

Analysis of a predicted nuclear localization signal: implications for the intracellular localization and function of the *Saccharomyces cerevisiae* RNA-binding protein Scp160

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ABSTRACT

Gene expression is controlled by RNA-binding proteins that modulate the synthesis, processing, transport and stability of various classes of RNA. Some RNA-binding proteins shuttle between the nucleus and cytoplasm and are thought to bind to RNA transcripts in the nucleus and remain bound during translocation to the cytoplasm. One RNA-binding protein that has been hypothesized to function in this manner is the *Saccharomyces cerevisiae* Scp160 protein. Although the steady-state localization of Scp160 is cytoplasmic, previous studies have identified putative nuclear localization (NLS) and nuclear export (NES) signals. The goal of this study was to test the hypothesis that Scp160 is a nucleocytoplasmic shuttling protein. We exploited a variety of yeast export mutants to capture any potential nuclear accumulation of Scp160 and found no evidence that Scp160 enters the nucleus. These localization studies were complemented by a mutational analysis of the predicted NLS. Results indicate that key basic residues within the predicted NLS of Scp160 can be altered without severely affecting Scp160 function. This finding has important implications for understanding the function of Scp160, which is likely limited to the cytoplasm. Additionally, our results provide strong evidence that the presence of a predicted nuclear localization signal within the sequence of a protein should not lead to the assumption that the protein enters the nucleus in the absence of additional experimental evidence.

INTRODUCTION

Eukaryotic cells are characterized by the separation of the genome and transcriptional machinery in the nucleus from the translational machinery in the cytoplasm. This separation is mediated by the double membrane of the nuclear envelope, which necessitates a system for transport between these discrete compartments. All macromolecular transport between the nucleus and the cytoplasm occurs via nuclear pore complexes (NPCs) embedded in the nuclear envelope (1,2). NPCs are massive multi-protein structures each composed of ~30 proteins, which are collectively referred to as nucleoporins (3). Although passive diffusion of molecules ≤ 40 kDa through NPCs can occur, most macromolecules require specific transport signal sequences to enter and exit the nucleus via the NPC (4).

Nuclear targeting signals mark proteins for transport into and out of the nucleus (4). The best-characterized nuclear targeting signal is the classical nuclear localization signal (cNLS), which consists of a monopartite or bipartite signal (5,6). The monopartite NLS contains a single cluster of four to six basic amino acids, while a bipartite NLS contains two stretches of basic amino acids separated by a stretch of 8–10 spacer residues (6,7). Nuclear export signals (NES) have also been identified. These targeting signals consist of a weak consensus of three to four hydrophobic residues (8). These classical targeting sequences can be, and often are, identified by computer searches; however, in many instances the function of these sequences is not experimentally verified.

Targeting signals are recognized by soluble nuclear transport receptors that shepherd cargo proteins through the nuclear pore complex (9). These receptors are termed karyopherins or importins/exportins. Many transport pathways involve direct cargo binding to receptors of

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the importin/karyopherin- β family. However, import of cargoes that contain a cNLS involves an adaptor protein, importin- α , that serves as an NLS receptor to recognize these cargoes in the cytoplasm (4). Importin- α and cNLS cargo interact with importin- β to form a trimeric import complex, which then translocates through the NPC (10). Once in the nucleus, a small GTPase, Ran-GTP (11,12), binds to importin- β causing a conformational change that results in dissociation of the import complex (13). The importin receptors are then recycled back to the cytoplasm to participate in subsequent rounds of import (14). In a similar manner, NES-dependent export involves the transport receptor, Crm1 (8,15). Like other export receptors of the importin- β family (16), Crm1 forms a trimeric complex with Ran-GTP and an export cargo (17). The trimeric complex is translocated through the NPC and once it reaches the cytoplasmic face of the NPC, Ran-GTP is hydrolyzed to Ran-GDP resulting in dissociation of the export complex (18).

Many proteins contain predicted NLS and NES sequences required for entering and exiting of the nucleus. Often, the function of these predicted sequences is assumed in the absence of supporting experimental data. One example of such a case is the coding sequence of the *Saccharomyces cerevisiae* control of ploidy protein of 160 kDa, Scp160. Although the exact function of Scp160 is unknown, it is thought to modulate gene expression through interacting with RNA substrates (19). Scp160 contains 14 predicted K-homology (KH) RNA-binding domains and also sequence motifs that have been hypothesized to function as both a cNLS and a classical NES (20,21). Since Scp160 associates with specific mRNA transcripts (19), it has been hypothesized to shuttle into the nucleus and then exit the nucleus in complexes with mRNA.

Despite the presence of a sequence noted as an NLS, the steady-state localization of Scp160 is clearly cytoplasmic (22–25). This cytoplasmic localization is consistent with at least two models: (i) Scp160 is solely restricted to the cytoplasm; or (ii) Scp160 shuttles into and out of the nucleus but the rate of export exceeds the rate of import. To distinguish between these two possibilities, we exploited yeast mutants that block different nuclear export pathways and assessed the localization of Scp160 in these mutant cells under conditions where these specific nuclear export pathways are blocked. If Scp160 does, in fact, shuttle between the nucleus and the cytoplasm, blocking its export would cause accumulation of the protein within the nucleus. However, if Scp160 does not shuttle, we would expect the localization to remain cytoplasmic despite blocked export pathways.

This general approach has been used previously to uncover the nucleocytoplasmic shuttling properties of other proteins. For example, the yeast poly(A)-binding protein 1, Pab1, has a well-characterized function in the cytoplasm where it binds to the poly(A) tail of mRNA transcripts and modulates their stability and translation (26). Consistent with these functions, Pab1 is localized to the cytoplasm at steady state (26). However, recent studies show that Pab1 accumulates within the nucleus of

cells that express a conditional mutant of the NES receptor, *crm1-1*, revealing the surprising finding that this protein with a well-characterized cytoplasmic function can enter the nucleus (27,28). These studies validate the use of export mutants to test whether Scp160 is also a shuttling protein.

The goal of this study was to carry out a functional analysis of the nuclear targeting signals present in the Scp160 protein. To accomplish this goal, we first exploited a number of conditional yeast mutants that impact nuclear export. We found that mutations that rendered each of the major export pathways nonfunctional did not cause accumulation of Scp160 within the nucleus, suggesting that Scp160 may not enter the nucleus. Second, we generated amino acid substitutions predicted to disrupt the function of the putative NLS of Scp160 and found that these mutations did not significantly impair Scp160 function, indicating that the putative NLS is not critical for the key cellular function of Scp160. These data challenge the model that Scp160 performs critical functions in the nucleus, and suggest that Scp160 functions are carried out exclusively in the cytoplasm.

MATERIALS AND METHODS

Plasmids and yeast strains

All yeast manipulations were performed according to standard protocols (29). All yeast strains used in this study are listed and described in Table 1. The wild-type yeast strain used for microscopy, JFY4493, was derived from the haploid parent strain W303 (MAT *a ura3-1 leu2-3 his3-11, 15 trp1-1 ade2-1 can1-100 RAD5+*, a gift from Dr R. Rothstein, Columbia University, NY, USA) by

Table 1. Strains and plasmids used in this study

Strains/plasmids	Description	Reference
ACY191	<i>mat α rpb1-1, ura2-52, his4-53</i>	(41)
ACY194	<i>mat α rat7-1, ura3-52, leu2Δ1, his3Δ200</i>	(46)
ACY372	<i>mat a crm1-3, ade2-1, ura3, leu2Δ1, his3, trp1</i>	(38)
ACY545	<i>mat α MEX67::KanMX4, ura3, leu2Δ1, his3Δ200, [+ mex67-5 LEU2]</i>	(42)
ACY1508	<i>mat α MLP1-RFP::KanMX4, ura3, leu2Δ1, his3Δ200, lys2</i>	(56)
JFY4247	<i>mat a SCP160::HIS3, EAP1::HIS3, ade2-1, ura3, leu2-3, his3-11, trp1-1, can1-100, [+ SCP160-URA3]</i>	(36)
JFY4493	<i>mat a FLAG-SCP160::SCP160, ade2-1, ura3-1, leu2-3, his3-11, trp1-1, can1-100</i>	(57)
pRS312	<i>TRP1, CEN, AMP</i>	(58)
pRS315	<i>LEU2, CEN, AMP</i>	(58)
pAC213	<i>SV40 NLS-PK1 NES-GFP, URA3, 2 μM, AMP</i>	(39)
pAC980	<i>ΔRGG Nab2-GFP, URA3, CEN, AMP</i>	(32)
JF4470	<i>SCP160-GFP, TRP1, CEN, AMP</i>	This study
JF4823	<i>FLAG-SCP160, LEU2, CEN, AMP</i>	This study
JF4843	<i>FLAG-SCP160-NLS_{mut}, LEU2, CEN, AMP</i>	This study

integration of a FLAG epitope onto the N-terminus of the Scp160 open reading frame. All recombinant DNA manipulations were performed according to standard procedures (30) utilizing the XL10-gold (Stratagene) strain of *Escherichia coli*. All mutagenesis was done using a QuikChange Site-Directed Mutagenesis Kit (Stratagene). Experiments were carried out at 34°C unless otherwise noted.

Lysate preparation and immunoblot analysis

Cells were grown to mid-log phase and harvested by centrifugation (5 min, 4000g at 4°C). Following two washes with ddH₂O, cells were lysed by vortex agitation with an equal volume of glass beads in 0.5 ml lysis buffer (25 mM Tris pH 7.2, 50 mM KCl, 30 mM MgCl₂, 1.5% Triton). Lysates were then transferred to a clean microfuge tube and centrifuged for 5 min at 3000g at 4°C. The supernatant was again transferred to a clean microfuge tube and centrifuged at 12000g for 10 min at 4°C.

Immunoblot analysis was performed essentially as described previously (22). Briefly, samples to be analyzed were mixed with sample buffer, boiled and run through a 10% SDS-polyacrylamide gel. The gel was then electroblotted onto a nitrocellulose membrane (Bio-Rad) and GFP-fusion proteins were detected by incubation of the membrane with a 1:10000 dilution of an α -GFP polyclonal antibody (31).

Direct fluorescence microscopy

Imaging was performed as described previously (32). In brief, yeast cells transformed with plasmids expressing GFP-fusion proteins were grown to mid-log phase in media lacking uracil and supplemented with adenine. Cultures were split and half was left at room temperature while the other half was shifted to 37°C for 2 h. Direct fluorescence and DIC images were collected using an Olympus BX epifluorescence microscope equipped with a Photometrics Quantix digital camera. IP Lab Spectrum software was used to capture images of cells. Prior to imaging, cells were incubated with 5 μ M Hoechst dye to visualize the chromatin within the nucleus.

Functional analysis of Scp160 variants *in vivo*

To assess the function of an Scp160 NLS variant (Scp160-NLS_{mut}) four lysine residues (321, 322, 331 and 333) within the predicted NLS were changed to alanines. We then tested whether this variant of Scp160 could rescue the synthetic lethality of an *Δscp160Δeap1* mutant using a standard plasmid shuffle assay (33). Briefly, Scp160-NLS_{mut} (*LEU2*) was transformed into an *Δscp160Δeap1* double mutant containing a wild-type *URA3* maintenance plasmid. Cells were inoculated into liquid medium and grown to saturation for 2 days. Cell numbers were equalized, serially diluted 1:10, and then spotted onto control plates of synthetic medium lacking leucine and test plates of synthetic medium lacking leucine and containing the drug 5-fluororotic acid (5-FOA). 5-FOA is toxic to cells containing the *URA3* gene product and, therefore, the only cells that grow on 5-FOA plates have lost the *URA3* plasmid and retain only the

LEU2 test plasmid (34). Test plasmids encoding a second wild-type copy of *SCP160* and empty vector (pRS315) were analyzed in parallel as positive and negative controls, respectively. Plates were incubated for two days at 34°C.

RESULTS

Scp160-GFP is localized to the cytoplasm at steady state

Figure 1A shows a schematic representation of the domain structure and postulated sequence motifs of Scp160. The predicted NES sequence is contained within the N-terminal portion of the protein, while the predicted NLS is located between KH domains three and four (KH3 and KH4), spanning a few residues into both KH3 and KH4. The amino acid sequence for each predicted transport signal is shown.

Figure 1B shows that, as previously reported (23–25), Scp160-GFP is localized to the cytoplasm and excluded from the nucleus. To confirm that the GFP signal reflects the localization of full-length Scp160, soluble lysates of wild-type cells expressing Scp160-GFP were subjected to immunoblot analysis with an α -GFP antibody (Figure 1C). Lysate from cells expressing NLS-NES-GFP served as a control for the specificity of the antibody. FLAG-Scp160-GFP migrated as a single band at ~200 kDa while NLS-NES-GFP migrated at 35 kDa.

As an additional control, we tested whether the GFP tag impacts the function of Scp160 by assaying the ability of Scp160-GFP to replace wild-type Scp160 *in vivo* using a plasmid shuffle assay. A *TRP1* plasmid containing *SCP160-GFP* was transformed into *Δscp160Δeap1* yeast containing a wild-type *URA3* maintenance plasmid of *SCP160*. Although Scp160 is not essential for cell viability (23), Scp160 becomes essential when the spindle pole body protein, Eap1 (35), is also deleted (36). Thus, we can employ this double mutant background where Scp160 is essential to rapidly assess Scp160 function. Transformants containing both the wild-type *URA3* plasmid and the *TRP1* test plasmid were streaked onto synthetic complete plates lacking uracil and tryptophan (control) and 5-FOA plates lacking tryptophan. No significant growth defect was observed in the Scp160-GFP versus the untagged Scp160 (data not shown). As expected, cells containing vector alone failed to grow, confirming the requirement for Scp160 in this genetic background.

Scp160 does not accumulate in the nucleus in a NES export mutant

To begin to assess whether Scp160 can enter the nucleus, we took advantage of the observation that Scp160 contains a predicted classical NES (20). If this NES mediates nuclear export of Scp160, then blocking the NES-dependent export pathway should cause nuclear accumulation of Scp160. To block this pathway, we utilized a conditional mutant of the NES receptor, Crm1 (37). *crm1-3* cells show defects in export of NES-cargo (38). Plasmids encoding Scp160-GFP or control cargoes either containing a classical NES, NLS-NES-GFP (39), or lacking an NES, Δ RGG-Nab2-GFP (32),

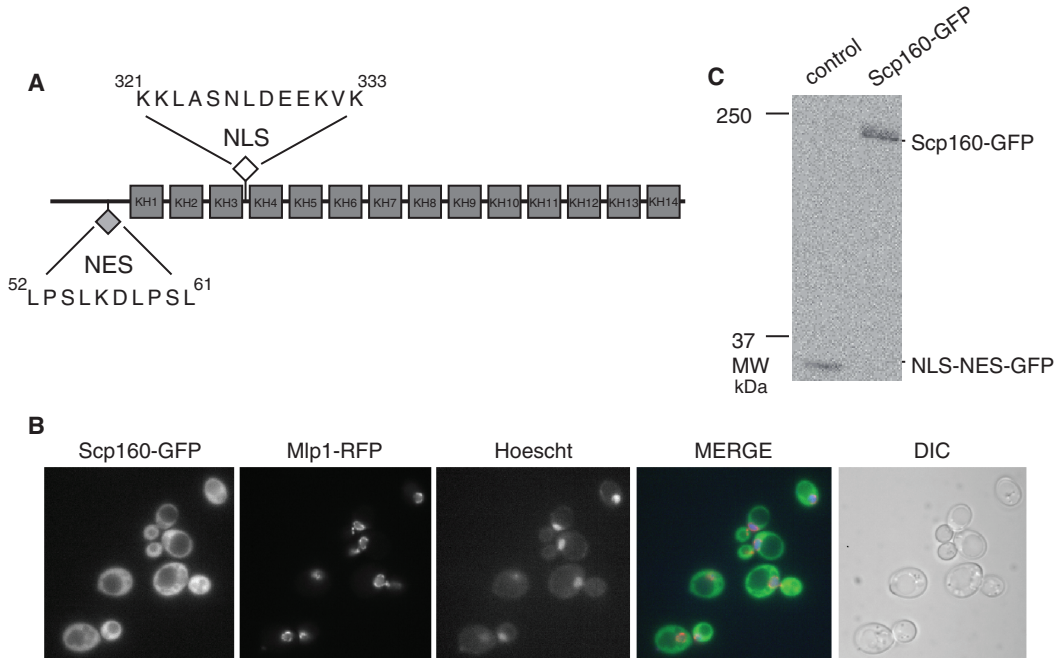


Figure 1. Scp160 is localized to the cytoplasm at steady state. (A) Schematic representation of Scp160 domain structure and location of putative nuclear targeting motifs. The predicted NLS is indicated by the filled diamond and the predicted NES is indicated by the open diamond. The sequences for the predicted NLS and NES motifs as well as their positions within the open reading frame are indicated. Numbered boxes represent the 14 KH domains. (B) The Scp160-GFP protein was expressed in wild-type cells that express a red-fluorescent protein-tagged nuclear rim protein, Mlp1 (56) to mark the position of the nucleus. Fluorescent protein localization was examined by direct fluorescence microscopy. Cells were also stained with Hoechst dye to mark the position of chromatin within the nucleus. A merged fluorescence image is shown as well as the corresponding DIC image. (C) Immunoblot analysis of GFP in protein lysate from wild-type cells transformed with plasmids encoding FLAG-Scp160-GFP (right lane) or a control NLS-NES-GFP (left lane). Migration of protein standards is indicated to the left of the image. The position of the bands corresponding to FLAG-Scp160-GFP and NLS-NES-GFP is indicated.

were transformed into wild type and *crm1-3* cells. Protein localization was assessed by direct fluorescence microscopy. Scp160 remained strictly localized to the cytoplasm in *crm1-3* cells at the non-permissive temperature with no apparent nuclear concentration (Figure 2). In contrast, the control NLS-NES-GFP accumulated in the nucleus of *crm1-3* cells. The localization of a control non-NES cargo protein, Δ RGG-Nab2-GFP, was not altered in the *crm1-3* cells compared to wild-type cells. These results indicate that either Scp160 does not enter the nucleus or its export is not dependent on the Crm1 export pathway.

Scp160 does not accumulate in the nucleus when poly(A) RNA export is blocked

Since Scp160 associates with mRNA (19), we hypothesized that Scp160 localization could depend on ongoing mRNA synthesis and mRNA export. A number of mRNA-binding proteins that undergo nucleocytoplasmic shuttling depend both on ongoing transcription and mRNA export (32,40). Therefore, we examined the steady-state localization of Scp160 in both *rpb1-1* cells where RNA polymerase II transcription is decreased (41) and *mex67-5* mutant cells where poly(A) RNA export from the nucleus is blocked (42,43). As controls we also examined the localization of NLS-NES-GFP, which is not affected by mRNA export (44), and

Δ RGG-Nab2-GFP, which depends on ongoing mRNA export and synthesis for export from the nucleus (44).

No nuclear accumulation of Scp160 was observed in either the *mex67-5* or *rpb1-1* cells (Figure 3). As expected, the control mRNA binding protein, Δ RGG-Nab2-GFP, accumulated in the nucleus of both *mex67-5* and *rpb1-1* cells following a shift to the non-permissive temperature. No change in the localization of NLS-NES-GFP was observed. These results indicate that either Scp160 does not enter the nucleus or its export does not depend on ongoing mRNA synthesis or mRNA export.

Scp160 does not accumulate in the nucleus of cells with an export-deficient NPC

As a final test of whether Scp160 enters the nucleus, we exploited a nuclear pore mutant that blocks all known nuclear export pathways, *rat7-1* (32,45–47). We hypothesized that if Scp160 enters the nucleus it should accumulate in the nucleus of *rat7-1* cells. Although *RAT7* (*NUP159*) is essential, the *rat7-1* allele is a temperature-sensitive allele that can be shifted to the non-permissive temperature to inactivate Rat7/Nup159 and block export pathways (46). We examined the localization of Scp160-GFP and control proteins, NLS-NES GFP and Δ RGG-Nab2-GFP, in *rat7-1* cells. Following the shift to the non-permissive temperature, Scp160-GFP remained cytoplasmic in *rat7-1* cells similar to wild-type cells (Figure 4). In contrast, NLS-NES-GFP

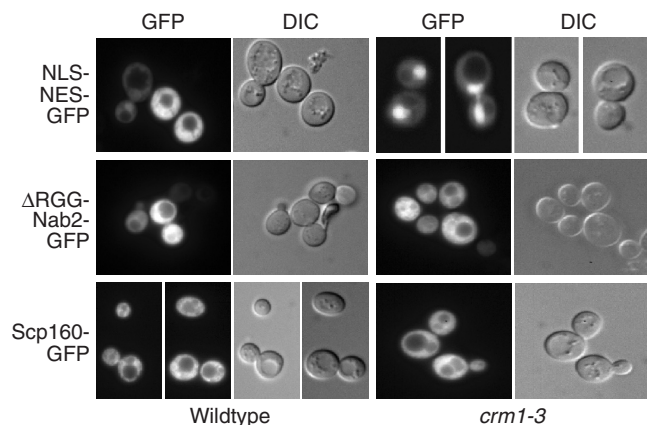


Figure 2. Localization of Scp160-GFP in *crm1-3* and wild-type cells. The localization of Scp160-GFP was examined in *crm1-3* cells following a 2 h shift to 37°C where NES-dependent nuclear export is blocked. Scp160 is localized to the cytoplasm in both wild-type and *crm1-1* cells. As controls, we also localized NLS-NES-GFP and Δ RGG-Nab2-GFP. As expected, NLS-NES-GFP accumulates in the nucleus of *crm1-1* cells, but Δ RGG-Nab2-GFP, which is exported via an mRNA export-dependent pathway, does not. Corresponding DIC images are shown.

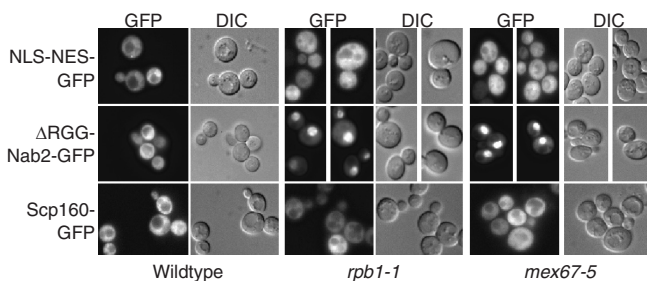


Figure 3. Localization of Scp160-GFP in *rpb1-1*, *mex67-5* and wild-type cells. The localization of Scp160-GFP was examined in wild-type, *rpb1-1* and *mex67-5* cells following a 1 h shift to 37°C. Results indicates that Scp160 is localized to the cytoplasm in both *rpb1-1* and *mex67-5* cells. As controls, we also localized NLS-NES-GFP and Δ RGG-Nab2-GFP. As expected, Δ RGG-Nab2-GFP, which is exported in an mRNA export-dependent manner, accumulates in the nuclei of both *rpb1-1* and *mex67-5* cells, but NLS-NES-GFP does not.

and Δ RGG-Nab2-GFP both accumulated in the nuclei of *rat7-1* cells. These results taken together with localization of Scp160 in other export mutants suggest that Scp160 does not enter the nucleus, at least under normal growth conditions.

In vivo function of an Scp160 NLS mutant

Based on its primary amino acid sequence, Scp160 has been predicted to contain a bipartite NLS (20). Like other previously characterized bipartite NLS sequences, Scp160 contains two stretches of basic amino acids separated by a short spacer region (Figure 5A). However, results of our localization studies suggest that the predicted NLS within Scp160 does not target the protein to the nucleus and, therefore, may not be functional. If this assessment is correct, amino acid substitutions within the key NLS

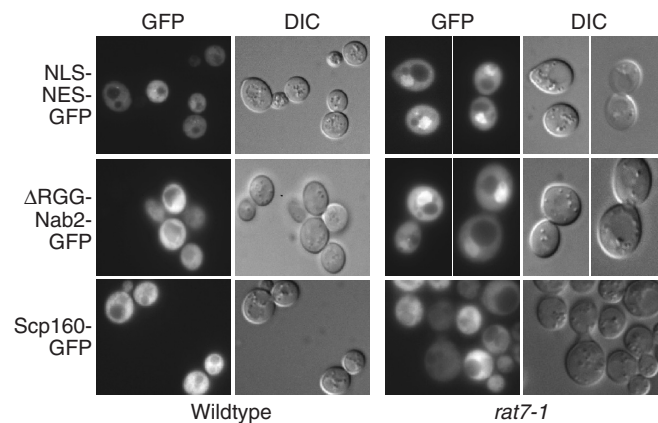


Figure 4. Localization of Scp160-GFP in *rat7-1* and wild-type cells. The localization of Scp160-GFP was examined in *rat7-1* cells following a 30 min shift to 37°C where all known transport pathways are blocked. As shown, Scp160 is localized to the cytoplasm in *rat7-1* cells. As controls, we also visualized NLS-NES-GFP and Δ RGG-Nab2-GFP. As expected both control proteins accumulate in the nuclei of *rat7-1* cells.

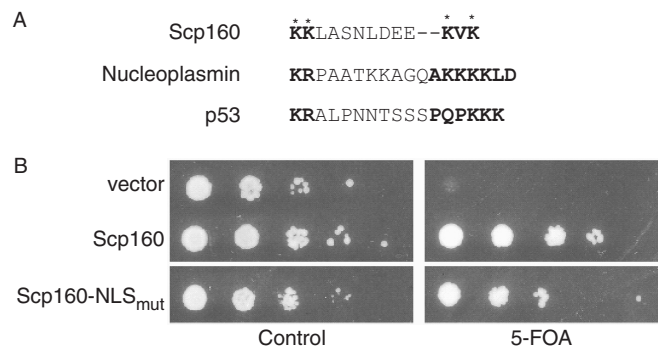


Figure 5. Functional analysis of Scp160-NLS_{mut}. (A) An alignment of the predicted NLS sequence from Scp160 with functional NLS sequences from nucleoplasmin and p53 is shown. The lysine residues that were changed to alanines to create Scp160-NLS_{mut} are indicated by asterisks. (B) To assess the functional importance of the predicted NLS within Scp160, we tested whether an Scp160 variant with a mutant NLS (Scp160-NLS_{mut}) could replace wild-type Scp160 in *Δscp160Δeap1* cells where Scp160 is required for viability (36). Cultures were grown to log phase and equal numbers of cells were serially diluted by orders of magnitude and spotted onto control plates lacking uracil and leucine (left) or test plates lacking leucine but containing 5-FOA (right). The top row shows cells carrying *LEU2* vector (pRS315); the middle row shows cells containing a wild-type copy of *Scp160*; the bottom row shows cells that express Scp160-NLS_{mut} as the sole copy of Scp160.

residues in the sequence should not significantly impact the function of Scp160. To test this hypothesis, four basic residues within the predicted NLS of Scp160 were changed to alanines to create Scp160-NLS_{mut} (residues changed are indicated by asterisks in Figure 5A). This Scp160 variant was then assayed for its ability to rescue the synthetic lethality of the *Δscp160Δeap1* cells. Growth of *Δscp160Δeap1* cells expressing Scp160-NLS_{mut} was not significantly affected compared to growth of these cells expressing wild-type Scp160 (Figure 5B). In contrast,

yeast-containing vector alone (pRS315) were unable to grow on 5-FOA medium. These results suggest that the predicted NLS does not affect the global *in vivo* function of Scp160.

DISCUSSION

This study was designed to test whether the yeast RNA-binding protein, Scp160, is a nucleocytoplasmic shuttling protein and hence to examine the function of the predicted nuclear targeting signals within Scp160. Previous studies have revealed that Scp160 is localized to the cytoplasm at steady state (22–25) and also provided evidence that Scp160 associates with specific mRNAs (19). Given the presence of sequences reminiscent of both an NLS and an NES motif (21), one possible mechanism for Scp160 function, consistent with other shuttling RNA binding proteins (26,32), is that Scp160 could enter the nucleus, associate with RNA substrates, and then accompany these RNA transcripts to the cytoplasm. However, results of our analysis indicate that Scp160 is not likely to enter the nucleus. Furthermore, the predicted NLS is not required for Scp160 function. Taken together, these experiments argue for an alternative model for Scp160 function. More generally, this study underlines the importance of experimental analysis to support the identification of putative nuclear transport signals in a protein of interest.

In this study, we localized Scp160 in a number of conditional mutants to test the hypothesis that Scp160 is able to move into the nucleus and accompany transcripts to the cytoplasm by virtue of its predicted NLS and NES signals. We localized Scp160 in mutants blocked in well-characterized export pathways, including NES-protein export (*crm1-3*), mRNA biosynthesis (*rbp1-1*), mRNA export (*mex67-5*) and all known export pathways (*rat7-1*). Despite its putative targeting sequences, Scp160 did not accumulate in the cell nucleus when any of these pathways were blocked. Furthermore, we have shown that amino acid changes within the predicted NLS of Scp160 do not severely affect Scp160 function *in vivo*. Due to the slight growth defect we observed when four lysines within the NLS were changed to alanines, we cannot eliminate the possibility that these residues are important for some other function of Scp160, however, taken together our data suggest that Scp160 does not enter the nucleus. We cannot, however, eliminate the possibility that Scp160 enters the nucleus under specific growth conditions not tested here. There is also the possibility that Scp160 export could depend on a pathway not examined here, but the nuclear pore mutant, which blocks all known export pathways, makes this interpretation of our results unlikely.

As a whole, these data suggest that Scp160 functions exclusively in the cytoplasm where it most likely associates with mRNA. A further extension of these findings is that the pleiotropic effects observed in *Δscp160* cells (20,23,48), including increased DNA content per cell, missegregation of nutritional markers through meiosis and abnormal cell morphology, are likely due to indirect

effects, perhaps caused by changes in expression of genes required for these pathways. Thus, deregulation of mRNA transcripts that bind to Scp160 may cause some of the phenotypes observed in *Δscp160* cells. Alternatively, some phenotypes may be due to loss of direct interaction of Scp160 with other proteins within the cell resulting in incompletely formed mRNP complexes. Taken with the localization studies described here, we conclude that the predicted nuclear targeting sequences in Scp160 are non-functional.

Scp160 is most closely related to the vigilin class of proteins, which, like Scp160, contain a large array of 14 to 15 repeated KH domains (21). Vigilins control gene expression through multiple mechanisms including regulating mRNA stability in the cytoplasm (49,50) and modulating heterochromatin formation in the nucleus (51,52). One piece of data that links Scp160 function with related proteins from higher eukaryotes is the finding that the *Drosophila* vigilin protein, Ddp1, can complement one phenotype associated with deletion of the *SCP160* gene in *S. cerevisiae* (48). Specifically, this study demonstrated that expression of Ddp1 in yeast cells lacking Scp160 could complement the ploidy defect observed when Scp160 is absent. However, this study did not test whether the Ddp1 entered the yeast nucleus and thus the mechanism underlying the phenotypic suppression remains unclear. Furthermore, given the vast differences in centromere sequence and organization in *S. cerevisiae* and *Drosophila* (53,54), it seems extremely unlikely that Ddp1 could complement the *Δscp160* yeast mutant by interacting with yeast centromeres. Thus, Ddp1 may share other evolutionarily conserved functions with Scp160 that have yet to be defined. Taken together, these findings suggest that the vigilin family of proteins controls gene expression through multiple mechanisms and that those functions that are located within the nucleus may be present only in higher eukaryotes. It is interesting that the role of vigilins in modulating heterochromatin is related to the RNAi pathway and that this pathway is absent from *S. cerevisiae* (55). Perhaps the nuclear function of vigilins arose in concert with the RNAi pathways.

Beyond the implications for Scp160 and vigilin function, this study highlights the importance of experimentally testing predicted targeting signals. Many proteins contain putative classical nuclear transport signals presumed to contribute to protein function in the absence of corroborating experimental evidence. Thus, caution must be used when incorporating *in silico* predictions into models for protein function.

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