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Trabajo Fin de Grado
Grado en Biología

Identification of nucleocytoplasmic transport signals in Rps15 and Ltv1, two proteins related to small ribosomal subunit export

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Abstract

Ribosomes are indispensable organelles for cell survival. Eukaryotic ribosomes are made up of two subunits (60S and 40S), which are independently exported from the nucleolus to the cytoplasm, where they are finally assembled to form the functional ribosome. Due to their large size, ribosomal subunits need nuclear transport receptors, or karyopherins (such as the export receptor XPO1), to move through the nuclear pore complex. Karyopherins recognize cargo proteins bearing nucleocytoplasmic transport signals (nuclear localization signals (NLSs) or nuclear export signals (NESs)). In addition to karyopherins, nucleocytoplasmic transport of the small ribosomal subunit (40S), requires other factors, such as the ribosomal protein Rps15 and the non-ribosomal protein Ltv1. Due to the role of these proteins in the export of small ribosomal subunit, we hypothesized that they could carry still uncharacterized nucleocytoplasmic transport signals. Here we describe the identification of three new functional nuclear transport signals: one NLS and one NES in Rps15, as well as one NLS in Ltv1. Our results provide new information on the specific amino acid sequences that contribute to the function of Ltv1 and Rps15 as adapters in the nuclear export of the small ribosomal subunit.

Resumen

Los ribosomas son orgánulos indispensables para la supervivencia de la célula. Los ribosomas eucariotas están compuestos por dos subunidades (60S y 40S), que se exportan desde el nucleolo hasta el citoplasma de forma independiente, donde son finalmente ensambladas para dar lugar al ribosoma funcional. Debido a su gran tamaño, las subunidades ribosómicas necesitan receptores de transporte nuclear, o carioferinas (como el receptor de exportación XPO1), para atravesar el complejo del poro nuclear. Las carioferinas reconocen proteínas "cargo" que portan señales de transporte nucleocitoplásmico (señales de localización nuclear (NLSs) o señales de exportación nuclear (NESs)). Además de las carioferinas, el transporte nucleocitoplásmico de la subunidad ribosómica pequeña (40S), requiere otros factores, como la proteína ribosómica Rps15 y la proteína no ribosómica Ltv1. Debido al importante papel que juegan en la exportación de la subunidad ribosómica pequeña, planteamos la hipótesis de que estas proteínas podrían poseer señales de transporte nucleocitoplásmico aún sin caracterizar. Aquí describimos la identificación de tres nuevas señales de transporte nuclear funcionales: una NLS y una NES en Rps15, así como una NLS en Ltv1. Nuestros resultados aportan nueva información sobre las secuencias de aminoácidos concretas que contribuyen al funcionamiento de Ltv1 y de Rps15 como adaptadores en la exportación nuclear de la subunidad ribosómica pequeña.

Introduction

Ribosomes are essential organelles for cell survival, as they are the machinery responsible for protein synthesis in the cytoplasm. Eukaryotic ribosomes are composed of a large (60S) subunit and a small (40S) subunit (Thomson *et al.*, 2013). The large subunit contains 5S ribosomal RNA (rRNA), 5.8S rRNA, and 25S/28S rRNA molecules (in yeast/ higher eukaryotes) as well as 46/47 ribosomal proteins (in yeast/ higher eukaryotes) whereas the small subunit contains 18S ribosomal RNA and 33 ribosomal proteins (De la Cruz *et al.*, 2015).

Ribosome synthesis begins in the nucleus with a 90S precursor that splits into two ribosomal pre-subunits: pre-60S and pre-40S. These pre-subunits must be transported to the cytoplasm, a process during which they progressively mature. Thus, the pre-60S and pre-40S subunits present in the nucleolus, mature into the pre-60S´ and pre-40S´ subunits present in the nucleoplasm. From the nucleoplasm, they are exported to the cytoplasm, where they further mature into pre-60S´´ and pre-40S´´ subunits, finally leading to the 60S and 40S mature subunits that will be assembled to form the functional (80S) eukaryotic ribosome (Figure 1) (Tschochner and Hurt., 2003).

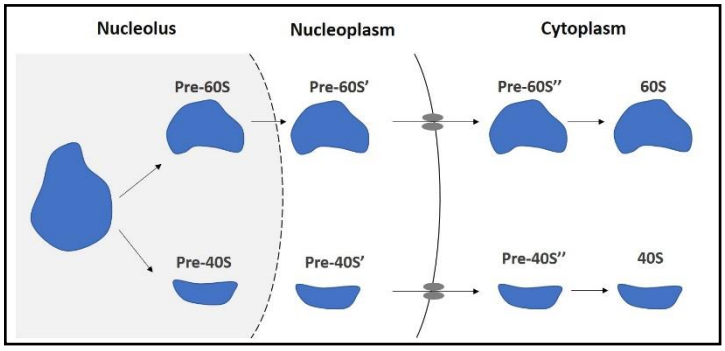

Figure 1. Schematic illustration of the process leading to the generation of mature eukaryotic ribosomal subunits. The synthesis of the 40S and 60S subunits begins with a 90S ribosomal precursor that separates into pre-40S and pre-60S in the nucleolus. Maturation of both pre-subunits continues in the nucleoplasm and they are then exported to the cytoplasm, where they complete the process to form mature 40S and 60S subunits that can be assembled intro a functional ribosome (Tschochner and Hurt, 2003).

As described above, a crucial step in ribosome synthesis is the transport of the immature subunits from the nucleus to the cytoplasm. For most proteins, including large ribonucleoprotein complexes, such as ribosomal subunits, transport between the nucleus and the cytoplasm occurs through large proteinaceous channels embedded in the nuclear envelope, known as nuclear pore complexes (NPCs). These channels are composed of multiple copies of around 30 different proteins known as nucleoporins (NUPs), which are organized in a structure with 3 stacked rings: the cytoplasmic, internal and nuclear rings (Figure 2A). Several filaments project towards the cytoplasm from the cytoplasmic ring and a basket-like structure emerges from the nuclear ring into the nucleus (Knockenhauer and Schwartz, 2016). Although small molecules can freely diffuse across the pore, the NUPs of the internal channel

form a meshwork that prevents the passage of proteins with a size over 30-60 kDa. These larger proteins, including the ribosomal subunits, can only pass through the NPC if they form a complex with members of a family of nuclear transport receptors called karyopherins (Sendino *et al.*, 2018).

Although some karyopherins can mediate bidirectional nucleocytoplasmic transport, most of them function only as nuclear import receptors (importins) or nuclear export receptors (exportins). The best-known nuclear import receptor is the Importinα/Importinβ heterodimer (Oka and Yoneda, 2018), whereas the best-characterized nuclear export receptor is the exportin XPO1 (Fung and Chook, 2014).

Karyopherins recognize and bind to particular amino acid sequences in the "cargo" proteins (i.e. the proteins that need to be transported). These amino acid sequences, which determine transport of the cargos, are globally termed "nucleocytoplasmic transport signals", and can function as nuclear localization signals (NLSs), that mediate binding to importins, or nuclear export signals (NESs), that mediate binding to exportins (Xu *et al.*, 2010). NLSs and NESs differ in their amino acid composition. NLSs recognized by the Importin α/Importin β heterodimer are generally peptide sequences with one or two clusters of basic amino acid residues (usually lysine and arginine), while the NESs recognized by XPO1 are peptide sequences with a series of hydrophobic residues (most frequently leucine) with a characteristic spacing between them (Xu *et al.*, 2010).

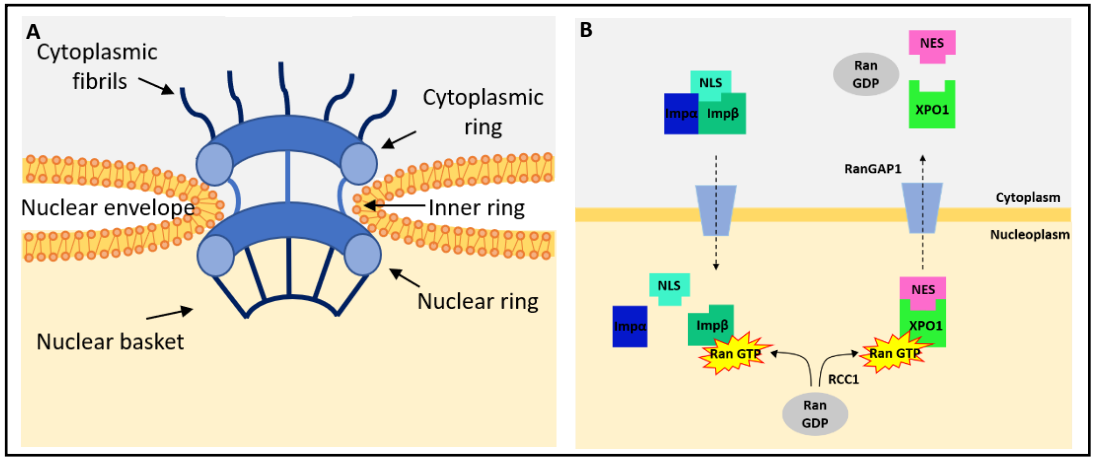

Figure 2. Nucleocytoplasmic transport of proteins mediated by karyopherins. A. Simplified illustration of the nuclear pore complex (NPC) and its main elements. Modified from Sendino *et al.,* 2018. **B.** Illustration of receptor-mediated protein transport through the NPC. Importin α/Importin β heterodimer mediates the import of a cargo protein with a NLS, whereas XPO1 mediates the export of a cargo protein with a NES. The GTPase Ran, bound to either GDP or GTP, determines transport directionality. Modified from Sendino *et al.,* 2018.

A crucial player in the process of nucleocytoplasmic transport is the small GTPase Ran, which regulates the binding and release of cargo proteins by karyopherins. A gradient of RanGTP/RanGDP is maintained across the nuclear envelope by two factors termed RanGAP1 and RCC1. The concentration of RanGTP is high in the nucleoplasm whereas the concentration of RanGDP is high in the cytoplasm. Importin/cargo complexes form in the cytoplasm and are disassembled in the nucleoplasm by RanGTP,

releasing the import cargo. On the contrary, exportin/cargo complexes assemble in the nucleoplasm, where they are stabilised by RanGTP, and are disassembled in the cytoplasm because of the hydrolysis of GTP, releasing the export cargo (Figure 2B) (Sendino *et al.*, 2018).

Due to the large size of ribosomal subunits (over 2.8 MDa the 60S, and over 1.4 MDa the 40S (Alberts *et al.*, 1999)) their movement across the nuclear pore complex requires the action of karyopherins (Moy and Silver, 1999; Sendino *et al.*, 2018), which in turn need to recognize target proteins bearing nucleocytoplasmic transport signals. Importantly, it has been shown that, in addition to karyopherins, the transport of the small ribosomal subunit from the nucleoplasm to the cytoplasm requires a series of ribosomal proteins, such as Rps15, and non-ribosomal proteins (also termed *trans*-acting factors), such as Ltv1, among others (Zemp and Kutay, 2007).

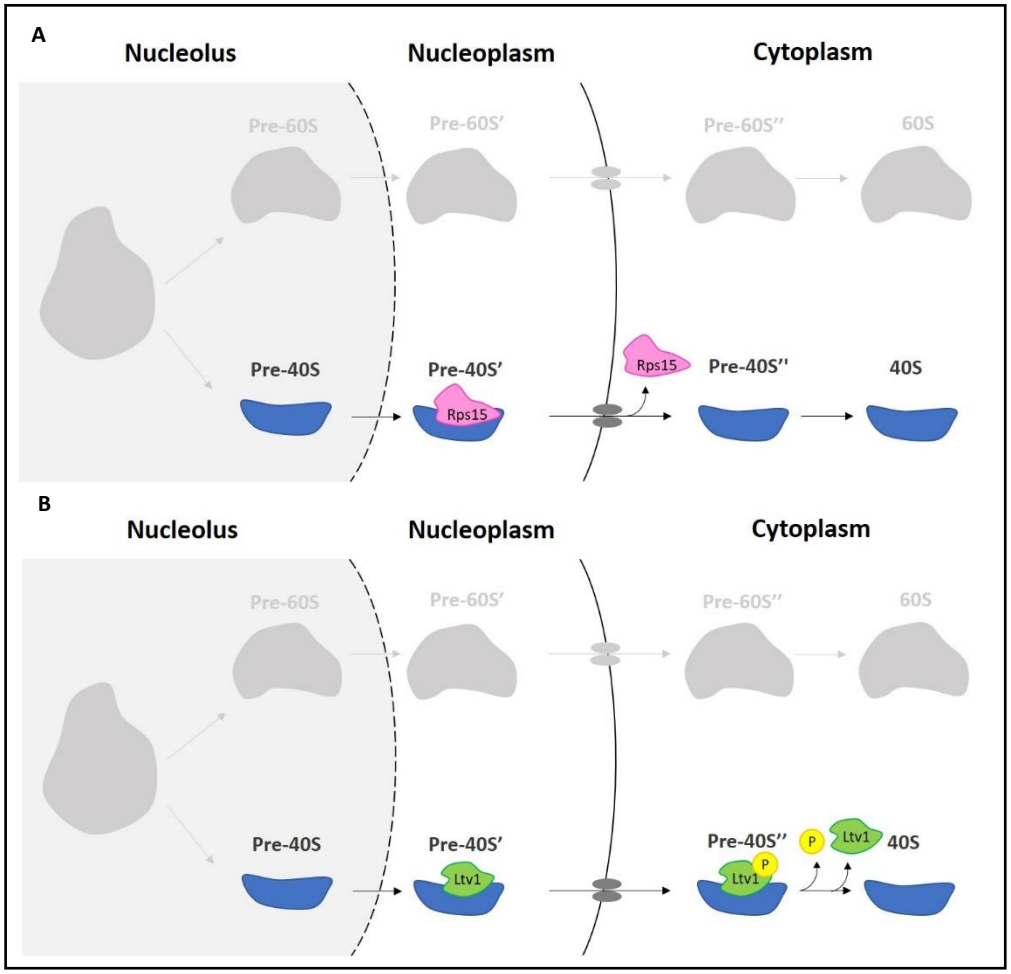

Figure 3. Role of Rps15 and Ltv1 in the formation of the 40S ribosomal subunit of *S. cerevisiae.* A. Rps15 is essential for the nuclear export of the pre-40S particle, but it is not essential for its maturation in the cytoplasm (Léger-Silvestre *et al.,* 2004). **B.** Ltv1 is a non-essential adapter for nuclear export of the pre-40S ribosomal subunit, and it is also involved on its cytoplasmic maturation (Seiser *et al.,* 2006).

Rps15 is a ribosomal protein essential for the transport but not for the maturation of the pre-40S ribosomal pre-subunit in the yeast *Saccharomyces cerevisiae* (Figure 3A) (Léger-Silvestre *et al.*, 2004)

and in mammalian cells (Rouquette *et al.*, 2005). Ltv1, on the other hand, is a non-ribosomal protein, which is involved in both the transport (as a non-essential adapter) and the maturation of the pre-40S particle in *S. cerevisiae* (Figure 3B) (Seiser *et al.*, 2006) and in mammalian cells (Ameismeier *et al.*, 2018).

Due to the important role that Ltv1 and Rps15 play in the transport of small ribosomal subunits to the cytoplasm, we hypothesized that these proteins might contain one or more nucleocytoplasmic transport signals (NESs or NLSs). While a NES has been already identified in Ltv1 (Figure 4A) (Merwin *et al.*, 2014), no transport signals have yet been identified in Rps15 (Figure 4B).

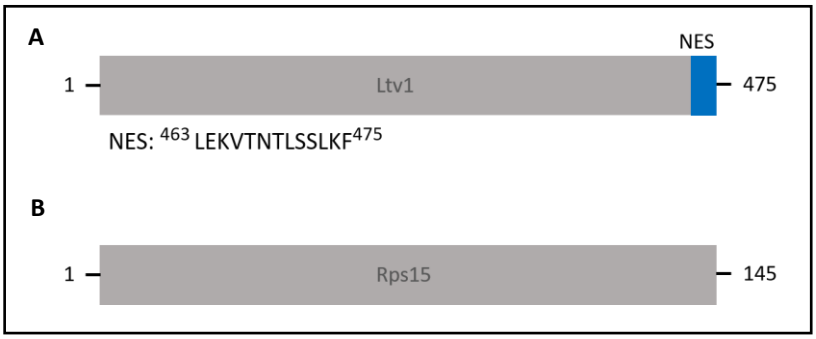

Figure 4. Schematic representation of Ltv1 and Rps15 illustrating current knowledge on their nucleocytoplasmic transport signals. A. Representation of Ltv1 indicating the position and amino acid sequence of an already identified NES motif (Merwin *et al.*, 2014). **B**. Representation of Rps15, showing that no NES or NLS motifs have been yet described.

The objective of the present study is to investigate if, besides the already identified NES of Ltv1, this protein and Rps15 bear other functional nucleocytoplasmic transport signals (NESs or NLSs).

Materials and Methods

1. Prediction of nuclear export and nuclear localization signals with bioinformatic tools

The protein sequences of human Ltv1 (475 amino acids; NCBI Reference Sequence: NP_116249.2) and Rps15 (145 amino acids; NCBI Reference Sequence: NP_001009.1) were retrieved using NCBI. The search for potential NESs was carried out with the following prediction tools: NetNES (La Cour *et al.*, 2004; http://www.cbs.dtu.dk/services/NetNES/), LocNES (Xu *et al.*, 2014; (http://prodata.swmed.edu/LocNES/LocNES.php) and WREGEX (Prieto *et al.*, 2014; http://ehubio.ehu.eus/wregex/), whereas the search for potential NLSs was carried out using PSORT II (Nakai and Horton, 1999; https://psort.hgc.jp/form2.html).

2. Cloning procedures

2.1. Design of a geneBlock DNA fragment encoding potential nuclear transport signals

The cDNA sequences coding for human Rps15 (NCBI Reference Sequence: NM_001018.4) and Ltv1 (NCBI Reference Sequence: NM_032860.5) proteins were obtained from NCBI. A geneBlock (gBlock), which is a commercially available synthetic double stranded DNA fragment, was designed to encode the amino acid sequences of the candidate NESs and NLSs. The sequence of the gBlock included Bam HI/Age I restriction sites flanking the cDNA of the candidate NESs and Hind III/Bam HI restriction sites flanking the cDNA of the candidate NLSs. These enzymes were chosen because the products of the digestion of the gBlock will be cloned into vectors opened with those enzymes. Using the Nebcutter webtool (http://nc2.neb.com/NEBcutter2/), we confirmed that there were no undesired cutting sites for these restriction enzymes elsewhere in the gBlock sequence. Then, a 387 nucleotide gBlock was purchased from IDT (Integrated DNA Technologies).

2.2. Digestion of the gBlock

Digestion of the gBlock with Bam HI, Age I and Hind III restriction enzymes (Thermo) was carried out in a single reaction using Bam HI buffer, whose suitability for simultaneous digestion with the three enzymes was confirmed using the "Thermo-digest" tool (https://www.thermofisher.com/es/es/home/brands/thermo-scientific/molecular-biology/thermo-scientific-restriction-modifying-enzymes/restriction-enzymes-thermo-scientific/double-digest-calculator-thermo-scientific.html). After digestion, DNA fragments were purified employing QIAquick ® PCR Purification Kit (Qiagen) following the manufacturer´s instructions.

2.3. Ligation and bacterial transformation

Purified gBlock fragments were cloned into Bam HI/Age I-digested Rev (1.4)-GFP vector (Henderson and Eleftheriou, 2000) (Figure 5A) and into Hind III/Bam HI-digested pEYFP-C1 vector (Clontech) (Figure 5B).

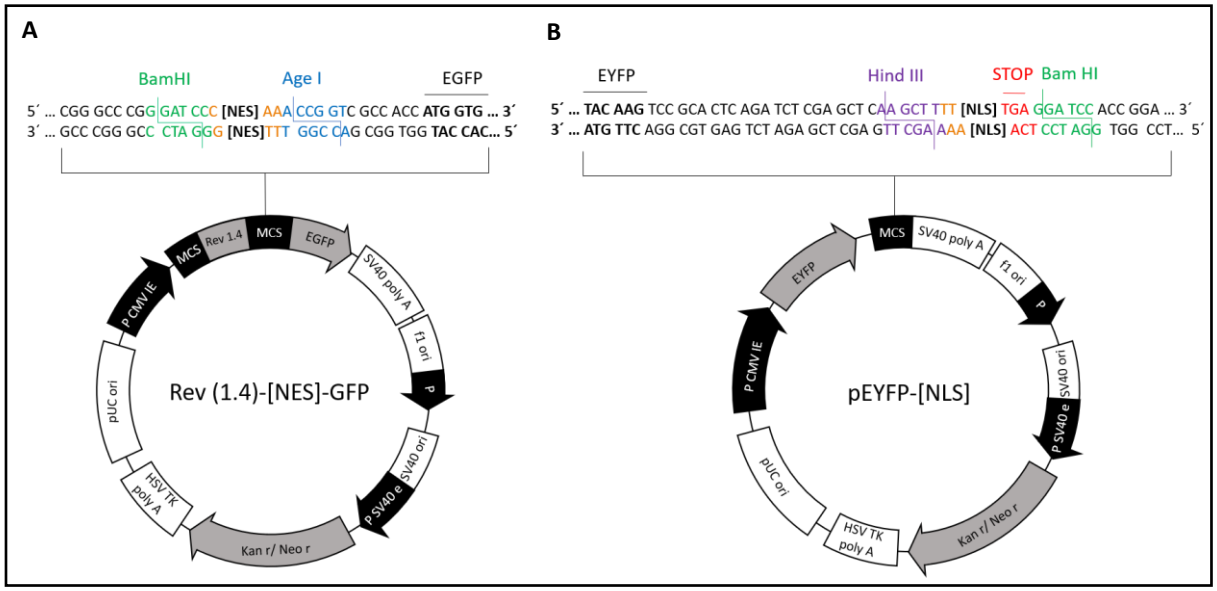

Figure 5. Details of the vectors employed for cloning. The Rev (1.4)-[NES]-GFP **(A)** and pEYFP-[NLS] **(B)** plasmid maps and multiple cloning sites (MCS) are shown. The arrows indicate the direction of the transcription from the promoters included in the plasmids. A detailed view of the MCS sequence is shown above the maps, with the used restriction sites indicated in green (Bam HI), blue (Age I) and purple (Hind III). Nucleotides added to maintain the reading frame are indicated in orange, and the STOP codon in red. Modified from the original vector maps provided by Clontech. EGFP: enhanced green fluorescent protein; EYFP: enhanced yellow fluorescent protein.

For each cloning, two separate ligations with different amounts of insert were set up, to increase the probabilities of obtaining the desired outcome (i.e. introduction of a single copy of the insert into the vector).

The ligation mix contained: water, 10x Ligase buffer, 10 mM ATP, digested vector, inserts and T4 DNA ligase (Thermo) in a final volume of 10 µl. As a control to estimate the potential religation capacity of the empty vectors, ligations without insert were simultaneously set up. Ligation reactions were performed overnight at 4 ºC.

The products of ligation reactions were transformed by heat shock (from ice to 37 ºC for 30 seconds and back to ice) into chemically competent *Escherichia coli* DH-5α bacteria. Then, 400 µl of transformed bacteria were plated on kanamycin-containing lysogeny broth (LB)-agar plates and incubated overnight at 37 ºC. Next day, 8 colonies from Rev (1.4)-GFP plates and 4 colonies from pEYFP-C1 plates were randomly selected, picked and grown overnight at 37 ºC in a tube with 10 ml of LB medium containing kanamycin. Then, the efficiency of the cloning procedure was evaluated using PCR to test the presence of inserts in the selected colonies.

2.4. Checking cloning efficiency by PCR and electrophoresis

A 500 µl aliquot from the 10 ml of bacterial culture was taken for DNA extraction using a quick protocol, as follows. The aliquots were centrifuged 5 minutes at 13000 rpm, the supernatant was removed and the bacterial pellet was resuspended in 50 µl of water. Resuspended bacteria were incubated at 100 ºC for 5 min, and then centrifuged for 3 min at 13000 rpm. The supernatant (containing bacterial and plasmid DNA) was transferred to a new tube. One µl of each supernatant was used as template DNA in PCR reactions with oligonucleotide primers (sequences not shown) flanking the cloning site in Rev (1.4)-GFP (vector for NES cloning) and pEYFP-C1 (vector for NLS cloning). The size of the amplified PCR products allows to determine the presence or absence of a cloned insert (Figure 6). For NES cloning, the UJAR 3 oligonucleotide was used as forward primer and the Rev seq oligonucleotide as reverse primer. In the case of the NLS cloning, UJAR 17 and UJAR 18bis oligonucleotides were employed as forward and reverse primers, respectively.

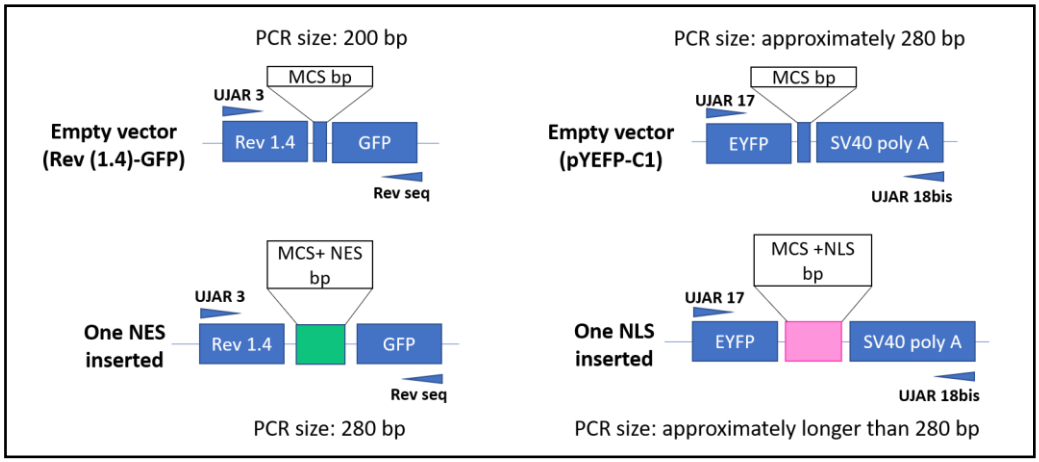

Figure 6. Schematic representation of the PCR approach used to check the presence of inserts, showing the expected size of amplified products. The size of the PCR product obtained with the empty vector (Rev (1.4)-GFP) is expected to be shorter than the size obtained with a plasmid containing the cloned NES. Similarly, the size of the PCR product obtained with the empty vector (pEYFP-C1) is expected to be shorter than the size obtained with a plasmid containing the cloned NLS. MCS: multiple cloning site.

Empty Rev (1.4)-GFP and pEYFP-C1 vector DNA were amplified as negative control (no insert present). A previously cloned Rev (1.4)-[ELM29NES]-GFP plasmid (Garcia-Santisteban *et al.*, 2012) was used as positive control for the presence of a single NES insert. No positive control for NLS insert was used because, while the NESs are always cloned as 19 amino acid segments, cloned NLSs have variable size.

The amplification products were analysed on a 1.5% agarose gel.

2.5. Plasmid miniprep, quantification and Sanger sequencing

Once the colonies that appeared to have acquired the intended inserts were identified by PCR, purified plasmid DNA from these colonies was obtained for use in subsequent steps (sequencing and transfection). Plasmid purification was carried out using QIAprep ® Spin Miniprep Kit (Qiagen) following manufacturer´s protocol.

A NanoDrop ™ spectrophotometer was used to determine the concentration of purified plasmid DNA. Finally, the presence of the correct cDNA encoding the candidate NES and NLS was confirmed using Sanger sequencing. For this purpose, aliquots of the plasmid DNA (at a concentration of 100 ng/µl) were sent to the STAB VIDA, a company that provides DNA sequencing services.

3. Cellular assays

After full confirmation of the successful cloning of candidate NESs and NLSs, cellular assays were carried out to experimentally determine if these predicted nuclear transport signals were functional.

3.1. Cell culture and transfection

HeLa cells were grown in DMEM (Dulbecco´s Modified Eagle´s Medium) supplemented with 10% foetal bovine serum, 100 units/ml penicillin and 100 µg/ml streptomycin (Invitrogen). For transfection, cells were seeded on glass coverslips in 12-well tissue culture plates. 24 h later, plasmids containing NES/ NLS candidate, as well as control plasmids, were transfected into the cells. As negative controls, the empty Rev (1.4)-GFP and pEYFP-C1 vectors were used. As positive controls for nuclear export and import, respectively, a plasmid containing a functional NES (Rev (1.4)-[ELM29NES]-GFP) (Garcia-Santisteban *et al.*, 2012) and a plasmid containing two functional NLSs (pEYFP-[2XNLS]) (Olazabal-Herrero *et al.*, 2019) were used. DNA transfections were carried out with X-tremeGENE ™ 9 DNA Transfection Reagent (Roche), according to the manufacturer´s protocol.

3.2. Nuclear export assay and evaluation of NLS function

To experimentally test the function of the predicted NESs, a nuclear export assay was carried out as previosuly described (Henderson and Eleftheriou, 2000). Briefly, 24 h after the transfection, cells expressing Rev (1.4)-GFP plasmids containing candidate NESs were treated for 3 h as indicated in Figure 7A. The treatment consisted of cycloheximide (CHX; Sigma) together with acinomycin D (ActD; Sigma) or CHX alone. CHX (at a final concentration of 10 µg/ml) is used to inhibit protein translation and, consequently, to prevent synthesis of new protein in the cytoplasm. This ensures that the fluorescent signal present in the cytoplasm is due to nuclear export. ActD (at a final concentration of 5µg/ml) was added to stop the nuclear import mediated by Rev (1.4) NLS (Henderson and Eleftheriou, 2000), thereby making nuclear export activity of weak NESs more noticeable.

On the other hand, the functionality of candidate NLSs was determined by comparing the localization of the fluorescent proteins fused to these sequences with the localization of a fluorescent protein bearing no NLS (pEYFP-C1, negative control) or fused to two copies of the SV40 large T antigen NLSs (pEYFP-[2XNLS], positive control) Figure 7B.

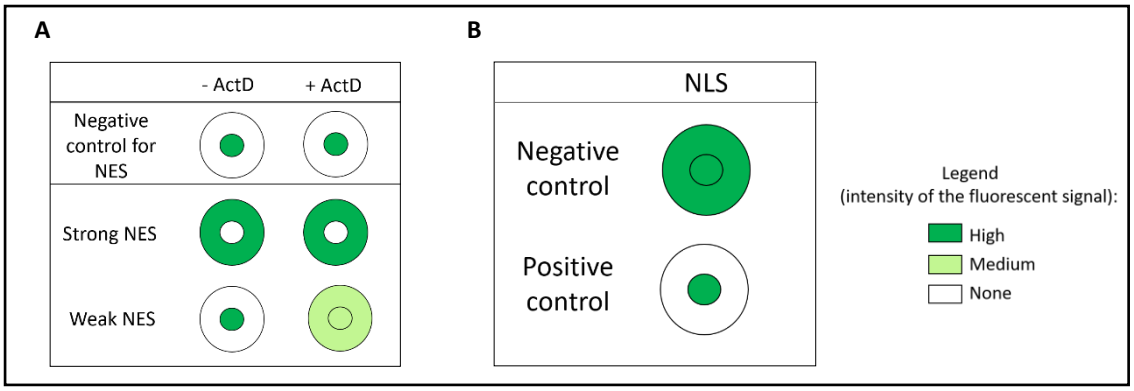

Figure 7. Schematic representations of the cellular assays used to evaluate the activity of nucleocytoplasmic transport signals. Transfected cells are depicted as circles, with the inner circle representing the nucleus. The darker is the colour, the higher is the intensity of the fluorescent signal in the corresponding compartment. **A**. Nuclear export assay for testing NESs activity. In the negative control (empty vector), the localization of the fluorescent protein is nuclear, both if cells are treated only with CHX (-ActD) or with CHX and ActD (+ActD). For strong NESs the localization of the fluorescent protein is cytoplasmic in both conditions. For active, but weak NESs, the cytoplasmic signal becomes more noticeable by blocking Rev (1.4)-mediated import with ActD treatment. **B**. Cellular transport assay for NLSs. In the negative control (empty vector), the localization of the fluorescent protein (EYFP) is nuclear+cytoplasmic, whereas in the positive control (plasmid with two functional NLSs), the localization of the fluorescent protein is nuclear.

3.3. Fluorescence microscopy analysis

Cells expressing fluorescent proteins were washed with PBS and fixed with 3.7% formaldehyde during 30 min. After washing with PBS again, cells were mounted onto microscope slides using a drop of VECTASHIELD ® mounting medium (Vector Laboratories), which contains DAPI to stain the cell nucleus. Cells were observed with a 20x objective using a Zeiss Axioskop fluorescence microscope to carry out the cell counting. To establish the level of activity of each NES, the localization of the fluorescent proteins (nuclear, nuclear+cytoplasmic or cytoplasmic) was determined in more than 200 cells per sample. Using this semiquantitative localization data, and the scoring method previously described (Henderson and Eleftheriou, 2000), functional NESs are assigned a score in a range between 1+ (the lowest, corresponding to very weak NESs) and 9+ (the highest, corresponding to very active NESs). On the other hand, the localization of the florescent proteins was also determined for the cells transfected with the NLS-encoding plasmids by counting 200 cells, but the NLS were classified only as functional or non-functional, with no assessment of NLS strength. The images were taken with a 40x objective using the NIS software (Nikon) and processed using Fiji (Fiji Is Just ImageJ; Schindelin *et al.*, 2012).

Results

In order to evaluate the potential presence of still uncharacterized NESs and NLSs in our target proteins, we carried out a series of steps, schematically illustrated in Figure 8: first, the amino acid sequences of Ltv1 and Rps15 were retrieved, next, candidate NESs and NLSs motifs were predicted using bioinformatic analysis and the predicted motifs were cloned. Finally, their function was experimentally tested using cellular transport assays.

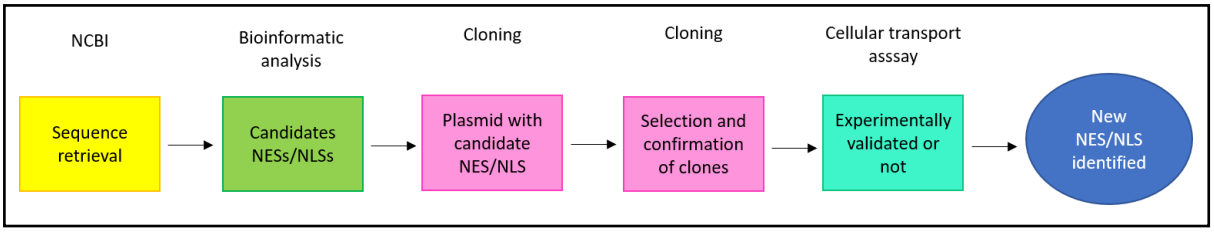

Figure 8. Flow diagram illustrating the steps of the analysis carried out to identify nucleocytoplasmic transport signals in the target proteins. In the first step, the protein sequence was retrieved using NCBI. Then, candidate NESs and NLSs were predicted by bioinformatic analysis, and cloned into suitable vectors. After selection and confirmation of correctly cloned NESs and NLSs, cellular transport assays were performed to experimentally test their functionality.

1. *In silico* prediction of nuclear export and nuclear localization signals in Rps15 and Ltv1

In order to determine the presence of potential nuclear transport signals in Rps15 and Ltv1 we carried out a bioinformatic prediction of candidate NESs and NLSs in these proteins using three NES prediction programs: NetNES, LocNES, WREGEX and one NLS prediction program: PSORT II.

Table 1. Candidate NESs and NLS for Rps15 and candidate NLS for Ltv1 predicted *in silico*.

.

	NES prediction with different tools (scores)			NLS prediction
Protein	NetNES	LocNES (score 0-1)	WREGEX (score 0-100)	PSORT II
Rps15	22-LDQLLDMSYEQL-33	8-KKRTFRKFTYRGVDL-22 (0.009)	26-LDMSYEQLMQL-36 (53.4)	50-RRKQHSLLKRLRKAKKE-66
	78-THLRPMII-85	71-EKPEVVKTHLRDMII-85 (0.162)	76-VKTHLRDMIIL-86 (43.3)	
		72-KPEVVKTHLRDMIIL-86 (O.309)	111-MIGHYLGEFSI-121 (35.1)	
		79-HLRDMIILPEMVGSM-93 (0.432)		
		82-DMIILPEMVGSMVGV-96 (0.052)		
		107-IKPEMIGHYLGEFSI-121 (0.033)		
Ltv1	Not analysed	Not analysed	Not analysed	427-KRARKQAIKEERKERRVEKKANKLAFKLEKRRQEK- 461

In the case of Rps15, we searched for both potential NESs and potential NLSs. In the case of Ltv1, we only searched for potential NLSs, because a functional NESs has been already described in

this protein (Merwin *et al.* 2014). As shown in Table 1, several candidate transport signals were identified with these *in silico* analyses. Since experimental testing of all these candidates was not feasible, those NESs predicted by more than one program and having the highest prediction scores, were selected to be tested. On the other hand, only one candidate NLS was predicted for each protein and both sequences were tested.

Thus, we selected the sequences indicated in Table 2 for experimental testing. Selected candidate include the Rps15 NES1, predicted with NetNES and WREGEX (Score 53.4), and the Rps15 NES2, predicted by NetNES, LocNES (Score 0.309) and WREGEX (Score 43.3). We also selected the Ltv1 NLS and the Rps15 NLS predicted with PSORT II (Table 2). The position of the selected candidate NESs and NLSs on each protein is schematically illustrated in Figure 9.

Table 2. Selected NESs and NLSs for Rps15 and for Ltv1. Several NES and NLS-flanking residues of Rps15 were also included to reach 19 amino acid for NESs and 20 amino acid for the NLS.

Rps15	Ltv1
NES1 (57 bp): 21-DLDQLLDMSYEQLMQLYSA-39	NLS (105 bp): 427-KRARKQAIKEERKERRVEKKANKLAFKLEKRRQEK- 461
NES2 (57 bp): 71-EKPEVVKTHLRDMIILPEM-89	
NLS (60 bp): 48-GLRRKQHSLLKRLRKAKKEA-67	

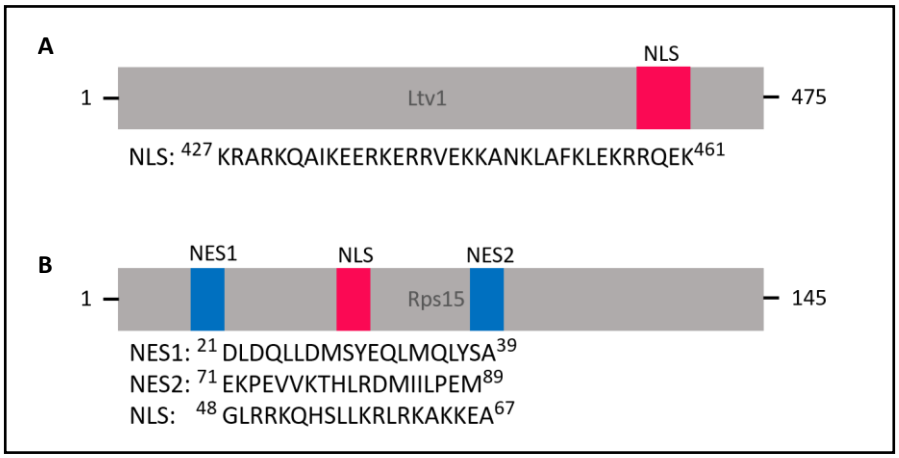

Figure 9. Schematic representation of Ltv1 and Rps15 proteins indicating the position and sequence of the predicted NESs and NLSs selected for experimental testing. A. Ltv1 showing the position and amino acid sequence of the potential NLS motif identified using the prediction webtools. **B**. Rps15 showing the position and amino acid sequence of the potential nuclear transport signals (NLS, NES1 and NES2) identified using the prediction webtools.

2. Cloning of predicted nuclear transport signals

As described in the Materials and Methods section, DNA sequences coding for selected candidate NESs and NLSs were obtained as a gBlock (Figure 10), and cloned into Rev (1.4)-GFP vector (for NESs) or into pEYFP-C1 vector (for NLSs).

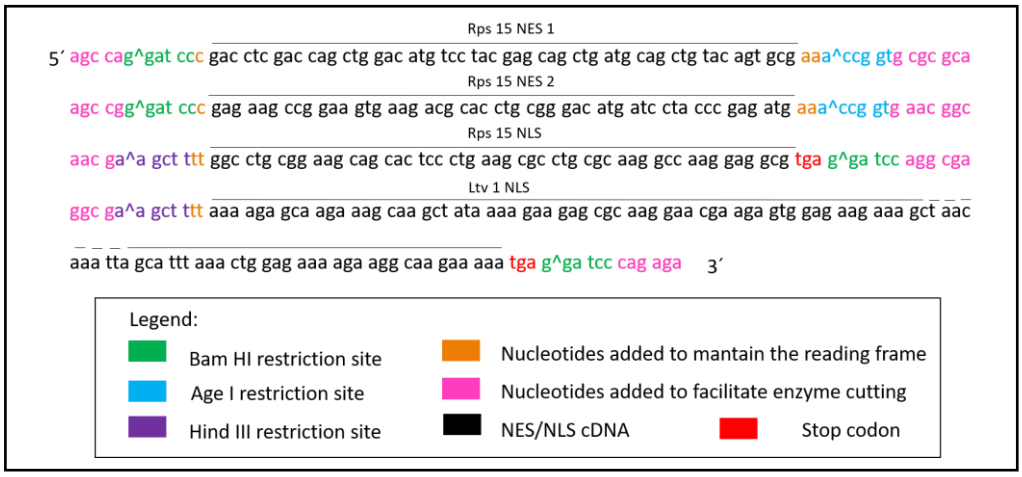

Figure 10. DNA sequence of the gBlock. Nucleotide colors indicate the different elements included in the designed DNA sequence, as detailed in the legend.

The aspect of the bacterial plates after transformation (Figure 11), suggested that the cloning procedure had worked correctly. Thus, there was little or no bacterial growth in the negative control plates (A and D), indicating a low religation capacity of the empty vectors. In contrast, many colonies were observed in the plates with bacteria transformed with insert-containing ligations, and there was a correlation between the amount of insert used and the number of colonies in the plates.

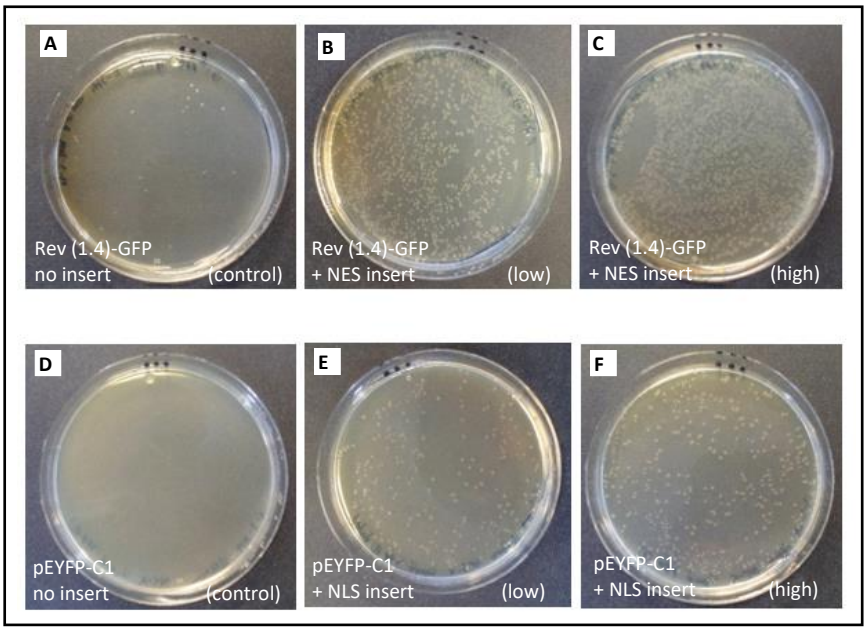

Figure 11. ***Escherichia coli*** **DH-5α bacterial colonies grow in LB-agar plates with kanamycin. Comparison of the densities in the plates according to the different transformations performed**. **A.** Negative control for NES cloning. **B.** Bacteria transformed with a ligation containing lower amount of NES insert. **C.** Bacteria transformed with a ligation containing high amount of NES insert. **D.** Negative control for NLS cloning. **E.** Bacteria transformed with a ligation containing lower amount of NLS insert. **F.** Bacteria transformed with a ligation containing high amount of NLS insert.

In order to screen for the presence of the desired insert, a mixture of genomic and plasmid DNA was extracted from several colonies (8 from the plate B in Figure 11 and 4 from the plate E in Figure 11) using a quick DNA extraction protocol. This DNA was amplified using PCR, and the size of the amplified PCR product was determined using agarose gel electrophoresis. For NESs, the expected amplicon size will be around 200 bp (the same as the negative control) if no insert is present and around 280 bp (the same as the positve control) if one copy of the insert is present. In the case of the NLS, the band size will be around 280 bp (the same as the negative control) is no insert is present, and variable bp (depending on the length of the cloned NLS, but always longer than 280 bp) if the insert is present.

As shown in Figure 12A, the size of the amplified fragment for the 8 bacterial colonies transformed with Rev (1.4)-[NES]-GFP was the same as that of the positive control. This result indicates that the 8 colonies tested had acquired one copy of the insert. On the other hand, the screening for correct NLS clones was not as successful. Thus, the size of the amplified fragments for the 4 bacterial colonies transformed with pEYFP-[NLS], suggested that only colony number 2 had acquired one copy of the insert. The size of the amplicon suggested that colony number 1 and 3 contained the empty vector whereas colony number 4 seemed to have acquired more than one insert units (Figure 12B). Thus, 8 new colonies were picked from the plate and their DNA was extracted and checked by PCR in order to identify more colonies containing one single copy of the NLS insert.

A first PCR reaction to test these 8 new colonies failed due to unknown technical issues (not shown). A second attempt, using a new *Taq* polymerase buffer and a new set of UJAR 17 and UJAR 18bis primers was successful. It was observed that only colonies 10 and 12 seemed to have acquired one NLS insert. The size of the amplified band suggested that colonies 5, 6, 7, 9 and 11 contained the empty vector and colony 8 seemed to have acquired more than one insert (Figure 12C).

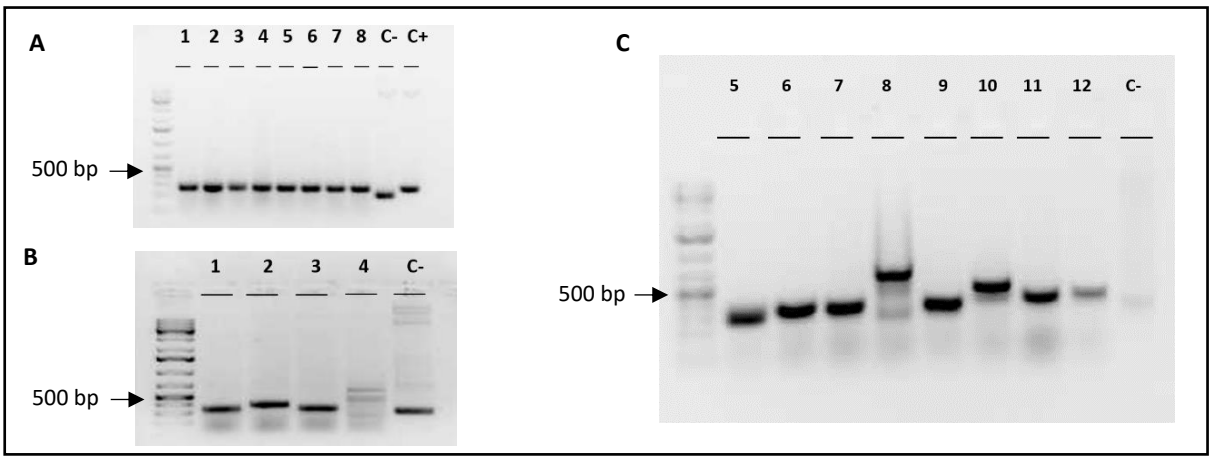

Figure 12. Results of agarose gel electrophoresis to screen the presence of desired inserts. A. Result of the analysis of bacterial colonies transformed with Rev (1.4)-[NES]-GFP containing either Rps15 NES1 or NES2. All colonies seemed to have acquired one single insert. **B**. Result of the analysis of bacterial colonies transformed with pEYFP-[NLS] containing either Rps15 NLS or Ltv1 NLS. Colony 2 seemed to have acquired one single insert. Colonies 1 and 3 seemed to be empty. Colony 4 seemed to have acquired more than one insert. **C**. Result of the analysis of eight additional bacterial colonies transformed with pEYFP-[NLS]. Colonies 10 and 12 seemed to have acquired one single insert. Colonies 5, 6, 7, 9 and 11 seemed to be empty. Colony 8 seemed to have acquired more than one insert.

In conclusion, NESs colonies 1, 2, 3 and 4, as well as NLS colonies 2, 10 and 12 were considered to most likely contain a single copy of the corresponding insert after the PCR screening, and were therefore selected for further analysis.

Plasmid DNA was purified from these colonies and sequenced using the Sanger method. Sequencing is necessary for two reasons. First, because there are two possible NESs and two possible NLS inserts, and it is necessary to identify the sequence cloned into each plasmid. And secondly, it is necessary to ensure that there are no mistakes in the cloned sequence (Figure 13).

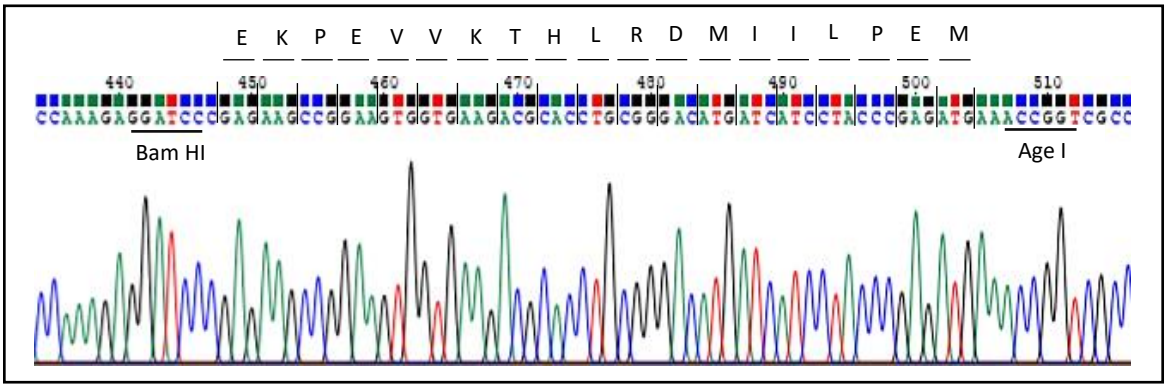

Figure 13. **Example of the results of Sanger sequencing obtained with one of the plasmids from NES cloning.** The result of the analysis shows that this plasmid (purified from colony 1) contains the DNA sequence encoding Rps15 NES2, and confirms that there are no unwanted mistakes. Rps15 NES2, amino acid sequence is shown above the DNA sequence. Following successful confirmation, this plasmid was named Rev (1.4)-[Rps15NES2]-GFP.

For NES cloning, DNA sequencing revealed that colonies 1, 2 and 3 had acquired one copy of Rps15 NES2, whereas colony 4 had acquired one copy of Rps15 NES1. For NLS cloning, DNA sequencing revealed that colonies 2 and 12 had incorporated one single copy of the insert encoding Ltv1 NLS. In all cases, the nucleotide sequences contained no unwanted mistakes. Of note, we found that colony 10 contained two tandem copies of Rps15 NLS. However, there was a stop codon at the end of the first copy (Figure 14) and thus, this plasmid would express EYFP fused to one single copy of Rps15 NLS. Therefore, we decided to use this plasmid in subsequent cellular experiments.

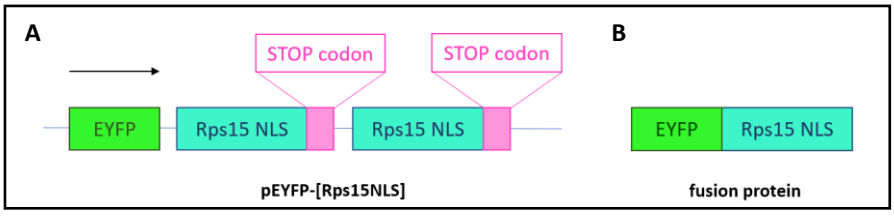

Figure 14. Plasmid with two tandem copies of Rps15 and the fusion protein expected to be expressed in cells transfected with this plasmid. A. Schematic representation of a fragment of the pEYFP-[Rps15NLS] plasmid, showing the two tandem copies of Rps15 NLS that have been cloned. The translation finishes with the stop codon after the first copy. Arrow indicates the direction of the translation. **B.** The protein product resulting from expression of this plasmid is the enhanced yellow fluorescent protein EYFP fused to one single copy of Rps15 NLS.

3. Evaluating the activity of candidate nuclear transport signals in Rps15 and Ltv1 using cellular assays

3.1. Evaluating Rps15 candidate NESs activity using an *in vivo* nuclear export assay

Hela cells were transfected with the Rev (1.4)-GFP derived plasmids containing Rps15 NES1 and NES2. Cells transfected with the empty Rev (1.4)-GFP vector were used as negative control, whereas cells transfected with a plasmid containing a known functional NES (Rev (1.4)-[ELM29NES]-GFP) (Garcia-Santisteban *et al*., 2012) were used as positive control. As shown in Figure 15, the localization of the fluorescent protein in the negative control cells was nuclear with prominent accumulation in the nucleoli. In positive control cells, the transfection rate was low, but the localization of the fluorescent protein in the few transfected cells observed in this sample was cytoplasmic (not shown). The localization of the protein encoded by the plasmid with Rps15 NES1 was nuclear+cytoplasmic or nuclear. On the other hand, the localization of the fluorescent protein in cells transfected with NES2 was nuclear, similar to the negative control.

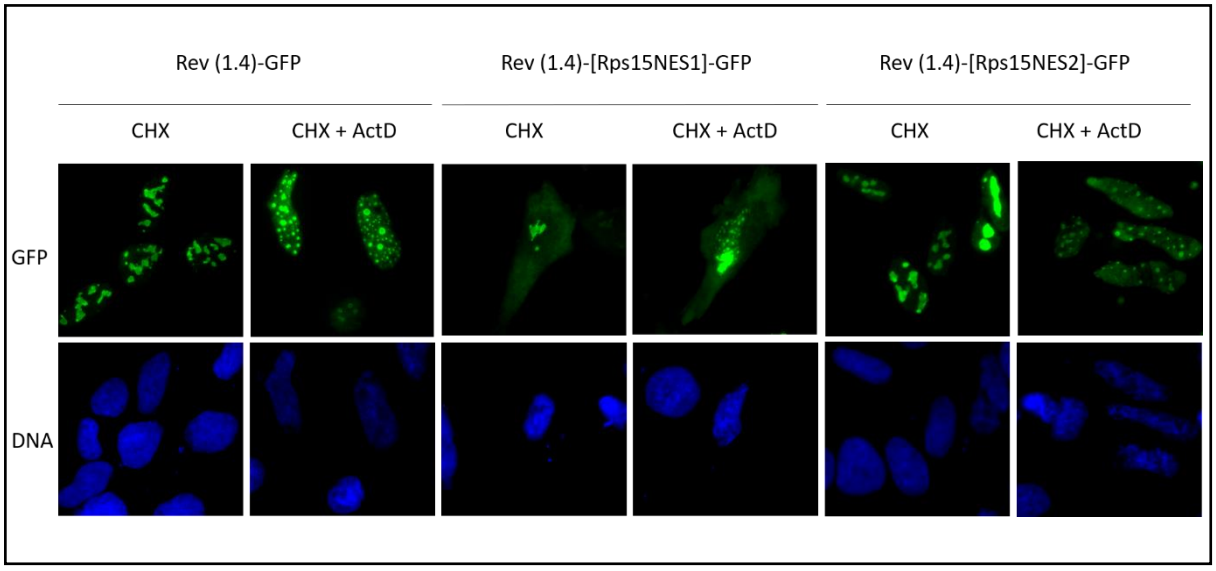

Figure 15. Results of the *in vivo* nuclear export assay for testing the activity of Rps15 NES1 and Rps15 NES2. Cells were treated for 3 h with CHX alone or with CHX and ActD. *Left.* Cells transfected with Rev (1.4)-GFP. *Centre.* Cells transfected with Rev (1.4)-[Rps15NES1]-GFP. *Right.* Cells transfected with Rev (1.4)-[Rps15NES2]-GFP.

To semi-quantitatively determine the proportion of cells with different subcellular localization of the fluorescent proteins, a cell counting procedure was carried out. As mentioned above, the transfection rate was low in positive control cells, and no cell counting was done in this sample. Nevertheless, the exclusively cytoplasmic localization of the fluorescent proteins in the few transfected cells indicated that the control was correct.

As shown in Figure 16, the localization of the fluorescent protein in the negative control and Rps15 NES2 samples, was almost exclusively nuclear even after treatment with ActD. In the case of

Rps15 NES1 the localization of the fluorescent protein was mainly nuclear in the absence of ActD, but it became partially nuclear+cytoplasmic in the presence of ActD, which suggests that this sequence is a weak NES.

In summary, the results of the nuclear export assay indicate that Rps15 NES 1 is functional with an activity level of 1+ (Henderson and Eleftheriou, 2000), while Rps15 NES2 is not functional.

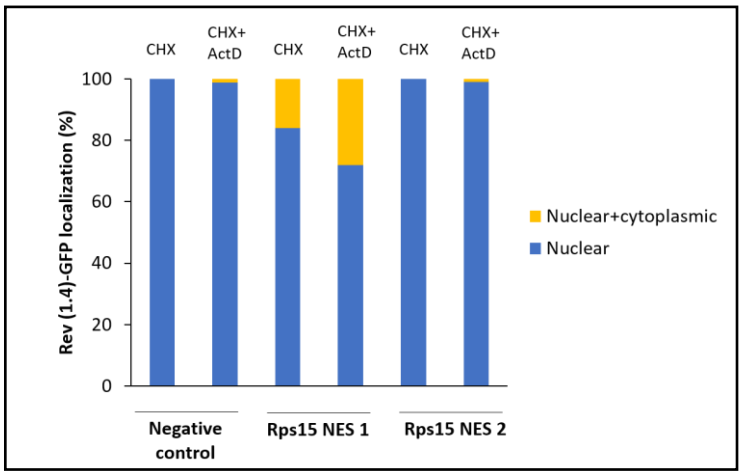

Figure 16. Graphs showing the percentages of cells with different subcellular localization of the fluorescent protein, Rev (1.4)-GFP. It is observed that Rps15 NES2 is not functional, showing the same localization as the negative control protein under both treatments. Rps15 NES1 is functional, with a nuclear expression of 84% nuclear and 16% nuclear+cytoplasmic under the treatment of CHX and an expression of 72% nuclear and a 28% nuclear+cytoplasmic after treatment with CHX + ActD.

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3.2. Evaluating the nuclear import activity of Rps15 and Ltv1 candidate NLSs

Hela cells were transfected with the pEYFP-C1 plasmids containing Rps15 NLS and Ltv1 NLS. As negative control, cells were transfected with the empty pEYFP-C1 vector. As positive control, cells transfected with a plasmid containing two functional NLSs (pEYFP-[2XNLS**]).** As shown in Figure 17, the localization of fluorescent protein (EYFP) was nuclear+cytoplasmic in the negative control cells. In the positive control cells, the fluorescent proteins were accumulated in the nucleus, more prominently in the nucleoli. Similar to the positive control, the localization of EYFP-Rps15 NLS and EYFP-Ltv1 NLS proteins was nuclear with higher intensity in the nucleoli.

A cell counting was carried out to establish the proportion of cells with each subcellular localization of fluorescent proteins. As shown in Figure 18, the localization of the fluorescent proteins was exclusively nuclear+cytoplasmic in the negative control cells. For the positive control cells, as well as the cells expressing EYFP fused to Rps15 NLS and Ltv1 NLS, the localization of the fluorescent proteins was exclusively nuclear.

These results clearly show that Rps15 NLS and Ltv1 NLS are functional nuclear localization signals that mediate nuclear import of the fused EYFP protein.

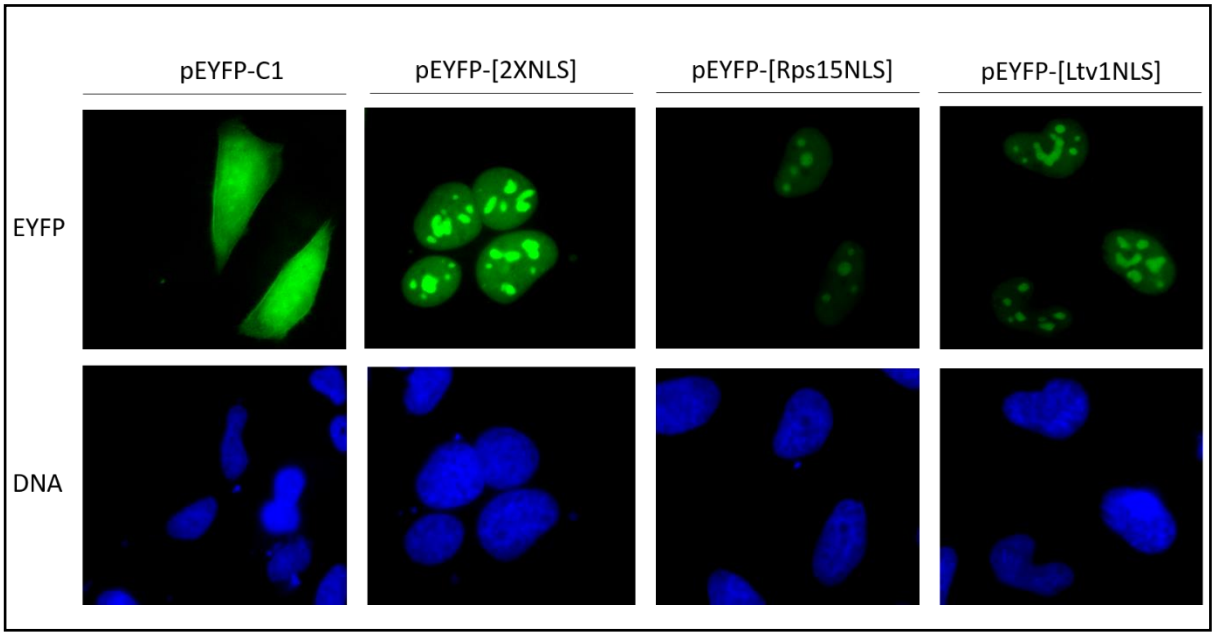

Figure 17. Results of the nuclear import activity of Rps15 NLS and Ltv1 NLS. *From left to right*: cells transfected with pEYFP-C1 (negative control), pEYFP-[2XNLS] (positive control), pEYFP-[Rps15NLS] and pEYFP-[Ltv1NLS].

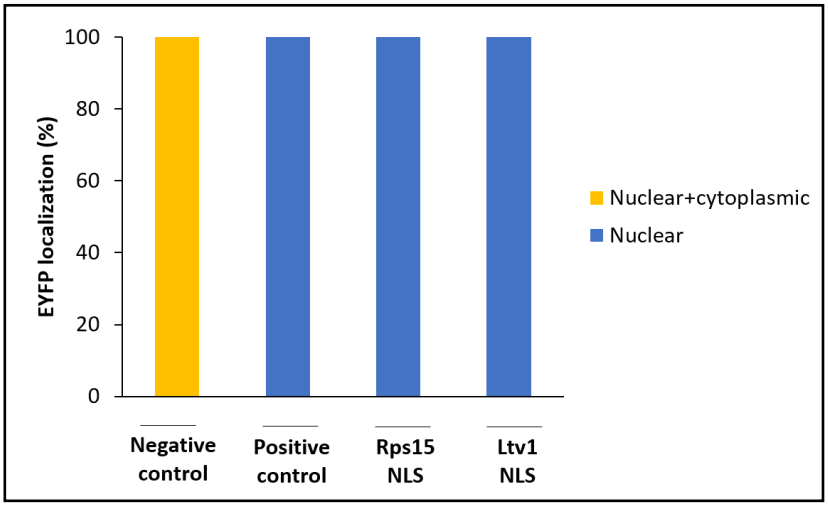

Figure 18. Graph indicating the percentage of cells with different subcellular localization of the fluorescent protein, EYFP. The negative control only has nuclear+cytoplasmic expression. The positive control, Rps15 NLS and Ltv1 NLS are functional, with 100% of cells showing nuclear localization of the fluorescent protein.

In conclusion, our results, combined with previous data showing that there is a functional NES in Ltv1 (Merwin *et al.*, 2014), indicate that both Ltv1 and Rps15 proteins have one functional NLS and one functional NES each (Figure 19).

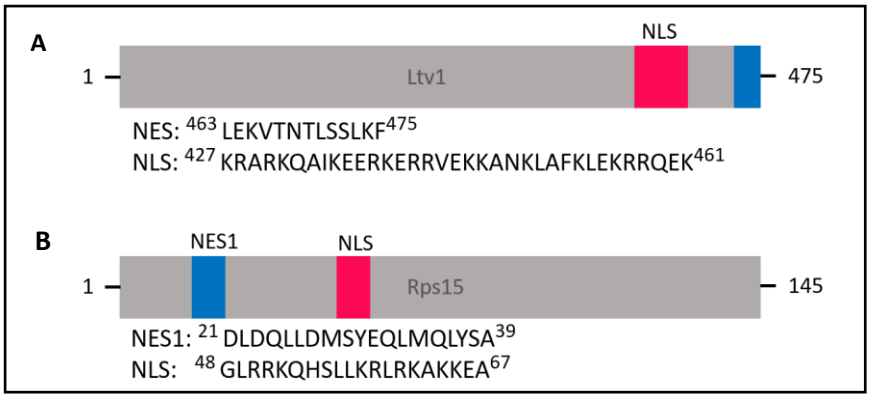

Figure 19. Schematic illustration of Ltv1 and Rps15 indicating the position and amino acid sequence of NESs and NLSs. The previously reported NES, as well as the novel nuclear transport signals identified in our study are shown. **A.** Ltv1 showing the position of all the transport signals identified and their amino acid sequence. **B.** Rps15 showing the position of all the transport signals described and their amino acid sequence.

Discussion

Ribosomes are vital for proper cell function. An essential step in ribosome synthesis is the correct transport of their subunits across the nuclear pore complex, a process that requires karyopherins as receptors and other proteins as auxiliary factors. Ltv1 and Rps15 proteins have been proposed, among others, as auxiliary factors involved in the export of the small ribosomal subunit to the cytoplasm (Zemp and Kutay, 2007). Ltv1 as a non-essential adaptor (Seiser *et al.*, 2006; Ameismeier *et al.*, 2018) and Rps15 as an essential adaptor (Léger-Silvestre *et al.*, 2004; Rouquette *et al.*, 2005).

The characterization nucleocytoplasmic transport signals that mediate nuclear import/export of the proteins involved in the transport of ribosomes can provide a better understanding of how they contribute to this process.

In the case of Ltv1, one NES had been already previously reported (Merwin *et al.*, 2014), but no NLS had been identified so far. However, it is obvious that Ltv1 needs to enter the nucleus first, in order to contribute to the export of the small ribosomal subunit. We show here that Ltv1 amino acid sequence 427-KRARKQAIKEERKERRVEKKANKLAFKLEKRRQEK-461 is a functional NLS, able to induce nuclear import of a fused EYFP protein. It remains to be established if this sequence is necessary for the nuclear import of the full-length Ltv1 protein. Site-directed mutagenesis studies should be carried out to examine this possibility. Remarkably, the NLS identified here locates close to the previously described NES, suggesting that the carboxy-terminal end of Ltv1 can be viewed as a nucleocytoplasmic transport module responsible for both import and export of the protein.

In the case of Rps15, no NES or NLS had been identified prior to our work. Our results suggest that Rps15 has one functional NES (21-DLDQLLDMSYEQLMQLYSA-39) and one functional NLS (48-GLRRKQHSLLKRLRKAKKEA-67). As above, site-directed mutagenesis should be carried out to confirm the relevance of these motifs in the context of full-length Rps15. Of note, Rps15 is a small protein that could inefficiently diffuse across the NPC. However, the presence of transport signals would allow for a faster and regulated transport of this protein, and can also be necessary for transport of Rps15 when it is bound to other proteins, forming larger complexes.

Altogether, these results, help to better understand the mechanistic details of the transport of the small ribosomal subunit through the nuclear pore complex, further establishing the implication of XPO1 and the importins, and describing the sequences of the adapter proteins that mediate this transport. It would be interesting to establish to what extent the new transport signals identified here are relevant for the proper function of Ltv1 and Rps15. In particular, it could be investigated whether mutating these NLS or NES signals alters the process of ribosome synthesis. As a reference, the effect of these mutations could be compared with the phenotypic effects of lacking the whole protein. In this regard,

yeast mutant cells lacking Ltv1 show a slower growth phenotype and a reduced export of the small ribosome subunit (Seiser *et al*., 2006). Similarly, it has been found that the synthesis of the small ribosome subunit is altered in cells with lower amount of Rps15 protein (Léger-Silvestre *et al.*, 2004). Unexpectedly, the export of the small ribosome subunit is not affected in mutants expressing a Ltv1 lacking its NES, suggesting that Ltv1 defective in export is still, at least partially, functional (Merwin *et al.*, 2014). It would be interesting to evaluate the ribosomal transport phenotype of cells expressing NLS mutant-Ltv1, as well as that of cells expressing NLS-mutant or NES-mutant Rps15.

The results presented here open new research lines that may increase our understanding about how these auxiliary transport factors contribute to the correct export and synthesis of the small ribosomal subunit.

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