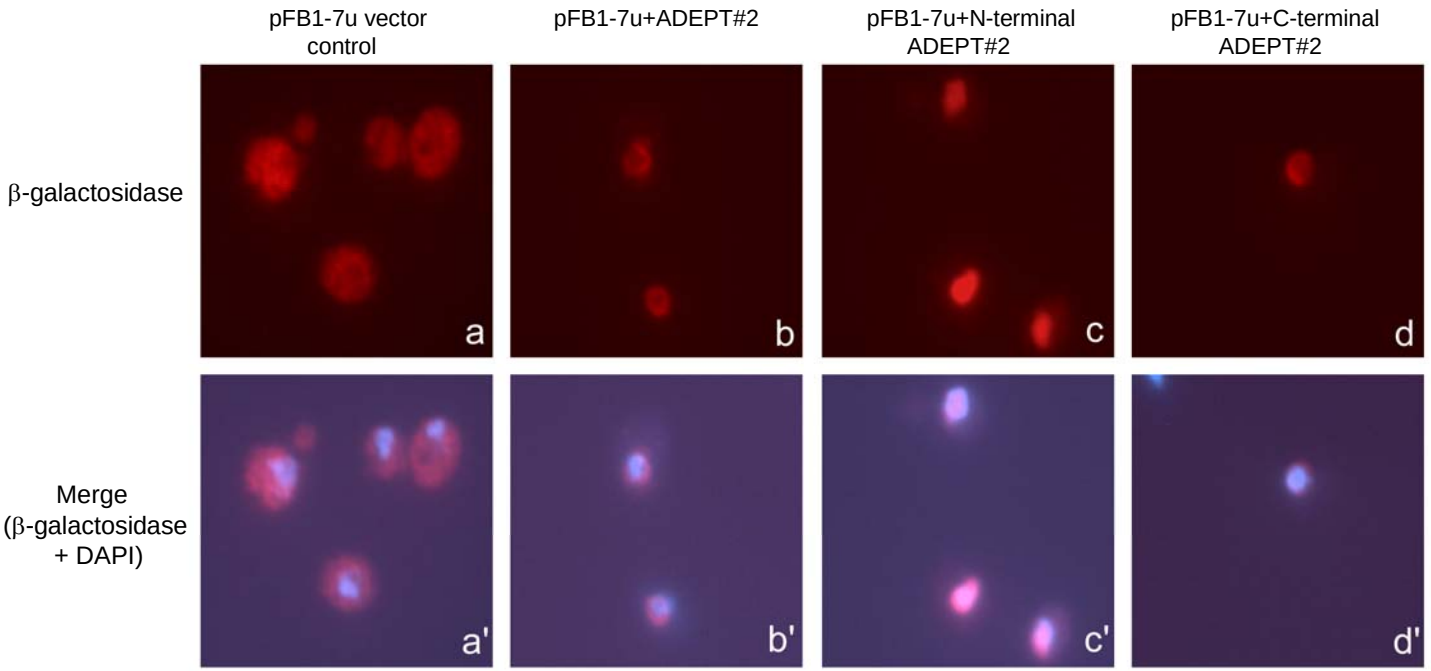
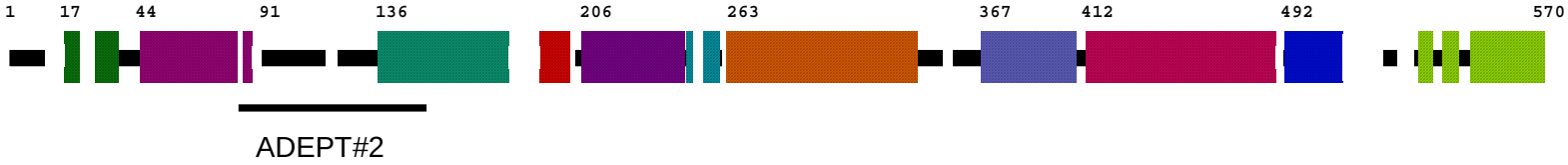
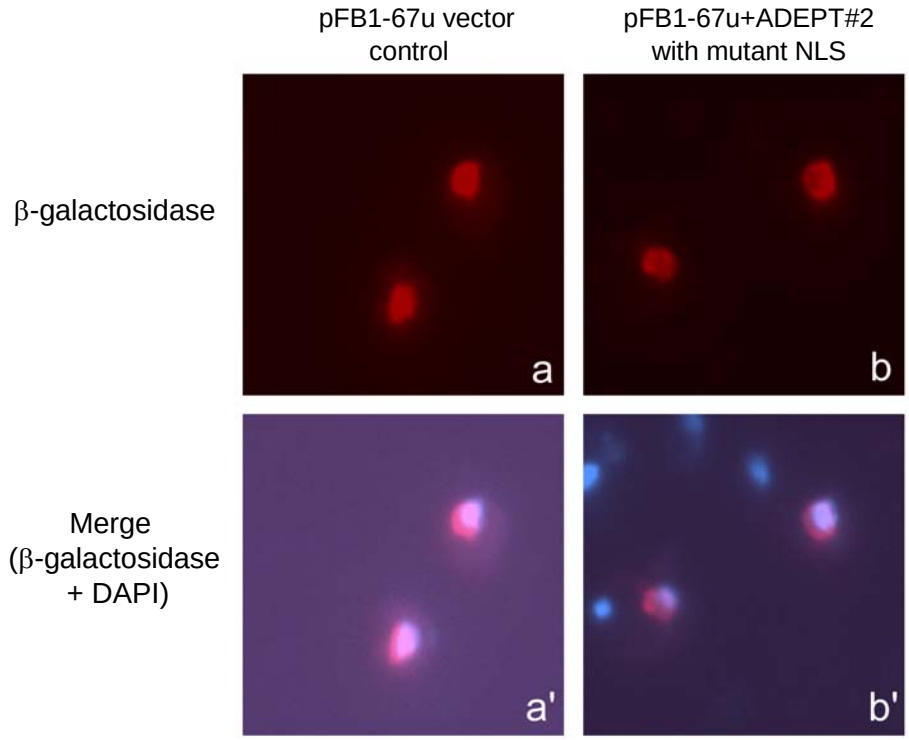

pFB1-7u vector control
pFB1-7u+ADEPT#2
pFB1-7u+N-terminal ADEPT#2
pFB1-7u+C-terminal ADEPT#2
β-galactosidase
Merge (β-galactosidase + DAPI)
a
b
c
d
a'
b'
c'
d'

Fig. 21: Subcellular localization of a pFB1-67u construct containing Trm1p ADEPT#2 with a mutant NLS transformed in the BY4741 strain. Trm1-β-galactosidase fusion proteins were localized by indirect immunofluorescence using affinity-purified rabbit polyclonal anti-β-galactosidase primary antibody (Hopper *et al.,* 1990) and Cy3-conjugated goat anti-rabbit IgG secondary antibody, each diluted 1:400. DAPI was used to detect DNA. (a) Cy3 image of pFB1-67u vector only control (a') Cy3 and DAPI merge of pFB1-67u vector only control (b) Cy3 image of pFB1-67u+ ADEPT#2mtNLS#1 (amino acids 73-151) (b') Cy3 and DAPI merge of pFB1-67u+ ADEPT#2mtNLS#1.


1
17
44
91
136
206
263
367
412
492
570
ADEPT#2


pFB1-67u vector control
pFB1-67u+ADEPT#2 with mutant NLS
β-galactosidase
Merge (β-galactosidase + DAPI)
a
b
a'
b'

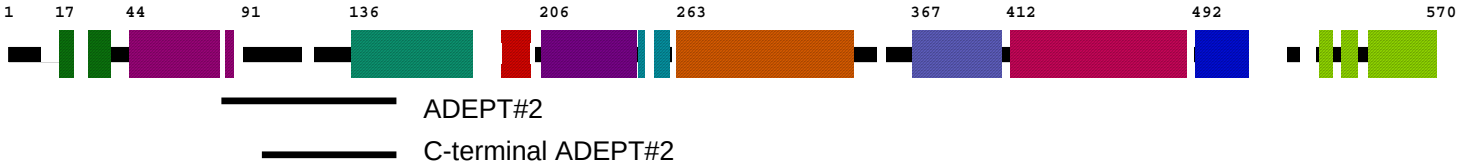
fusion proteins in *trm1* cells (Winzeler *et al.,* 1999). Both the Trm1 (amino acids 73-151)-β-galactosidase and Trm1 (amino acids 89-151)-β-galactosidase fusion proteins were maintained at the INM in the *trm1* mutant cells (Fig. 22 b and b', c and c', respectively) indicating this region of Trm1p-II is sufficient on its own to mediate targeting to the INM.

All results from experiments described in this chapter are summarized in Table 13.

DISCUSSION

When ADEPT#1 or ADEPT#3 was deleted from Trm1-II-GFP, the protein was released from the INM and accumulated in the nucleoplasm indicating these regions of Trm1p-II have a role in targeting the protein to the INM. For ADEPT#1, this result is consistent with previous data that indicated the N-terminal 48 amino acids of Trm1p were necessary, both for targeting to the mitochondria as well as the INM (Ellis *et al.,* 1989). However, neither ADEPT#1 nor ADEPT#3, necessary for maintaining Trm1-II-GFP at the INM by the deletion studies, were capable, alone or in combination, to target a fusion protein to the INM, indicating these regions are not sufficient for INM targeting of Trm1p-II. It is possible that removing these *cis*-acting sequences from endogenous Trm1p-II prevents their proper folding and/or interactions with additional regions of Trm1p-II, therefore preventing their function as targeting motifs. This could be a problem if ADEPT#1 and ADEPT#3 were required to physically interact with one another, as these regions were fused in tandem in the pFB1-67u vector. However, this does not

Fig. 22: Subcellular localization of a pFB1-7u construct containing Trm1p ADEPT#2 transformed in the *trm1* strain. Trm1-β-galactosidase fusion proteins were localized by indirect immunofluorescence using affinity-purified rabbit polyclonal anti-β-galactosidase primary antibody (Hopper *et al.,* 1990) and Cy3-conjugated goat anti-rabbit IgG secondary antibody, each diluted 1:400. (a) Cy3 image of pFB1-7u+ADEPT#2 (amino acids 73-151)/BY4741 (b) Cy3 image of pFB1-7u+ADEPT#2 (amino acids 73-151)/*trm1*.


1
17
44
91
136
206
263
367
412
492
570
ADEPT#2
C-terminal ADEPT#2

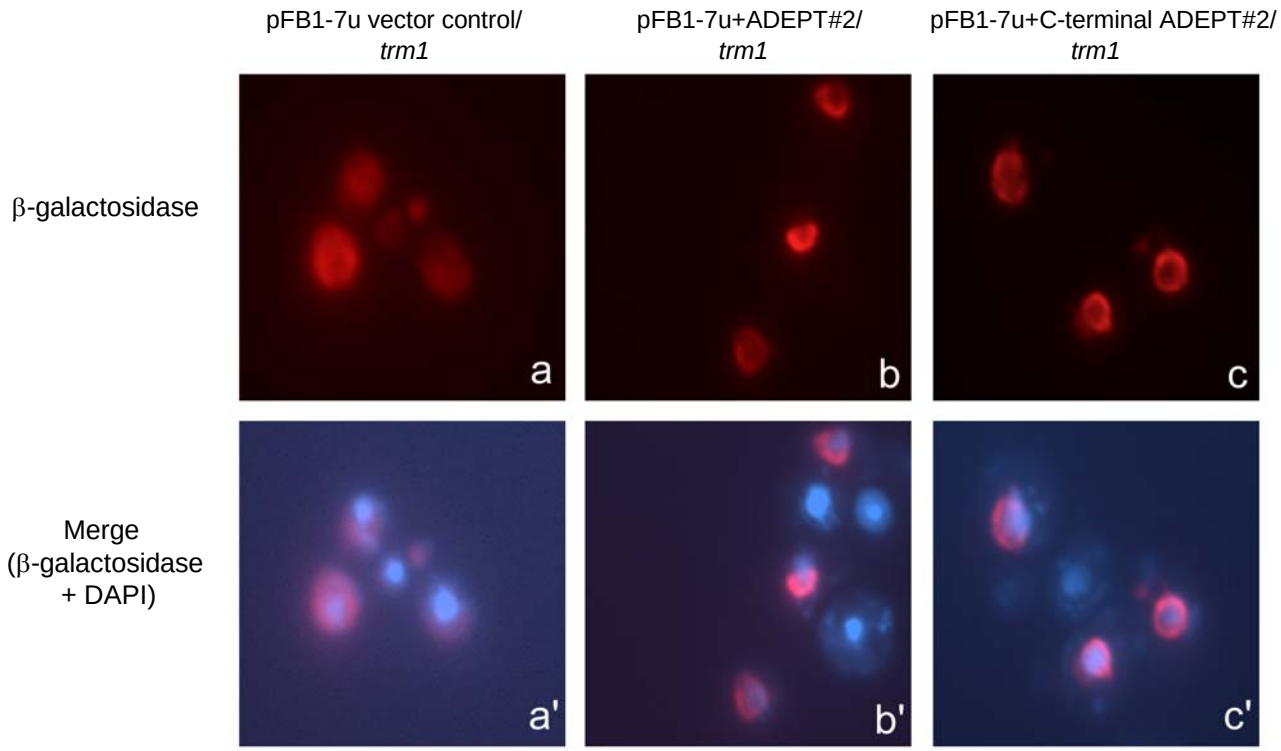

pFB1-7u vector control/
trm1
pFB1-7u+ADEPT#2/
trm1
pFB1-7u+C-terminal ADEPT#2/
trm1
β-galactosidase
Merge
(β-galactosidase
+ DAPI)
a
b
c
a'
b'
c'

Table 13: Results from all experiments reported in Chapter 4.

Construct	Independent:		Localization
	Cultures	Transformations	
Trm1-II-GFP	1	2	Nuclear rim
ADEPT#1Δ-GFP	1	2	Nuclear
mtNLS#1-GFP	1	2	Cytoplasmic
ADEPT#2Δ-GFP	1	2	Cytoplasmic
ADEPT#3Δ-GFP	1	2	Nuclear
ADEPT#4Δ-GFP	1	2	Nuclear rim
pFB1-67u vector only	1	2	Nuclear
pFB1-67u+ADEPT#3	1	2	Nuclear
pFB1-7u+ADEPT#4	1	2	Nuclear
pFB1-7u+ADEPT#3/ADEPT#4	1	2	Nuclear
pFB1-7u+ADEPT#3/ADEPT#1	1	2	Cytoplasmic (punctate, possibly membranes)
pFB1-7u	1	2	Cytoplasmic
pFB1-7u+ADEPT#2	1	2	Nuclear rim
pFB1-7u+N-terminal ADEPT#2	1	2	Nuclear
pFB1-7u+C-terminal ADEPT#2	1	2	Nuclear rim
pFB1-67u+mtNLSADEPT#2	1	2	Nuclear rim
pFB1-7u+ADEPT#2/*trm1*	1	2	Nuclear rim
pFB1-7u+C-terminal ADEPT#2/*trm1*	1	2	Nuclear rim

appear to be as we identified a single *cis*-acting region of Trm1p-II, surrounding ADEPT#2 (amino acids 89-151) capable of targeting a fusion protein to the INM. This is the first reported *cis*-acting sequence capable of conferring INM targeting of a peripheral INM protein. Preliminary BLAST searches using amino acids 89-151 of Trm1p-II did not identify this motif in other proteins, including known INM proteins. However, this analysis needs to be repeated with varying stringency for homology. Also, it is possible that INM targeting sequences share a common secondary structure(s), though no similarity in primary sequence is evident, thus explained by the lack of results from the BLAST search.

ADEPT#2 (amino acids 89-151) of Trm1p was sufficient to target β-galactosidase to the INM. However, it differed from the localization of the full length Trm1-II-GFP as it was not uniformly distributed around the entire nucleus. It often formed 'half-moon' or cap structures at the nuclear periphery, rather than a continuous pattern around the entire nucleus. Interestingly, when amino acids 73-151 of Trm1p were fused to tandem copies of EGFP (pIG vectors; Butterfield-Gerson *et al.,* 2005), a single dot structure was observed surrounding the DAPI staining, presumably on the INM, in both live and fixed cells (Shaheen and Hopper, unpublished). This suggests that: 1) there may be additional regions of Trm1p that regulate INM targeting and their absence makes targeting less efficient, or 2) targeting is a two-step process, requiring initial targeting to the INM and subsequent spreading from a central targeting site (3-step process when including initial translocation to the nuclear interior [via active transport through the central channel of the NPC]). Previous studies have been carried out to correlate these dot structures with a particular region of the nucleus, such as the nucleolus (data not shown) or the spindle

pole body (Murthi and Hopper, unpublished); however to date, no correlation has been made between the Trm1p-II dots and a particular subnuclear domain.

We showed in Chapter 3 the presence of a putative NLS within ADEPT#4. Although this putative NLS fit the first criteria for a NLS (Dingwall and Laskey, 1991), that it is capable of targeting a cytoplasmic protein, β-galactosidase to the nucleus, the role of this NLS in targeting the endogenous protein to the nucleus is unclear. One possibility was in the context of the endogenous protein, this NLS may be weak and thus no phenotype would be observed upon its deletion. When ADEPT#4 was deleted from Trm1-II-GFP, the protein was maintained in the nucleus, specifically in the ring-structures characteristic of localization at the INM, similar to the endogenous protein. Additionally, when Rose *et al.* (1992) altered NLS#1 within ADEPT#2 of Trm1p-II, the majority of the protein accumulated in the cytoplasm, rather than the nucleus. This suggests that the putative NLS within ADEPT#4 is dispensable for nuclear targeting of Trm1p-II and that nuclear targeting is predominantly, if not entirely, regulated by NLS#1 under the particular growth conditions employed. It remains possible that the putative NLS functions under different growth conditions than those tested. Interestingly, Trm1p with mutant NLS#1 was localized in discrete patches within the cytoplasm, suggesting possible membrane association (Rose *et al.,* 1992). It is possible that preventing Trm1p-II from targeting to the nucleus and binding specifically to the INM results in the binding of the protein indiscriminately to any available membrane.

The INM targeting motif is directly adjacent to NLS#1 within ADEPT#2. However, mutating the five basic residues of NLS#1 to acidic residues (Rose *et al.,* 1992) did not prevent amino acids 73-151-β-galactosidase from targeting to the INM, indicating

this region is dispensable for targeting. We showed that the N-terminal 15 amino acids are dispensable for targeting to the INM, as amino acids 89-151-β-galactosidase was just as effectively targeted to the INM as the 73-151-β-galactosidase fusion protein. Therefore, it is the residues downstream of NLS#1, from amino acids 103-151 that are sufficient for targeting to the INM. ADEPT#2, as identified in the original ADEPT analysis (Stanford *et al.,* 2000) mapped this region from amino acids 91-136. Therefore, we predict that a smaller region of ADEPT#2, corresponding to amino acids 89-136, may be the minimal sequence for INM targeting.

Using a peptide plot obtained from *Saccharomyces* Genome Database (yeastgenome.org) of the Wisconsin Sequence Analysis Package, we examined the hydrophobicity of the amino acids within ADEPT#2. ADEPT#2 contains the known NLS of Trm1p (amino acids 95-102). An NLS must be exposed on the surface of the protein in order to be recognized by the karyopherin. This NLS is enriched in basic amino acids that are hydrophilic and likely exposed on the surface of the protein as would be expected. However, the region downstream from the NLS (amino acids 130-160) appears to shift toward hydrophobic and thus may be buried within the protein. Our proposed model for Trm1p-II targeting to the INM has amino acids 89-151 of Trm1p-II interacting at the membrane with an unidentified protein or possibly a lipid. We suggest that in order for this region to be exposed for interaction with the unknown membrane protein or lipid, Trm1p may require a post-translational modification that would change its confirmation, thus exposing the proposed INM targeting motif within ADEPT#2.

Previous studies by Murthi and Hopper (2005) showed that Trm1p-II is acetylated in the cytoplasm by Nat C, an N-terminal acertyltransferase complex composed of three

subunits, Mak3p, Mak10p and Mak31p. In mutants of the individual Nat C subunits, *mak3*, *mak10,* and *mak31*, Trm1-II-GFP was not acetylated and was mislocalized from the INM to the nucleoplasm. These results suggested a role for N-terminal acetylation in targeting to the INM. Although Trm1p-II is not acetylated in *mak3* cells, it still targets to the nucleus, likely by NLS#1 (amino acids 95-102), though it is not capable of targeting/binding to the INM. Trm1p-II may require acetylation at its N-terminus to induce a conformational change thereby exposing ADEPT#2 for binding at the INM (Murthi and Hopper, 2005). By this model, the β-galactosidase fusion protein containing amino acids 89-151 was sufficient for targeting to the INM since it is liberated from the context of the endogenous protein and exposed for binding to the INM.

An independent model for targeting Trm1p-II to the INM can be proposed if we assume that amino acids 89-151 are not buried within Trm1p-II, but rather exposed on the surface due to their proximity to the exposed NLS#1. As Trm1p-II is translated in the cytoplasm, NLS#1 would be recognized by its karyopherin, likely importin β, to be imported to the nucleus. Importin β is a large protein which may effectively mask the INM targeting motif surrounding the NLS, preventing its function until Trm1p-II is targeted to the nucleoplasm and released by importin β. Evidence to support this model is that mutating NLS#1 to prevent nuclear targeting resulted in accumulation of Trm1-II-GFP in discrete patches within the cytoplasm, possibly due to membrane binding. Mutating the known NLS within Trm1p would prevent importin β from binding to Trm1p thus allowing exposure of the INM targeting motif which may mediate binding to membranes indiscriminately. It is also possible that Trm1p was targeted to the mitochondria when the NLS within ADEPT#2 was mutated as a result of the presence of

a mitochondrial targeting sequence within ADEPT#1. Furthermore, acetylation may contribute to membrane targeting. If Trm1p-II is prevented from going to the nucleus, it may bind to membranes in the cytoplasm, including those of the Golgi, ER, and mitochondria. However, when importin β binds to Trm1p-II, it prevents the acetyl group from binding to membranes in the cytoplasm, and results in targeting to the nucleoplasm (and subsequent targeting to the INM may be mediated by the INM targeting motif and/or the acetyl group). It is also possible that acetylation of Trm1p-II may contribute to its distribution around the entire INM forming the characteristic ring structures observed in both live and fixed cells.

CHAPTER FIVE

Membrane Tether

ABSTRACT

We performed competition experiments to elucidate the membrane-binding partner of Trm1p-II. We anticipated that self-competition by Trm1p-II for its membrane-binding partner would generate a nucleoplasmic pool of Trm1-II-GFP. Instead, these experiments resulted in aberrant nuclear structures and translocation of Trm1-II-GFP from the INM to the ER. By a different approach, a genome-wide study found Trm1-II-GFP was mislocalized from the INM to the nucleoplasm in *ice2* cells (Murthi and Hopper, 2005). As Murthi and Hopper (2005) proposed, Ice2p may be the membrane-binding partner of Trm1p-II or a regulator of the membrane-binding partner. Therefore, we examined the localization of the amino acids 73–151-β-galactosidase fusion protein in *ice2* cells to determine whether INM localization is disrupted. We found that, in contrast to full-length Trm1-II-GFP, the amino acids 73–151-β-galactosidase fusion protein was maintained at the INM in *ice2* cells, though enriched on one side of the INM rather than evenly distributed around the INM. This indicates Ice2p may function indirectly to target or maintain the full-length Trm1p-II at the INM. These results are not consistent with Ice2p functioning as the actual membrane tether for Trm1p-II. We suggest that Ice2p may regulate a protein required for spreading Trm1p-II around the INM, explaining why no phenotype is observed for the amino acids 73–151-β-galactosidase fusion protein in *ice2* cells. The 73–151-β-galactosidase this fusion protein does not spread around the entire INM even in wild-type cells, in contrast to the full-length protein.

INTRODUCTION

Previous studies on Trm1p focused on identifying *cis*-acting sequences involved in its cellular targeting to the mitochondria and nucleus (Stanford *et al.,* 2000; Ellis *et al.,* 1987; 1989; Rose *et al.,* 1992; 1995). In this thesis, we focused on mapping additional regions of Trm1p-II that mediate targeting, both to the nucleus and INM (see Chapters 3 and 4). However, we also wanted to elucidate *trans*-acting factors to delineate a mechanism for targeting a peripheral protein specifically to the INM. Trm1p-II requires proteins, including, though likely not limited to, karyopherins to ultimately target to the nucleus. We reason that Trm1p-II requires additional unidentified proteins to target within the nucleus, specifically to the INM. To understand targeting of Trm1p-II *in vivo*, we must elucidate transient and stable interacting partners contributing to its final localization at the INM.

A powerful method for identifying protein-protein interactions is a two-hybrid screen (Fields and Sternglanz, 1994). However, our lab previously attempted several two-hybrid screens using Trm1p as the bait. These efforts failed to identify any candidate interactions (N.C. Martin, University of Louisville, personal communication to AKH), perhaps because the interactor is an integral membrane protein not readily uncovered by the standard two-hybrid method (Fields and Sternglanz, 1994). Therefore, we proposed alternative strategies to identify *trans*-acting factors involved in targeting Trm1p-II to the INM.

Collections of temperature-sensitive mutants were screened to find those that mislocalized Trm1-GFP (Kao and Hopper, unpublished). The rationale was that deletion of the membrane binding partner of Trm1p would cause the protein to accumulate in the

nucleoplasm rather that at the INM. However, after screening approximately 200 mutants, none were found to cause nucleoplasmic accumulation of Trm1-GFP. The concurrent development of several collections in *S. cerevisiae* made it feasible to perform studies more efficiently on a genome-wide scale. Therefore, our strategies to identify *trans*-acting partners of Trm1p-II were modified to take advantage of these new tools.

Murthi and Hopper (2005) undertook a genome-wide study screening the approximately 4700 members of the unessential yeast deletion collection (Winzeler *et al.,* 1999) for mutants that caused mislocalization of Trm1-II-GFP. Several mutants were identified that caused mislocalization of Trm1-II-GFP from the INM into the nucleoplasm, including Mak31p, Mak10p, and Mak3p, subunits of an N-terminal acetyltransferease complex called Nat C. The Nat C complex could be involved in Trm1p-II targeting to the INM directly, by acetylating Trm1-II-GFP itself, or indirectly, by acetylating a secondary protein necessary for targeting Trm1-II-GFP to the INM. Murthi and Hopper (2005) went on to demonstrate that Trm1p-II was N-acetylated in wild-type cells, though likely not in *mak3* cells, indicating the Nat C complex acetylates Trm1p-II. Furthermore, this modification was found to be necessary for targeting Trm1-II-GFP to the INM, as altering the N-terminal amino acids to prevent its recognition by Nat C caused nucleoplasmic accumulation of Trm1-II-GFP. Although N-terminal acetylation had been implicated in targeting to membranes previously, including those of the Golgi and chloroplast, this was the first report of N-terminal acetylation contributing to targeting a protein to the INM (Behnia *et al.,* 2004; Setty *et al.,* 2004; Pesaresi *et al.,* 2003; Murthi and Hopper, 2005).

Murthi and Hopper (2005) also found Trm1-II-GFP mislocalized in *ice2* cells. When Ice2p was identified in this screen, little was known about its cellular function. As its deletion resulted in predominant nucleoplasmic accumulation of Trm1-II-GFP, Murthi and Hopper (2005) proposed Ice2p may either be an INM binding partner of Trm1p-II or a regulator of the INM binding partner. Since Ice2-GFP locates to the ER (Huh *et al.,* 2003) rather than the nucleus, the data support a role for Ice2p in regulating targeting of the INM tether of Trm1p-II from the ER to the INM. By this model, deletion of *ICE2* prevents efficient targeting of the INM tether of Trm1p-II resulting in accumulation of Trm1p-II in the nucleoplasm. However, as the ER is continuous with the nuclear membrane, it remains possible that Ice2p is present in multiple cellular compartments.

Current studies in our lab are utilizing Trm1-II-GFP to screen two essential yeast collections (Mnaimneh *et al.,* 2004; C. Boone, University of Toronto, personal communication to AKH) for mislocalization of the protein (Harchar and Hopper, unpublished). These collections contain the majority of the remaining ~1500 essential genes not previously screened for mislocalization of Trm1-II-GFP. With the completion of these studies, the entire *S. cerevisiae* genome will have been assayed for its role in targeting of Trm1-II-GFP to the INM.

Murthi and Hopper (2005) carried out GST-pulldowns using Trm1-II-GST to purify Trm1p-II from both wild-type and *mak3* cells. As Trm1-II-GFP is nucleoplasmic in *mak3* cells, it is possible that Trm1p-II is prevented from interacting with its INM binding partner. Therefore, the goal of these experiments was to identify a protein(s) that interacted with Trm1-II-GST in the wild-type strain, but was missing from the *mak3* cells. However, these experiments failed to reveal any candidate proteins. As an

alternative strategy to identify the INM binding partner of Trm1p-II, cross-linking studies are currently being employed in both wild-type and *mak3* cells. To date, one candidate protein has been isolated, though its identity remains unknown (Murthi and Hopper, unpublished).

We proposed competition experiments to elucidate proteins able to compete with Trm1p-II for binding at the INM. Such an approach theoretically could either identify the INM binding partner of Trm1p-II or other soluble proteins that share the INM binding partner. For these competition studies to work, we had to determine whether the membrane-binding partner of Trm1p-II could be saturated. Therefore, we first attempted to demonstrate that Trm1p-II could compete with itself for binding at the INM, indicating the binding partner was the limiting factor in these experiments. We predicted a shift in localization of Trm1-II-GFP from the INM into the nucleoplasm by Trm1p-II competing for binding to the INM binding partner when these proteins were cotransformed. Our hypothesis was Trm1-II-GFP would localize to the INM in the absence of Trm1p competitor; however, upon expression of increasing amounts of Trm1p-II, Trm1-II-GFP would be released from the INM into the nucleoplasm. Our lab successfully utilized a similar competition strategy in the past for another protein, Mod5p (Benko *et al.,* 2000). Upon verification that Trm1p-II could compete with itself, our intent was to transform libraries of highly expressed genes into cells expressing Trm1-II-GFP to identify other proteins resulting in mislocalization of Trm1-II-GFP. Proteins identified in this assay would be further characterized to determine whether their effect on Trm1p-II localization was direct or indirect.

Following up studies carried out by Murthi and Hopper (2005), analyses were performed to characterize the possible interaction between Trm1p-II and Ice2p. As Ice2p has been implicated in having a role in targeting Trm1p-II to the INM (Murthi and Hopper, 2005), we were interested in further investigating the function of this protein. In Chapter 4, we described identification of an INM targeting motif in Trm1p-II sufficient to target β-galactosidase to the INM. We wanted to determine if a β-galactosidase fusion protein containing amino acids 73–151 of Trm1p-II was also mislocalized in *ice2* cells, similar to the phenotype observed with full-length Trm1-II-GFP. If Ice2p is the INM binding partner of Trm1p-II, then we predict the amino acids 73–151-β-galactosidase fusion protein would also accumulate in the nucleoplasm, rather than at the INM, in *ice2* cells.

METHODS

Trm1p constructs for competition experiments

Trm1-II-GFP was digested from pGP19 (Trm1-II-GFP in pRS416; generated by G. Peng) by *Nae*I and *Sac*I to produce an approximately 3200 bp digest fragment. This fragment was ligated into pRS414 (Sikorski and Heiter, 1989), a centromeric vector maintained in approximately 1 copy/cell. This plasmid, Trm1-II-GFP-414, containing the *TRP1* gene, which allows yeast cells auxotrophic for tryptophan to be rendered prototrophic, was transformed into W303α cells using the one-step yeast transformation protocol, described in Chapter 2. Cells containing the plasmid were selected by growing on media lacking tryptophan.

TRM1 without its endogenous promoter was amplified by PCR using oligonucleotides TRM5 and TRM3B to produce an approximately 1700 bp fragment that was ligated into the multi-copy vector, pEMBLyex4, maintained in approximately 10–50 copies/cell. pEMBLyex4 contains both a *CYC1* promoter, allowing constitutive expression of Trm1p-II, and *GAL10*, a galactose-inducible promoter, which allows for regulation of expression by altering the carbon source. In the presence of glucose, Trm1p-II is constitutively expressed. Upon shift to media containing galactose, the cells induce expression of *GAL10*-regulated genes. pEMBLyex4 contains both the *URA3* gene, allowing yeast cells auxotrophic for uracil to become prototrophic, as well as a defective *LEU2* gene (*Leu2Δ*), allowing yeast cells auxotrophic for leucine to become prototrophic. The plasmid, Trm1p-EMBL, was transformed into W303α cells already possessing the Trm1-II-GFP-414 plasmid, using the one-step yeast transformation protocol, described in Chapter 2. Cells containing both plasmids were selected by growing on media lacking both uracil and tryptophan (with glucose as the carbon source).

Inducing expression of the competitor

Yeast cultures were grown initially on media lacking uracil and tryptophan to select for transformants possessing both plasmids. This media contained glucose allowing constitutive expression of *TRM1* from Trm1-EMBL. The cultures were subsequently shifted from media lacking uracil and tryptophan with glucose to media lacking uracil and tryptophan with galactose to induce expression of Trm1p 1000-fold via the *GAL10* promoter of pEMBLyex4, relative to the constitutive *CYC1* promoter. As pEMBLex4 contains the *Leu2Δ* gene that selects for approximately 100 copies of the plasmid/cell

(Erhart and Hellenberg, 1983), cells could be selected on media lacking both leucine and tryptophan with glucose. These cells could then be shifted to media lacking both leucine and tryptophan with galactose to induce expression of Trm1p an additional 1000-fold. By growing the cells containing both Trm1-II-GFP-414 and Trm1-EMBL on various media, the expression of competitor Trm1p can be manipulated to varying levels.

ADEPT#2 in pFB1-7u for *ice2* experiments

Amino acids 73–151 were amplified with oligonucleotides KAS021 and KAS022 and flanked with *Bgl*II sites for insertion into pFB1-7u. The construction of this plasmid was described previously in Chapter 4.

Indirect immunofluorescence

Indirect immunofluorescence was performed using the procedure of Pringle *et al.* (1990) described in Chapter 2. To localize Trm1-II-GFP in the competition experiments, cells were fixed for approximately 25 min. Mouse monoclonal anti-GFP antibody (Roche) was used at 1:400 dilution, mouse anti-Nsp1p antibody (Tolerico *et al.,* 1999) was used at 1:10,000 dilution, and rabbit anti-Kar2p antibody (Scidmore *et al.,* 1993) was used at 1:3500 dilution. The secondary antibody was Cy3-conjugated goat anti-mouse IgG or Cy3-conjugated goat anti-rabbit IgG, each diluted 1:400.

To localize the β-galactosidase fusion protein in the *ice2* experiments, cells were fixed for approximately 45 min. Affinity-purified rabbit polyclonal anti-β-galactosidase antibody (Hopper *et al.,* 1990) was used at 1:400 dilution to localize the fusion protein. The secondary antibody was Cy3-conjugated goat anti-rabbit IgG, used at 1:400 dilution.

RESULTS

Competition of Trm1-II-GFP from the INM with full-length Trm1p

Trm1-II-GFP was expressed via a centromere-containing vector, maintained in approximately 1 copy/cell, under the control of its endogenous promoter in W303α cells. Increasing levels of a Trm1p competitor were expressed simultaneously within the W303α cells expressing the GFP-tagged protein. The localization of Trm1-II-GFP was monitored by indirect immunofluorescence using a commercial mouse anti-GFP antibody (Roche). This strategy is summarized in Fig. 23, including the relative copy number of the competitor plasmid in each cell and the relative levels of the competitor Trm1p-II.

The hypothesis for this competition experiment was that increasing the levels of untagged Trm1p would compete for binding at the INM with Trm1-II-GFP causing release of the GFP-tagged protein into the nucleoplasm. This prediction was based on the assumption that the binding sites at the INM for Trm1p-II were saturable. However, the results were far more complex, with Trm1-II-GFP localizing in a pattern characteristic of a known ER resident protein in *S. cerevisiae*, Kar2p (Fig. 24). To compare the localization of Trm1-II-GFP when exposed to varying levels of Trm1p competitor to the localization of Kar2p under the same conditions, we visualized the ER using an antibody for Kar2p (Scidmore *et al.,* 1993). The localization pattern of Kar2p was similar to that for Trm1-II-GFP when highly overexpressed (Fig 24. l vs d). This result suggested that Trm1p was translocated from the INM to the ER and plasma membrane as increasing levels of competitor protein were produced.

To explain the results, we proposed that there is a second binding partner for Trm1p-II within the nucleoplasm that functions to retain the protein within the nucleus.

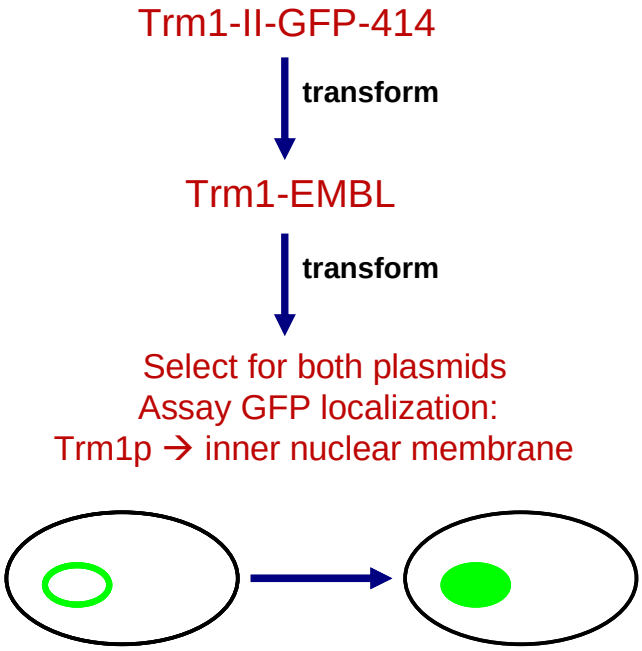
Instead of competing for binding at the INM, we may have saturated binding of this internal tether, thereby allowing Trm1p-II to move retrograde from the INM to the continuous perinuclear ER, remaining bound to its INM partner. Once in the perinuclear ER, Trm1p-II could be translocated to the cortical ER, lining the plasma membrane, thus explaining the localization of Trm1-II-GFP near the plasma membrane (Fig. 24). Similar results have been observed upon overexpression of vertebrate and *S. cerevisiae* integral INM proteins. MAN1 accumulates in the ER (Li *et al.,* 2000; Wu *et al.,* 2002), while emerin can be found both in the ER and at the plasma membrane in vertebrates (Ostlund *et al.,* 1999); Brr6p, Spo7p, and Nem1p all accumulate in the ER and at the plasma membrane upon overexpression in *S. cerevisiae* (de Bruyn Kops and Guthrie, 2001; Siniossoglou *et al.,* 1998). Brr6p, Spo7p, and Nem1p are all predicted to be relatively small proteins, less than 50 kDa compared to Trm1p-II which is predicted to be approximately 63 kDa or Trm1-II-GFP which is predicted to be approximately 89 kDa.

To verify that the overexpression of Trm1p did not alter overall cell morphology, we assayed the localization of the NPC using mouse monoclonal anti-Nsp1p antibody (Tolerico *et al.,* 1999) and the ER using rabbit anti-Kar2p antibody (Scidmore *et al.,* 1993). The NPC and ER looked similar in cells expressing the Trm1p competitor relative to control cells expressing the pEMBLex4 vector alone, even in cells possessing the highest level of overexpressed Trm1p (Fig. 24).

Although these competition experiments changed the way we thought about Trm1p-II localization within the nucleus, the results indicate that the competition strategy

Fig. 23: Strategy for Trm1p competition experiments.

Two plasmids, Trm1-II-GFP-414 and Trm1-EMBL, were cotransformed into W303α cells. Increasing amounts of Trm1p were expressed by induction of *TRM1* in the Trm1-EMBL, under *GAL10/CYC1* control. The table lists the relative copy number of the competitor plasmid, Trm1-EMBL, in each cell after growth on various media. We assayed for altered localization of Trm1-II-GFP in the presence of increasing amounts of Trm1p to ultimately provide an assay for elucidating the inner nuclear membrane-binding partner of Trm1p. We hypothesized Trm1-II-GFP would be competed from the INM into the nucleoplasm with increasing levels of expression of the competitor from Trm1-EMBL.



Expected Plasmid Copy Number

0-2	pRS414
5-20	pEMBLyex on –uracil + glucose
~100	pEMBLyex on –leucine + glucose
5-20x10^3	pEMBLyex on –uracil + galactose
100x10^3	pEMBLyex on –leucine + galactose

Table 14: Sequence of the oligonucleotides used for amplification of *TRM1* fragment for competition experiments.

Name	**Oligonucleotide sequence**
TRM5	5-TACGTCTAGAGAAGGTTTCTTCAGGATC-3
TRM3B	5-ACTTAAGCTTACTGCCCTCCTGATTAC-3

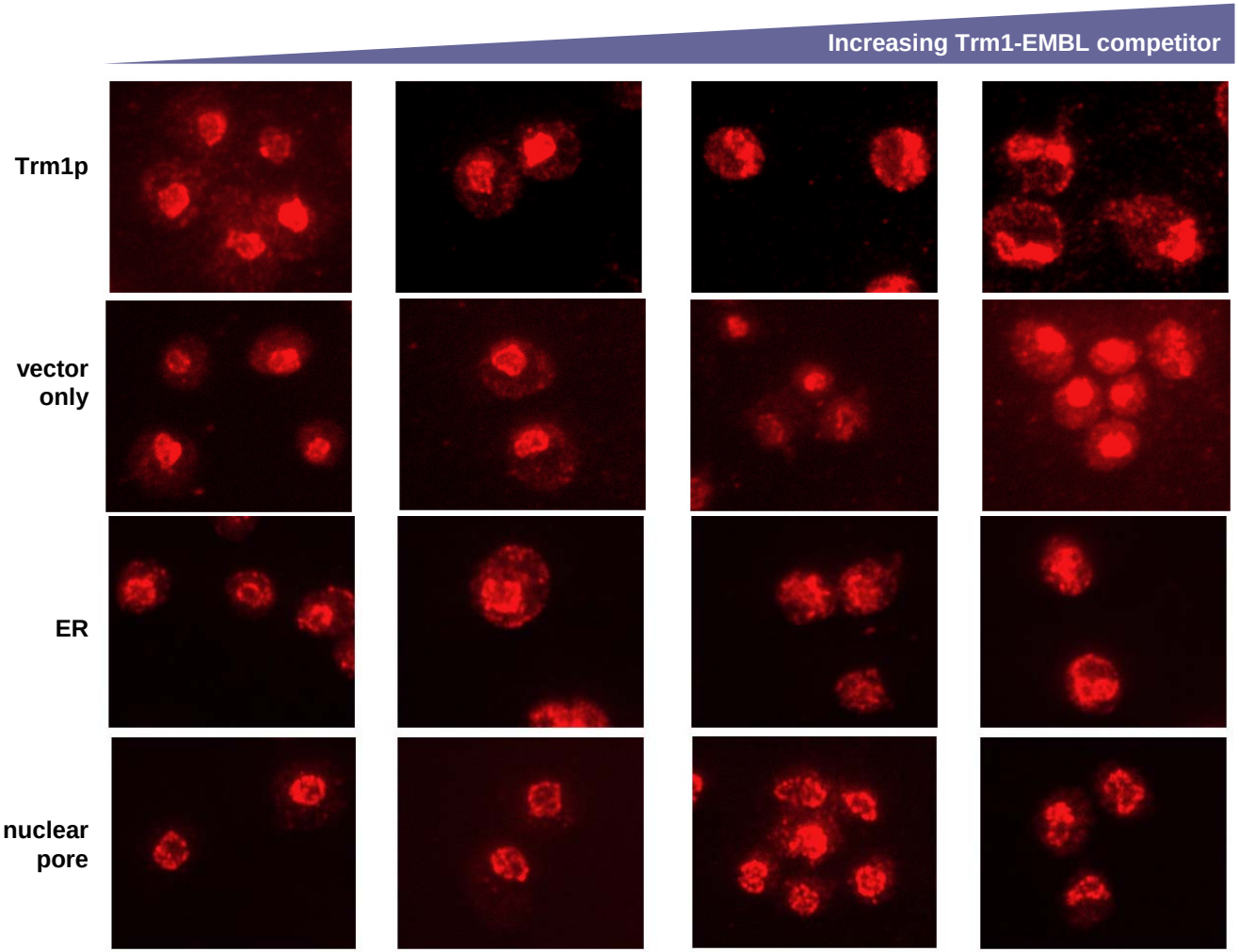
Table 15: Oligonucleotide pairs and DNA template used to amplify *TRM1* fragment for competition experiments.

PCR product	Oligonucleotides	Template	Template Source
TRM1 w/o promoter	TRM5/3B	pRS416-Trm1-HC3	G. Peng

Table 16: Plasmids used in the competition experiments and the proteins encoded.

Plasmid	**Encoded Protein**
Trm1-GFP-414	*TRM1* w/promoter and termination sequences fused to GFP
Trm1-EMBL	*TRM1*, amino acids 17-570, under *GAL10/CYC1* control

Fig. 24: Competition of Trm1-II-GFP with full-length Trm1p in W303α cells. Indirect immunofluorescence using mouse anti-GFP primary antibody (Roche), mouse anti-Nsp1 primary antibody (Tolerico *et al.,* 1999), rabbit anti-Kar2 primary antibody (Scidmore *et al.,* 1993), and Cy3 conjugated goat anti-mouse or anti-rabbit secondary antibody. Subcellular localization of Trm1-II-GFP upon induction of increasing levels of Trm1p-II competitor. Localization of NPCs and ER upon increasing levels of Trm1p-II competitor. (a-d) Cy3 images of pEMBLex4 vector control (e-h) Cy3 images of Trm1-II-GFP (i-l) Cy3 images of NPCs (m-p) Cy3 images of ER.


Increasing Trm1-EMBL competitor
Trm1p
vector only
ER
nuclear pore

would not succeed in identifying Trm1p-II interacting proteins responsible for INM tethering. However, our model for Trm1p-II targeting to the INM has been revised in light of the results obtained from these competition experiments. We propose that there are two binding partners, one at the INM and another within the nucleoplasm, that are both necessary to maintain Trm1p-II at the INM. This is similar to integral INM proteins in vertebrates that bind to stable nuclear components, such as chromatin binding proteins, the nuclear lamins, or chromatin, to maintain their nuclear localization (Vaughan *et al.,* 2001; Poliodaki *et al.,* 2001; Libotte *et al.,* 2005; Zhang *et al.,* 2005).

Competition of Trm1-II-GFP with Trm1-β-galactosidase fragments

The competition experiments with full-length Trm1p-II suggested that the INM binding partner of Trm1p-II was not limiting. However, we hypothesized that overexpressing Trm1p released Trm1-II-GFP from the INM to the ONM/ER by disrupting binding of Trm1-II-GFP to a tether in the nucleoplasm. To help identify the *cis*-acting region of Trm1p that may interact with the nucleoplasmic tether, we tested whether a fusion protein, sufficient for targeting to the INM, is able to cause translocation of Trm1-II-GFP to the ONM/ER as well. We predict that the fusion protein, amino acid 89–151-β-galactosidase, which contains the INM targeting information, likely does not contain the *cis*-acting sequence required for binding to the nuclear tether (to maintain nuclear localization) and thus, should not cause mislocalization of Trm1-II-GFP from the INM to the ONM/ER. However, it is possible that the Trm1 amino acid 89–151-β-galactosidase fusion protein would compete for binding at the membrane, forcing release of Trm1-II-GFP into the nucleoplasm. To test this hypothesis, we overexpressed 89–151

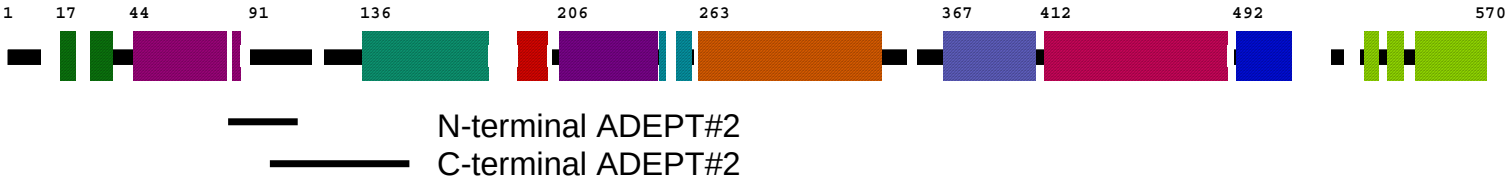
-β-galactosidase in the presence of Trm1-II-GFP and followed the localization of Trm1-II-GFP. Expression of the β-galalctosidase fusion proteins cannot be regulated by induction with galactose, although the protein is highly expressed within cells. As a negative control, amino acid 73–108-β-galactosidase, which accumulates in the nucleoplasm rather than at the INM, was transformed into W303α cells as well. We predict this fusion protein would not effect the localization of Trm1-II-GFP as it does not contain the *cis*-acting INM targeting motif and likely does not interact with the proposed tether within the nucleoplasm.

We did not observe any mislocalization of Trm1-II-GFP upon cotransformation of either of the Trm1-β-galactosidase fusion proteins (Fig. 25). Rather, Trm1-II-GFP was maintained at the INM in cells overexpressing either Trm1 amino acids 89–151-β-galactosidase or 73–108-β-galactosidase fusion proteins.

Localization of Trm1-β-galactosidase within *ice2* cells

As Murthi and Hopper (2005) showed that Trm1-II-GFP is mislocalized from the INM to the nucleoplasm in *ice2* cells, we examined the localization of amino acid 73–151-β-galactosidase in *ice2* cells. Contrary to that observed for the endogenous protein (Murthi and Hopper, 2005), the fusion protein was maintained at the INM in *ice2* cells (Fig. 26), suggesting that Ice2p most likely is not the INM binding partner of Trm1p-II, one of two models suggested by Murthi and Hopper (2005). Additionally, Ice2p was shown to localize to the ER (Huh *et al.,* 2003) and have a role in cortical ER inheritance (de Martin *et al.,* 2005), suggesting that its function may be indirect in targeting

Fig. 25: Competition of Trm1-II-GFP with pFB1-7u constructs containing Trm1p ADEPT#2 fragments in W303α cells. Subcellular localization of pFB1-7u constructs containing fragments of Trm1p transformed in W303α strain. Trm1-β-galactosidase fusion proteins were localized by indirect immunofluorescence using affinity-purified rabbit anti-β-galactosidase primary antibody (Hopper *et al.,* 1990) and Cy3 conjugated goat anti-rabbit IgG secondary antibody, each diluted 1:400. DAPI used to detect DNA. (a) Cy3 image of pFB1-7u vector control (b) Cy3 and DAPI merge of pFB1-7u vector control (c) Cy3 image of pFB1-7u+ADEPT#2 (amino acids 89–151) (d) Cy3 and DAPI merge of pFB1-7u+C-terminal ADEPT#2 (amino acids 89–151) (e) Cy3 image of pFB1-7u+ADEPT#2 (amino acids 73–108) (f) Cy3 and DAPI merge of pFB1-7u+C-terminal ADEPT#2 (amino acids 73–108).


1
17
44
91
136
206
263
367
412
492
570
N-terminal ADEPT#2
C-terminal ADEPT#2

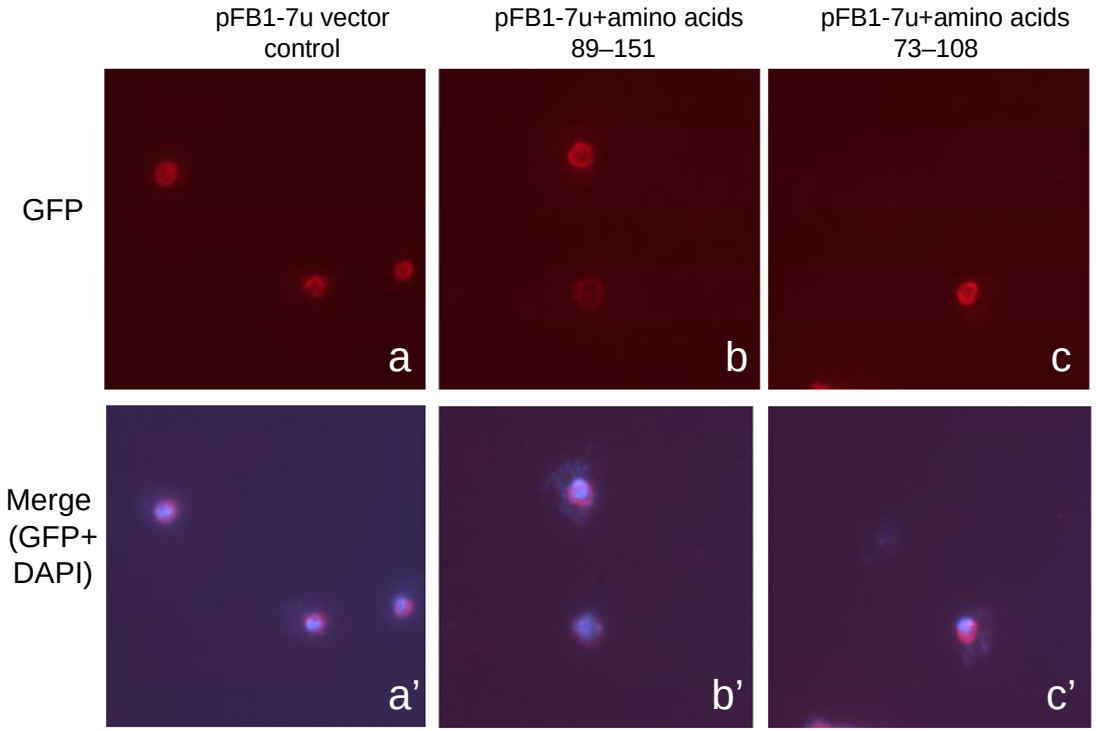
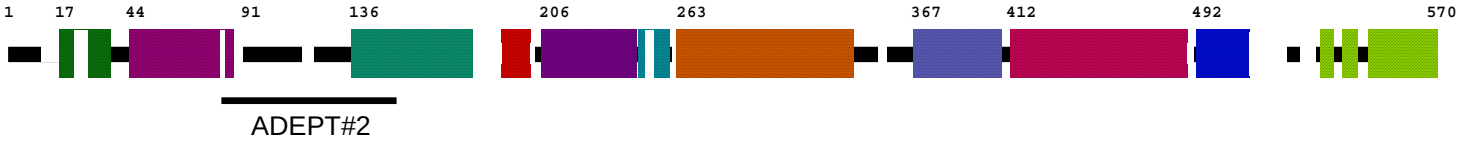

pFB1-7u vector control
pFB1-7u+amino acids 89–151
pFB1-7u+amino acids 73–108
GFP
Merge (GFP+ DAPI)
a
b
c
a'
b'
c'

Fig. 26: Subcellular localization of pFB1-7u constructs containing Trm1p ADEPT#2 fragments transformed in the *ice2* strain. Trm1–β-galactosidase fusion proteins were localized by indirect immunofluorescence using affinity-purified rabbit anti-β-galactosidase primary antibody (Hopper *et al.,* 1990) and Cy3 conjugated goat anti-rabbit IgG secondary antibody, each diluted 1:400. DAPI used to detect DNA. (a) Cy3 image of pFB1-7u vector control (a') Cy3 and DAPI merge of pFB1-7u vector control (b) Cy3 image of pFB1-7u+ADEPT#2 (amino acids 73–151) (b') Cy3 and DAPI merge of pFB1-7u+ADEPT#2 (c) Cy3 image of pFB1-7u+C-terminal ADEPT#2 (amino acids 89–151) (c') Cy3 and DAPI merge of pFB1-7u+C-terminal ADEPT#2.


1
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ADEPT#2

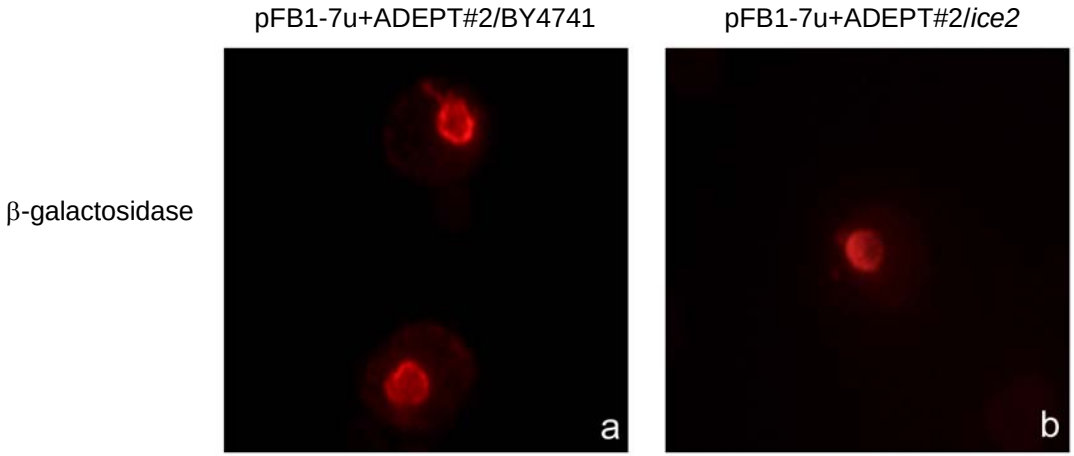

pFB1-7u+ADEPT#2/BY4741
pFB1-7u+ADEPT#2/ice2
β-galactosidase
a
b

Table 17. Results from all experiments reported in Chapter 5.

Construct	Independent:		Localization
	Cultures	Transformations	
Trm1-II-GFP/Trm1-EMBL/W303 (-ura, glucose)	1	2	Nuclear rim
Trm1-II-GFP/Trm1-EMBL/W303 (-ura, glucose)	1	2	Nuclear rim
Trm1-II-GFP/Trm1-EMBL/W303 (-ura, galactose)	1	2	Aberrant nuclear; possibly ER and plasma membrane
Trm1-II-GFP/Trm1-EMBL/W303 (-ura, galactose)	1	2	Aberrant nuclear; possibly ER and plasma membrane
Trm1-II-GFP/pFB1-7u vector/W303	1	2	Nuclear rim
Trm1-II-GFP/pFB1-7u+ADEPT#2 (amino acids 73–108)/W303	1	2	Nuclear rim
Trm1-II-GFP/pFB1-7u+ADEPT#2 (amino acids 89–151)/W303	1	2	Nuclear rim
pFB1-7u+ADEPT#2 (amino acids 73–151)/BY4741	1	2	Nuclear rim
pFB1-7u+ADEPT#2 (amino acids 73–151)/*ice2*	1	2	Nuclear rim

Trm1p-II to the INM.

We propose Ice2p, instead, functions to spread the endogenous protein throughout the INM. As the 73–151-β-galactosidase fusion protein does not spread throughout the INM in BY4741 wild-type cells, no phenotype is observed in the *ice2* cells. Ice2p may not be involved directly in spreading Trm1p-II around the INM, but rather is required for efficient targeting of an unidentified protein involved in Trm1p-II spreading to the INM from the ER.

DISCUSSION

The data presented thus far suggest that Trm1p-II likely has two tethers within the nucleus: one at the INM (based on the nucleoplasmic localization of the Trm1 ADEPT#3 deletion) and another within the nucleoplasm (based on the competition results that showed Trm1p to be located at the INM and ER when overexpressed). This is similar to vertebrate integral INM proteins that are maintained in the nucleus via binding to stable nuclear components, such as chromatin, chromatin binding proteins, or the nuclear lamina (Ostlund *et al.,* 2006; Poliodaki *et al.,* 2001; Vaughan *et al.,* 2001).

Tertiary structure likely plays a role in movement through the lateral channels of the NPC. Trm1-II-GFP (approximately 89 kDa) far exceeds the size limitation (approximately 60 kDa; Hinshaw *et al.,* 1992; Akey and Radermacher, 1993) predicted for the lateral channels in vertebrates. Three alternatives can explain how Trm1-II-GFP translocates to the ER from the INM. First, although Trm1-II-GFP is predicted to be too large to translocate through the approximately 10 nm lateral channels, based on size predictions from vertebrate NPCs, its structure may actually allow its retrograde

movement to the ER. It is possible that Trm1-II-GFP becomes unfolded allowing its movement through the lateral channels to the continuous ER. It is known that ATP is required for translocation of integral INM proteins from the ER to the INM. In the model proposed by Ohba *et al.* (2004), energy is necessary to allow restructuring of the NPC to expose the lateral channels for protein translocation. However, another possibility is that ATP is needed to unfold a protein to allow its movement through the lateral channel. The lateral channels of the *S. cerevisiae* NPC may be bigger than those of vertebrate NPCs. Furthermore, since no studies have been conducted in *S. cerevisiae* to investigate the structural limitations of the lateral channels, it is possible that these channels are larger in *S. cerevisiae* cells than vertebrate cells, allowing larger proteins and macromolecules to traverse. Second, though a pathway similar to that of the "diffusion-retention" model has been proposed in *S. cerevisiae* for targeting of an integral protein to the INM (King *et al.,* 2006), it is possible that yeast have a unique pathway, divergent from the "diffusion-retention" model proposed for vertebrate INM integral proteins (Smith and Blobel, 1993; Soullam and Worman, 1993; 1995). Third, it is possible that the ONM and INM fuse allowing proteins to be exchanged freely between these membranes. Upon overexpression in *S. cerevisiae*, Trm1-II-GFP is "forced" from the INM to the ONM as binding to the nuclear tether become limiting. Once translocated to the ONM, Trm1-II-GFP would be free to diffuse to the perinuclear ER continuous with the ONM. Trm1-II-GFP could then be translocated to the cortical ER that lines the plasma membrane, producing a phenotype similar to that observed for Trm1-II-GFP in the competition experiments.

The competition experiments did not produce the predicted results. This can be explained by several models. First, if the membrane-binding partner is shared between the INM and ONM, this would account for Trm1-II-GFP being localized in both membranes upon overexpression. Second, Trm1-II-GFP may have a nuclear tether within the nucleoplasm that retains the protein within the nucleus, preventing its translocation to the ONM or ER. Thus, it may be this tether that is limiting, rather than the membrane-binding partner, and as Trm1p was overproduced in the competition experiments, Trm1-II-GFP was released from its nuclear tether and free to move to the ONM and ER.

In an attempt to verify the presence of a nuclear tether, we performed the competition experiments with fusion proteins containing various fragments of ADEPT#2 of Trm1p-II. However, these experiments were also unsuccessful in competing Trm1-II-GFP from the INM into the nucleoplasm. No alterations in the localization of Trm1-II-GFP were observed when the amino acids 73–151-β-galactosidase or 73–108-β-galactosidase fusion proteins were coexpressed with Trm1-II-GFP. It is possible that neither of these fusion proteins contain the *cis*-acting sequence of Trm1p-II necessary for interaction with the nucleoplasmic tether. However, it is also possible that the *cis*-acting sequence of Trm1p required to interact with the nucleoplasmic tether was present in these ADEPT#2 fragments, this sequence was not in a structural confirmation to allow its interaction with the nucleoplasmic tether.

Our goal for the competition experiments was to exploit this technique to identify proteins that could disrupt INM localization of Trm1-II-GFP, possibly as a means to identify the INM binding partner, or elucidate other proteins that share the INM binding partner, to learn more about INM targeting in general. However, this strategy was

unsuccessful for the purpose of identifying *trans*-acting factors involved in Trm1p-II INM localization.

Ice2p was identified in a concurrent study wherein the unessential *S. cerevisiae* collection was screened for mutants that mislocalize Trm1-II-GFP (Murthi and Hopper, 2005). The role of Ice2p in INM targeting of Trm1p-II was unclear, as the only known function of this protein is in ER inheritance (deMartin *et al.,* 2005). We were interested in delineating a role for Ice2p in INM targeting of Trm1p-II and thus additional experiments were performed utilizing the fusion protein capable of targeting β-galactosidase to the INM. The amino acid 73–151-β-galactosidase fusion protein was maintained at the INM in *ice2* cells, suggesting Ice2p performs an indirect or secondary function in targeting Trm1p-II to the INM. The only difference in localization of full-length Trm1p-II relative to the amino acid 73–151-β-galactosidase fusion protein is that the fusion protein does not spread around the entire INM. We propose that INM targeting is a two-step process, requiring targeting to a specific site on the INM and subsequent spreading around the INM. The cortical ER is disrupted in *ice2* cells, possibly preventing efficient targeting of an unidentified protein to the nucleus, where it functions to regulate spreading of Trm1p-II around the INM.

CHAPTER SIX

GENERAL DISCUSSION

In this thesis, we used Trm1p-II as a reporter to identify *cis*-acting sequences and *trans*-acting factors involved in protein targeting to the INM. Although most peripheral INM proteins possess NLSs directing them to the nucleus, no motif has been identified directing these proteins specifically to the INM.

To date, the only peripheral INM proteins studied extensively, with respect to targeting, have been the vertebrate nuclear lamins. It is possible that the nuclear lamins target to the INM by virtue of their interaction with integral INM proteins, including LBR, LAPs, and emerin. The opposite has been proposed for integral INM proteins that are retained at the INM by their interaction with stable peripheral proteins, including the nuclear lamins (Vaughan *et al.,* 2001).

The molecular structure of the nucleus is highly complex and ordered. This organelle is further divided into subdomains, including the nucleolus, the INM, the speckles, and Cajal bodies, specialized for various functions. It remains unclear how these subnuclear regions are delineated from one another, as no membranes separate them. In addition, the nucleus is a dynamic organelle in higher eukaryotes, requiring reassembly after each round of mitosis or meiosis.

Protein targeting to the nucleus has been aided greatly by the elucidation of the molecular structure of the NPC (Rout *et al.,* 2000; Cronshaw *et al.,* 2002). Co-crystallization of karyopherins and nuclear pore proteins indicate elaborate interactions are necessary for translocation through the central channel of the nuclear pore. Although

we know much about *cis*-acting sequences, such as NLSs, and *trans*-acting factors, such as the members of the importin β family, involved in transport between the nucleus and cytoplasm, we do not fully understand the mechanism that allows movement through the NPC.

Protein targeting is critical to the cellular function of most proteins. Incorrect localization of a functional protein can be just as devastating to the cell as a mutant non-functional protein. Many diseases are attributed to mislocalization of nuclear proteins, both integral INM proteins, such as LBR, emerin, and MAN1, as well as peripheral INM proteins, such as the differentially regulated nuclear lamins, lamins A and C.

Diseases caused by mislocalization of nuclear proteins

Mutations in nuclear proteins, including lamin A, LBR, emerin, and MAN1 result in an ever-increasing family of diverse diseases, collectively referred to as "laminopathies". To date, there are approximately 20 diseases that fall into the family of laminopathies. Most of the nuclear proteins implicated in these disorders are ubiquitously expressed, which raises the question of how specific tissues are affected. Fully understanding the structure and maintenance of the nucleus is of critical importance if we are to understand the pathogenesis of these disorders.

Emery-Dreifuss muscular dystrophy arises from mutations in either the gene encoding the integral INM protein, emerin (*EMD*; which results in the X-linked form), or the gene encoding the peripheral proteins, lamins A and C (*LMNA*; which results in the autosomal dominant form)(Reviewed in Ostlund and Worman, 2003). A rare autosomal recessive form of Emery-Dreifuss muscular dystrophy resulting from the inheritance of

two mutant alleles of *LMNA* has also been reported (Raffaeli Di Barletta *et al.,* 2000). Both the X-linked and autosomal dominant form can result in muscle contractures, rigidity of the spine, and muscle weakness (particularly in the arms and legs). However, this disorder exhibits significant clinical variability, presenting early in childhood in some patients and in adulthood (usually in the third decade) in others and often with different combinations of symptoms. Ultimately, cardiomyopathy and conduction defects (often in the atrioventricular node) lead to death.

Dunnigan-type familial partial lipodystrophy is an autosomal dominant disorder resulting from mutations in the gene encoding lamins A and C. This disorder impairs the body from properly maintaining subcutaneous fat, particularly in the extremities, while accumulating fat in the neck and face; these effects often begin in puberty (Köbberling *et al.,* 1986). In addition, affected individuals often exhibit high levels of triglycerides, which leads to episodes of acute pancreatitis. Further, this disorder is often coupled with insulin resistance and diabetes, particularly in females, suggesting an endocrine connection (Vigouroux *et al.,* 2000).

Hutchinson-Gilford Progeria is an autosomal dominant disorder resulting from mutations in the gene encoding lamins A and C (De Sandre-Giovannoli *et al.,* 2003). This disorder is characterized by premature aging (thinning of skin, loss of subcutaneous fat, inability to gain weight [failure to thrive]) with individuals developing premature atherosclerosis, which ultimately results in death (often from a stroke and/or myocardial infarction). Atypical Werner's syndrome is similar in its clinical features to those of Hutchinson-Gilford Progeria, though often less severe. Atypical Werner's syndrome is an

autosomal recessive disorder caused by mutations in the gene encoding lamins A and C and presents clinically with features of premature aging (Chen *et al.,* 2003).

Pelger-Huet anomaly is an autosomal dominant disorder caused by mutations in the gene encoding the integral INM protein LBR (*LBR*) (Hoffmann *et al.,* 2002). This disorder is relatively benign in heterozygous individuals, resulting in structural abnormalities in granulocytes that do not affect the function of these cells. In contrast, homozygous individuals may exhibit skeletal anomalies (polydactyly, short metacarpals, short upper limbs, and/or short stature), developmental delays, and seizures (Shultz *et al.,* 2003).

Greenberg skeletal dysplasia (also known as Hydrops-ectopic calcification-"moth-eaten" [HEM] skeletal dysplasia) is an autosomal recessive disorder resulting from a mutation in the gene encoding LBR. This disorder can result in abnormal bone calcification, short limbs, and dwarfism and is lethal *in utero* (Worman and Courvalin, 2005). In addition, these individual exhibit a defect in cholesterol biosynthesis, resulting from a deficiency of the enzyme, 3β-hydroxysterol-Δ^{14}-reductase (Kelley *et al.,* 2001).

The most recent additions to this growing list of diseases include osteopoikilosis, Buschke-Ollendorff syndrome, and melorheostosis. These autosomal dominant disorders all result from various mutations (usually loss-of-function) in the gene for the integral INM protein, MAN1. The common characteristic of these disorders are their affects on bone (Hellemans *et al.,* 2004). Osteopoikilosis is an asymptomatic disorder presenting as areas of increased density within bones upon x-ray examination. Buschke-Ollendorff syndrome can lead to malignant transformations of bone (into osteosarcoma, chondrosarcoma, and giant cell tumor) while often accompanied by aortic stenosis and

diabetes. Melorheostosis is a progressive disorder resulting from the widening of the cortex within bone; severity of symptoms is relative to the age of onset. This disorder can cause severe pain and physical deformity, and may require amputation of affected limbs in extreme situations.

Understanding the molecular basis of "laminopathies" will require elucidating critical interactions between proteins within the nucleus. For example, deleting the genes encoding both MAN1 and emerin is synthetically lethal in *C. elegans* (Liu *et al.,* 2003). Studies in model organisms, such as those described here in *S. cerevisiae*, allow for identification of the complex genetic interactions within cells that cannot be easily observed in humans.

As mentioned previously in Chapter 1, the nucleus breaks down and reassembles with each round of mitosis in higher eukaryotic cells. Nuclear disassembly initiates at the end of prophase and requires several cellular events: fragmentation of the nuclear membrane, dissociation of the NPCs, and depolymerization of the nuclear lamina. During mitosis, there is a parallel breakdown of the ER, which is contiguous with the nuclear membrane, with this organelle also fragmenting into vesicles. The NPCs dissociate into subunits. The nuclear lamina disassembles; B-type lamins remain associated with the nuclear membrane vesicles, while lamins A and C are released as free dimers into the cytoplasm. The chromosomes condense and the nucleolus disappears.

Reassembly of the nucleus occurs following telophase. As the chromosomes decondense, the nuclear membrane begins to reform by binding of the membrane vesicles to the chromosomes, a process that likely involves both lamins and integral INM proteins. As the vesicles fuse around the chromosomes, the NPCs begin to reassemble.

The nuclear lamina reforms as the chromosomes continue to decondense. The membrane vesicles, which are now bound to the chromosomes, fuse together forming an intact nuclear membrane. At this point, the nucleus excludes cytoplasmic molecules. However, the newly reformed nucleus begins to selectively import nuclear proteins from the cytoplasm via active transport through the central channel of the NPCs. Finally, the nucleolus reassembles as the chromosomes decondense and transcription begins, completing mitosis.

The process of nuclear breakdown and reassembly has been studied in live cells using fluorescence recovery after photobleaching (FRAP) techniques (Ellenberg *et al.,* 1997). During interphase, LBR-GFP was immobilized in the nuclear envelope, likely via its known interaction with heterochromatin and/or lamins. A subpopulation of LBR-GFP within ER membranes, by contrast, was entirely mobile and diffused rapidly and freely. Time-lapse imaging revealed LBR-GFP redistributed into the contiguous ER membrane during prometaphase; LBR-GFP was highly mobile during at this time within the ER membrane. At the end of mitosis, nuclear membrane reformation coincided with immobilization of LBR-GFP at contact sites with chromatin. LBR-GFP-containing ER membranes then wrapped around chromatin, compartmentalizing the nuclear material. Expansion of the nuclear envelope followed over the next 30-80 min.

Regulation of nuclear localization sequences

The continued identification of novel NLSs indicates there are likely undiscovered motifs. These novel motifs exhibit divergent characteristics that those of the prototypic NLSs, originally identified in SV40 large T antigen and nucleoplasmin. As

more resident nuclear proteins are identified, additional NLSs are likely to be elucidated, requiring continued reevaluation of our understanding of the nuclear-cytoplasmic transport process.

Sorting of proteins to organelles can be regulated in various ways. NLS masking can prevent recognition by the import machinery, either by protein folding preventing exposure of the signal or binding of a *trans*-acting factor to hide this signal. Post-translational modifications also contribute greatly to regulation of NLSs *in vivo*. Many proteins, including transcription factors, are phosphorylated and subsequently targeted to the nucleus.

Sorting isozymes are of particular interest in targeting studies, as these proteins possess multiple targeting signals. How the cell distinguishes the predominant signal is unclear. In the case of Trm1p, the two forms of the protein are targeted to the mitochondria and nucleus, although the mechanism regulating the partitioning of the protein is not known. Trm1p-I, the long form possessing both the mitochondrial targeting signal and the NLS, is targeted exclusively to the mitochondria. Perhaps there is a competition between the mitochondrial targeting signal and the NLS. Alternatively, the most N-terminal signal may be cotranslationally recognized and the protein is directed toward this organelle early on.

Acetylation

The role of protein modification in cellular targeting has been raised by recent findings showing N-terminal acetylation is necessary for targeting Arl3p to the Golgi in *S. cerevisiae* and chloroplast precursor proteins to the chloroplast in *Arabidopsis* (Behnia

et al., 2004; Setty *et al.,* 2004; Pesaresi *et al.,* 2003). This suggests acetylation may provide a role in targeting to membranes in general. Murthi and Hopper (2005) have shown this modification is necessary for targeting Trm1p-II to the INM as well. Trm1p-II is acetylated at the N-terminus by the Nat C complex composed of Mak3p, Mak10p and Mak31p (Murthi and Hopper, 2005). Murthi and Hopper (2005) demonstrated that preventing this modification, either by deletion of the genes encoding various subunits of the Nat C complex or altering the N-terminus of Trm1p-II to prevent its recognition by the Nat C complex, resulted in mislocalization of Trm1-II-GFP from the INM into the nucleoplasm. This suggested that N-terminal acetylation is necessary for targeting Trm1p-II to the INM. However, acetylation does not appear to be sufficient to direct Trm1-II-GFP to the INM. Similarly, N-terminal acetylation is not sufficient for targeting to either the Golgi or chloroplast (Behnia *et al.,* 2004; Setty *et al.,* 2004; Pesaresi *et al.,* 2003). This suggests acetylation may provide a role in targeting to membranes in general.

Endoplasmic reticulum connection

The competition experiments described in Chapter 5, as well as studies performed by Murthi and Hopper (2005) found the ER to have a role in protein targeting. Ice2p, an integral protein of the ER (Huh *et al.,* 2003), was uncovered by a genome-wide screen in *S. cerevisiae*. In *ice2* cells, full-length Trm1-II-GFP was mislocalized from the INM into the nucleoplasm, suggesting Ice2p contributed to the INM localization of Trm1p-II. In addition, the competition experiments showed that Trm1-II-GFP was transported to the perinuclear and cortical ER upon overexpression. Although vertebrate INM proteins have been shown to be translocated to the continuous ER upon overexpression, likely by

retrograde movement through the lateral channels of the NPC, no model for retrograde movement from the INM to the ER has been proposed for *S. cerevisiae*.

Targeting Trm1p-II to the inner nuclear membrane

Targeting to the INM, in the case of Trm1p-II, likely requires both *cis*-acting sequences as well as *trans*-acting factors specifying protein modification. The requirement for N-terminal acetylation explains a previous result where deleting amino acids 1-48 from Trm1p caused accumulation of the protein in the nucleoplasm (Rose *et al.,* 1992). However, a fusion protein containing amino acids 1-213 of Trm1p, which contains the N-terminus as well as the internal INM targeting motif within amino acids 89-151, does not localize to the INM but rather the nucleoplasm (Rose *et al.,* 1992). This implies a region of Trm1p-II downstream from amino acid 213 is necessary for correct targeting to the INM. As ADEPT#3 is necessary for Trm1-II-GFP INM targeting, this region may be involved.

The current model for Trm1p-II targeting has Trm1p-II synthesized in the cytoplasm where it is exposed to the cytoplasmic Nat C complex consisting of Mak3p, Mak10p and Mak31p that recognizes the N-terminal sequence of Trm1p-II, Met-Leu-Lys. Murthi and Hopper (2005) showed the presence of an acetyl group on the N-terminus of Trm1p-II. Our model proposes this modification allows the N-terminus of Trm1p-II to interact with a downstream region of Trm1p-II, perhaps ADEPT#3, resulting in a conformational change exposing the *cis*-acting targeting sequence within ADEPT#2. The INM-targeting region within ADEPT#2 may be downstream of NLS#1; this is suggested by the fact that Trm1p-II located to the nucleoplasm in *mak3* cells, indicating

the NLS within ADEPT#2 was exposed. However, Trm1p-II was unable to bind and be retained at the INM, potentially due to lack of exposure of the INM-targeting region in ADEPT#2. NLS#1 (amino acids 95-102) of Trm1p-II is recognized by its importin, most likely importin β /Kap95p via its adaptor, importin α/ Srp1p/Kap60p, based on its sequence similarity to that of the NLS within the SV40 Large T Antigen. Binding of importin β masks the acetyl group at the N-terminus, which may mediate interaction with membranes, thereby preventing inappropriate interaction with membranes in the cytoplasm. As the INM targeting motif of Trm1p-II surround the NLS within ADEPT#2, this region would be effectively masked by importin β as well, preventing its interaction with membranes in the cytoplasm. Trm1p-II is transported through the central channel of the NPC to the nucleoplasm, where importin β releases the protein exposing both the N-terminal acetyl group and the internal INM targeting motif. Once in the nucleus, Trm1p-II is targeted via the 60-amino acid ADEPT#2 (amino acids 89-151) targeting motif to the INM, where it interacts with its binding partner, either an unidentified protein or a lipid.

Why such elaborate targeting mechanisms?

The targeting of Trm1p-II to the INM is not a necessary requirement for its *in vivo* function (Rose *et al.,* 1992). As Trm1p can function to modify pre-tRNAs in the cytoplasm if prevented from targeting to the nucleus, why are there such elaborate targeting mechanism for this protein? We propose it is advantageous to the cell, at least under certain conditions, to maintain tRNA modification enzymes in the same cellular compartment.

trm11 and *trm1* genetically interact, as double mutants exhibit slow growth phenotypes at 30^{o}C. Purushothaman *et al*. (2005) showed that formation of Gm34, catalyzed by Trm7p, depends on correct tRNA structure as tRNAs lacking both m2G10, catalyzed by a complex of Trm11p-Trm112p, and m22G26, catalyzed by Trm1p, are not modified by Trm17p, possibly as a result in inappropriate folding of tRNA. Although m22G26 is an unessential modification of tRNA, disrupting multiple tRNA modifications in combination can inhibit growth. Shaheen and Hopper (2005) have demonstrated that tRNA is able to move retrograde from the cytoplasm to the nucleus and they have suggested that this may allow for proofreading of tRNA. Under certain conditions, it may be advantageous for the cell to concentrate the tRNA machinery, including tRNAs and their modification enzymes, within the nucleus. We need to consider the localization of proteins with respect to a dynamic cell, where proteins are constantly being shuttled, degraded, and synthesized.

Fig. 27: Model for targeting Trm1p-II to the inner nuclear membrane.

(1) Trm1p-II is translated in the cytoplasm

(2) In the cytoplasm, Trm1p-II is acetylated at its N-terminus by the Nat C complex composed of Mak3p, Mak10p and Mak31p

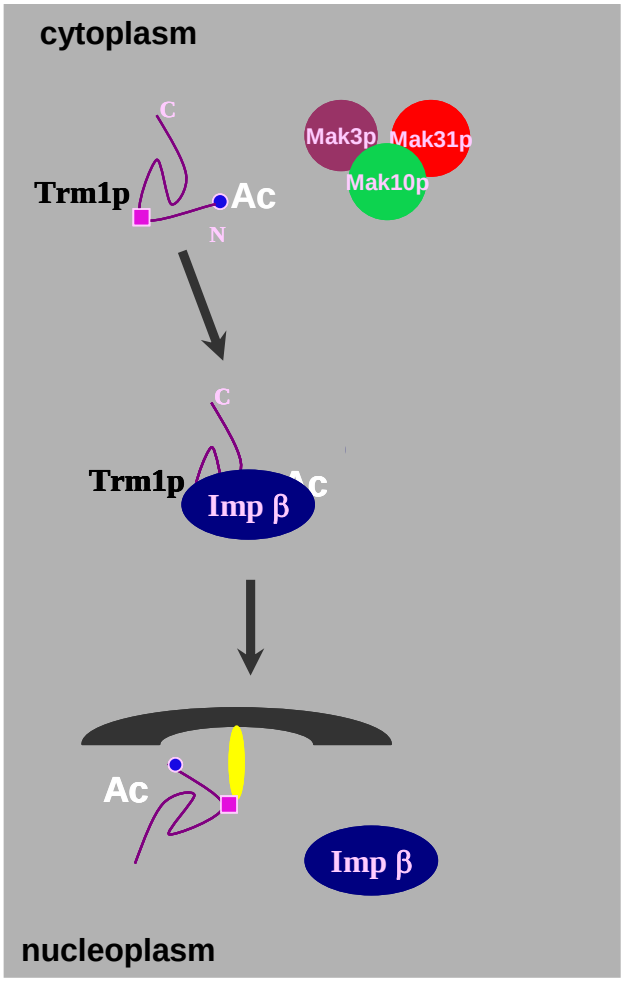
(3) N-terminal acetylation results in a conformational change in Trm1p-II exposing the inner nuclear membrane (INM) targeting sequence (amino acids 89-151) within ADEPT#2

(4) Importin β binds to NLS#1 masking both the N-terminus and INM targeting motif of Trm1p-II, preventing their interaction with membranes in the cytoplasm

(5) Trm1p-II targets to the nucleus via importin β which recognizes NLS#1 (amino acids 95-102); NLS#2 may function *in vivo* and/or under different growth conditions than those tested

(6) Importin β dissociates from Trm1p-II, exposing the acetyl group at the N-terminus and the INM targeting motif (amino acids 89-151), allowing Trm1p-II to target to the INM

(7) Trm1p-II spreads from an initial binding site at the INM throughout the entire INM (likely aided by a yet unidentified protein regulated by Ice2p?)



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Meetings Attended
Dynamic Organization of Nuclear Function, Cold Spring Harbor Laboratory, October 2004
Yeast Cell Biology, Cold Spring Harbor Laboratory, August 2003
Yeast Cell Biology, University of Wisconsin–Madison, July 2002
Society for Integrative and Comparative Biology, Denver, Colorado, January 1999

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