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SEARCH AND CHARACTERIZATION OF NUCLEOCYTOPLASMIC TRANSPORT SIGNALS IN THE c-CBL ONCOPROTEIN

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Abstract: Most proteins cannot cross the nuclear envelope by diffusion, instead they need to be actively transported by specific nuclear transport (import or export) receptors that recognize and bind specific nucleocytoplasmic transport signals in proteins. Nuclear import receptors recognize Nuclear Localization Signals (NLSs) and transport their cargo proteins to the nucleus, while nuclear export receptors recognize Nuclear Export Signals (NESs) and transport cargos to the cytoplasm. The best-studied export receptor is CRM1, which mediates the nuclear export of cargos bearing "leucine-rich" NES motifs. CRM1-mediated nuclear export is crucial for proper protein localization and to maintain cellular homeostasis. Therefore, CRM1 malfunction can be a trigger for different human diseases, and identifying CRM1 cargo proteins might be important to better understand related pathologies. However, the full CRM1 cargo spectrum is not yet completely clear. A global proteomics analysis, proposed a long list of potential CRM1 cargoes, but many remain to be validated. In the present work, a protein from this list, c-CBL, was selected for further study, as the mechanisms and signals that might modulate its subcellular distribution are still unkown. We aimed to identify novel nucleocytoplasmic transport signals in this protein. For that purpose, *in silico* predictions and nuclear export assays were carried out. We report here two novel functional NESs in c-CBL, and discuss the importance of this finding in the context of c-CBL's transport, stressing the need for further research on c-CBL's nucleocytoplasmic transport to better understand and use c-CBL as a novel target for different therapies.

Laburpena: Proteina gehienek ezin dute gaineztadura nuklearra difusioz zeharkatu, eta nukleoko garraio-hartzaile espezifikoen bidez igaro behar dute modu aktiboan (inportazioz edo esportazioz). Hartzaile horiek proteinen garraio nukleozitoplasmatikorako seinale espezifikoak ezagutu eta lotzen dituzte. Zehazki, inportazio-hartzaileek nukleorako kokapen-seinaleak (NLS) ezagutzen dituzte eta proteinak nukleora garraiatzen dituzte; esportazio-hartzaileek, berriz, nukleotiko esportazio-seinaleak (NESak) ezagutu eta proteinak zitoplasmara garraiatzen dituzte. Gehien ikertu den esportazio-hartzailea CRM1 da, "leuzinatan aberatsak diren NESak" dituzten proteinak zitoplasmara esportatzen dituena. CRM1 bidezko esportazioa ezinbestekoa da proteinen kokapen egokirako eta zelulako homeostasia mantentzeko. Ondorioz, CRM1-en akatsek zenbait giza gaixotasun eragin ditzakete. Horregatik, CRM1-en bidez esportatzen diren proteinak identifikatzea garrantzizkoa izan daiteke horiei loturiko gaixotasunak hobeto ulertzeko. Hala ere, CRM1-en bidez esportatzen diren proteinen espektroa (zein proteina garraiatzen diren CRM1 bidez) ez da guztiz argia oraindik. Proteomika-analisi global batek CRM1-en bidez esportatzen diren proteina potentzialen zerrenda proposatu zuen, baina horietako asko balioztatzeke daude. Lan honetan, zerrendako proteina bat, c-CBL, hautatu zen sakonago ikertzeko. Izan ere, proteina horren kokapen zelularra eraentzen duten mekanismo eta sekuentziak ezezagunak dira eta beraz, gure helburua proteina honetan garraio nukleozitoplasmatikorako sekuentzia berriak bilatzea zen. Horretarako, *in silico* iragarpenak egin genituen, esportazio-entseguekin batera. Lan honetan bi NES funtzional berri deskribatu dira eta aurkikuntza honek c-CBL-ren garraioan duen garrantziaz eztabaidatzen da. Azkenik, c-CBL terapia ezberdinetan itu berri gisa erabili ahal izateko, bere garraioaren inguruko ikerketa gehiagoren beharra azpimarratzen da.

Key words: CRM1, c-CBL, NES, NLS, In silico, Nuclear export assay.

1. INTRODUCTION

Eukaryotic cells have a distinctive structure that acts as a boundary between the cytoplasm and the nucleus: the nuclear envelope (NE). Although the NE isolates the nuclear content, it is not strictly closed. In fact, it bears some multiproteic openings all along its surface, called nuclear pore complexes (NPCs). NPCs are cylindrical and symmetrical structures made up of about 30 different proteins called nucleoporins that allow the bidirectional transport of various cellular components between the nucleus and the cytoplasm (Hampoelz et al., 2019). This nucleocytoplasmic transport is essential for a variety of cellular processes, such as transcriptional regulation and cell cycle control, and it is therefore responsible for cellular homeostasis (Paci et al., 2021).

Importantly, not all molecules are transported through the NPCs in the very same way. Small molecules can diffuse, but molecules larger than 30 kDa –most proteins– cannot cross the pore via diffusion; their only way of shuttling across the NPCs is by binding to specific nuclear transport receptors (NTRs) (Kimura & Imamoto, 2014). NTRs are members of the karyopherin family, a group of soluble proteins that recognize specific amino acid sequence motifs in cargo proteins, bind to them, and help the protein into or out of the nucleus (Cautain et al., 2015). Nuclear import receptors (importins) recognize sequence motifs called Nuclear Localization Signals (NLSs) responsible for the transport towards the nucleus, whereas nuclear export receptors (exportins) recognize sequence motifs called Nuclear Export Signals (NESs) responsible for the transport towards the cytoplasm.

On the one hand, the most widely studied import receptor is the α/β importin heterodimer, which mediates the nuclear import of cargos bearing a "classical" NLS motif: a sequence characterized by the presence of one or two regions containing basic amino acids. On the other hand, the most widely known export receptor is CRM1 (also called XPO1), which mediates the nuclear export of cargos bearing "leucine-rich" NES motifs: short sequences with a characteristic spacing of hydrophobic amino acid residues (Meyer et al., 1996) (Fig. 1).

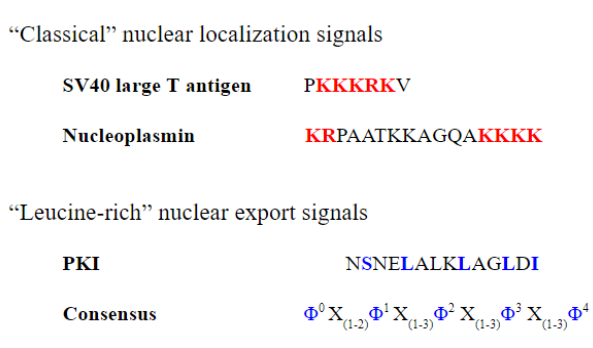

Figure 1. Examples of nucleocytoplasmic transport signals. The basic residues that characterize "classical" NLSs are highlighted in red and characteristic hydrophobic residues of "leucine-rich" NESs (represented by Φ in the consensus) are highlighted in blue. Prototypical NLSs, containing one or two clusters of basic amino acids, can be found in the simian virus 40 (SV40) large T antigen or nucleoplasmin proteins, respectively (Kalderon et al., 1984). One of the best-studied examples of NESs can be found in protein kinase inhibitor (PKI) (Wen et al., 1995).

Besides NPCs and NTRs, another key component of the nuclear transport process is the Ran GTPase. A RanGDP/RanGTP gradient across the nuclear envelope enables the binding and release of cargos in the nucleus or the cytoplasm and thus, determines the direction of the transport. This gradient (high concentration of RanGDP in the cytoplasm and high concentration of RanGTP in the nucleus) is maintained thanks to the cytoplasmic GTPase activating protein RanGAP1 and the nuclear chromatin-bound nucleotide exchange factor RCC1. RanGTP promotes the disassembly of the importin/cargo complex and, contrarily, it stabilizes the exportin/cargo complex by forming a trimeric complex (exportin/RanGTP/cargo). Therefore, the importin/cargo complex is assembled in the cytoplasm –where RanGTP concentration is low– and disassembled in the nucleus, while the exportin/cargo complex is assembled in the nucleus –where RanGTP concentration is high– and disassembled in the cytoplasm (Pemberton et al., 2005) (Fig. 2).

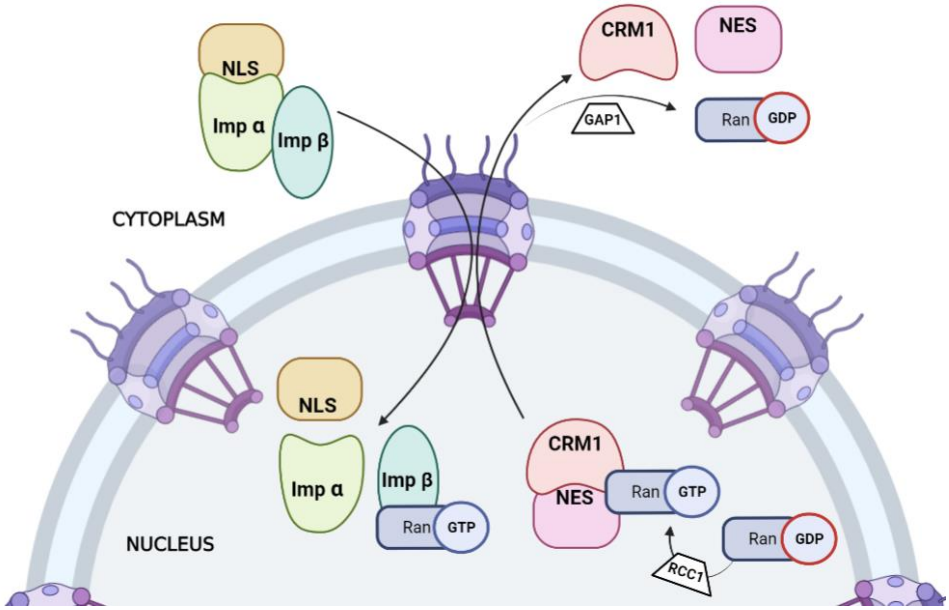

Figure 2. Receptor-mediated nucleocytoplasmic transport of proteins. Illustrative overview of Importinα/Importinβ-mediated nuclear import of a cargo protein bearing a "classical" NLS (left) and CRM1-mediated nuclear export of a cargo protein bearing a "leucine-rich" NES (right). Imp: Importin; CRM1: exportin 1; GAP1: GTPase activating protein 1; RCC1: nucleotide exchange factor (Regulator of Chromosome Condensation 1).

Since NESs and the CRM1-mediated nuclear export pathway play an important role in protein localization and cellular homeostasis, their malfunction (e.g., by mutations or loss of activity) can be a trigger for different illnesses (Lee et al., 2020). In this regard, oncogenic mutations in the *XPO1* gene, encoding CRM1, have been detected in certain hematological malignancies, suggesting that CRM1 may be a therapeutic target in cancer (Gravina et al., 2014). For that reason, identifying and characterizing CRM1 cargo proteins related to cancer might be key to better understand and tackle various anomalies and malignancies (Sendino et al., 2018). Despite the importance of this question, it is still not completely

clear how broad the CRM1 cargo spectrum really is, and which set of cellular processes are directly or indirectly controlled by CRM1-dependent nuclear export. Some light on this issue was provided by a global proteomics analysis of CRM1-mediated export (Kirli et al., 2015), which was based mainly on CRM1 affinity chromatography and mass-spectrometric analyses. In their report, Kirli and colleagues described a list of potential CRM1 cargoes –1050 in human HeLa cells–, constituting what was termed the "CRM1-dependent nuclear exportome". Each of the putative cargoes was categorized according to different parameters indicating their probability of really interacting with CRM1. The categories range from 'A' –the most probable cargoes– to 'non-binders'. Importantly, even though this global study provided a crucial resource to study CRM1-mediated export, most of the putative cargoes in the list still remain to be confirmed.

In the present work, c-CBL protein, an 'A' category putative CRM1 cargo in Kirli and colleagues' study, was chosen for further study. This protein, encoded by the Casitas B-lineage Lymphoma proto-oncogene, is an E3 ubiquitin ligase, which accepts ubiquitin from specific E2 ubiquitin-conjugating enzymes and then transfers it to diverse substrates, promoting their degradation by the proteasome (Lock et al., 2006). Regarding its structure (Fig. 3), c-CBL is a 120-kDa protein of 906 amino acids that contains 3 highly conserved domains: Tyrosine Kinase-Binding (TKB), Really Interesting New Gene (RING) finger and Linker (L) domains. The TKB domain allows binding to phosphorylated tyrosines found in its interacting proteins, while the RING finger is the domain involved in the recruitment and later transfer of ubiquitin moieties from E2 enzymes to target proteins; the linker comprises the region between the TKB and RING finger domains. Apart from these main domains, c-CBL has an extensive proline-rich region, as well as an ubiquitin-associated (UBA) domain, implicated in ubiquitin binding, and a Leuzine Zipper (LZ) domain that mediates self-oligomerization. In addition to these structural motifs, c-CBL shows many phosphotyrosine and phosphoserine residues that allow interactions with a plethora of proteins in ways that still remain poorly detailed (Meng et al., 1999; Rubi et al., 2005; Schmidt & Dikic, 2005; Rafiq et al., 2014; Dong et al., 2016).

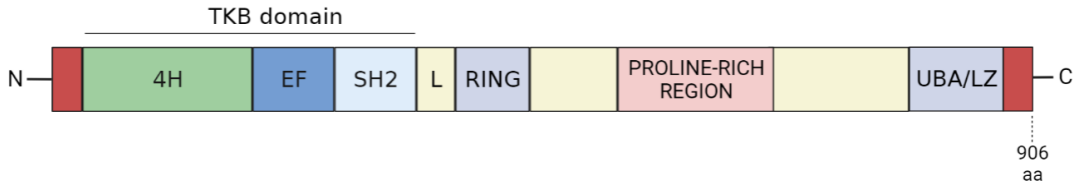

Figure 3. **Structure and domains of c-CBL protein**. The three main domains of c-CBL are: tyrosine kinase-binding (TKB), linker (L) and RING finger domains. The TKB domain is composed of 3 interacting domains comprising a four-helix bundle (4H), a Ca^{2+}-binding EF hand (EF), and a variant Src homology region 2 (SH2) domain that is connected to the RING finger by a short linker domain (L). c-CBL also contains an extensive proline-rich region, as well as ubiquitin-associated (UBA) and leuzine zipper (LZ) domains in its carboxyl terminus.

The activity of c-CBL is regulated by non-receptor tyrosine kinases (NRTKs) of the SRC family, which, upon activation via external stimuli, carry out phosphorylation of tyrosine residues releasing c-CBL from its autoinhibited conformation (Deckert et al., 1998; Nihal et al., 2016). Recent findings have shown that, apart from ubiquitin-ligase enzymatic functions, c-CBL also acts as an adaptor protein, in a constitutive or phosphorylation-dependent way (Bartkiewicz et al., 1999). Given its dual function as an enzyme and adaptor protein, c-CBL is involved in numerous intracellular signalling pathways, in which it can act as either negative or positive regulator (Schmidt & Dikic, 2005). Through its E3 ubiquitin ligase activity, c-CBL negatively regulates several receptor and non-receptor tyrosine kinases involved in the control of the immune system, cell proliferation, differentiation and apoptosis (Joazeiro et al., 1999). These substrates are recognized and polyubiquitinated by activated c-CBL, which leads to their subsequent degradation by the proteasome, thus terminating their signalling and downregulating their corresponding pathways (Levkowitz et al., 2000). On the other hand, through its adaptor role, c-CBL can bind to different cytoplasmic proteins enabling connections that positively contribute to signal transduction in pathways associated to the regulation of the actin cytoskeleton (Meng et al., 1998), but also to cell proliferation and survival (Garcia-Guzman et al., 2000).

c-CBL dysfunction contributes to many human diseases, including immune disorders, a Noonan syndrome-like condition, and a great number of malignancies (Katzav et al., 2015; Martinelli et al., 2010), in which can act either as an oncoprotein or tumor suppressor protein, depending on the cancer type and its specific pathogenic pathway (Tsygankov et al., 2001). It must be noted, though, that in some cases functional redundancy with other proteins, such as CBL-b, can overcome the outcome of c-CBL defects (Purev et al., 2009).

As for most proteins, subcellular localization is a crucial aspect of c-CBL biology. Although c-CBL can be associated to the plasma membrane, cilia and Golgi apparatus (Scaife et al., 2000) its location has been reported as mainly cytoplasmic (Thul et al., 2017). Nevertheless, some studies suggest that it may locate to the nucleus of certain cell types in specific developmental stages (Denis et al., 1999).

Despite the crucial role that subcellular localization may play in c-CBL function, the mechanisms that might modulate its nucleocytoplasmic transport remain to be further clarified. In this regard, we hypothesize that c-CBL could have specific amino acid motifs that function as nucleocytoplasmic transport signals to ensure that this protein is properly localized to carry out its cellular functions. Thus, we aim to identify potential NLSs and NESs in c-CBL protein sequence by using *in silico* predictions and cellular assays.

2. MATERIAL AND METHODS

2.1. *In silico prediction of potential nucleocytoplasmic transport signals*

The amino acid sequence of human c-CBL protein (sized 906 aa) was retrieved in FASTA format from the *Ensembl* genome browser (Hubbard, 2002), and was analyzed using bioinformatics tools to predict potential nucleocytoplasmic transport signals. NES prediction was carried out using Wregex (Prieto et al., 2014) and LocNES (Xu et al., 2015) predictors. NLS prediction, on the other hand, was carried out using Wregex, and PSortII (Horton et al., 2005) tools. In order to select *in silico*-predicted NES motifs for experimental testing, they were ranked according to the score provided by Wregex and locNES. Selected candidate NES (cNES) motifs were experimentally tested using a nuclear export assay based on the Rev(1.4)-GFP reporter (Henderson & Eleftheriou, 2000).

2.2. *The Rev(1.4)-GFP nuclear export reporter*

The Rev(1.4)-GFP reporter plasmid (Fig.4) encodes a chimeric protein composed of a mutant HIV-1 Rev protein, termed Rev(1.4), fused to GFP (a green fluorescent protein that allows to easily determine the localization of the reporter in the cell using fluorescence microscopy). Wild-type Rev bears a NLS and a NES, but the Rev(1.4) mutant used here bears a non-functional NES. As a result, the export-defective Rev(1.4)-GFP protein accumulates in the nucleus due to Rev NLS-mediated nuclear import. Therefore, export activity of candidate NES motifs (cloned between the Rev and the GFP moieties) can be determined by their ability to prompt cytoplasmic relocation of the otherwise nuclear Rev(1.4)-GFP reporter (Henderson & Eleftheriou, 2000).

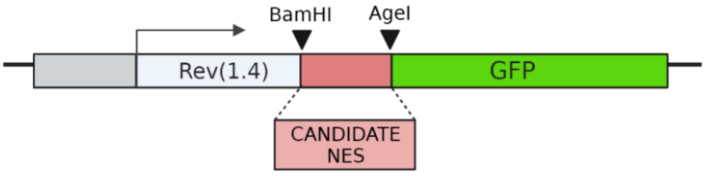

Figure 4. **Schematic representation of Rev(1.4)-GFP reporter plasmid.** The Rev(1.4)-GFP vector encodes a mutant version of HIV Rev and GFP proteins, which are separated by a region with BamHI and AgeI restriction sites. Therefore, candidate NESs are cloned between the Rev and GFP domains, and export activity of the cloned motifs is determined by their ability to relocate the Rev-GFP fusion reporter to the cytoplasm.

2.3. *gBlock™ design and cloning procedures*

In order to clone predicted candidate NES motifs, a gBlock containing DNA sequences encoding these motifs plus a few flanking residues was designed and purchased from IDT company (Integrated DNA Technologies, Coralville, IA, USA). gBlocks are commercially available double-stranded DNA fragments of 125–3000 bp in length, that allow easy gene construction or modification. For the design, the amino acid sequences of cNESs were first turned into DNA sequences using Sequence Manipulation

Suite (Stothard, 2000) as a tool for reverse translation. This hypothetic cDNA sequence was contrasted with the real cDNA sequences, obtained from the coding domain sequence (CCDS) stocked in *Ensembl*. Next, DNA sequences providing restriction sites for the enzymes BshT1 (an AgeI isoschizomer) and BamHI, along with random-sequence spacers were added to the NES-coding sequence, taking care to avoid altering the reading frame. The gBlock was finally assessed using NEBcutter software (Vincze, 2003) to ensure it had no more than 3 restriction sites for each enzyme (i.e., that no additional restriction sites for BshTI and BamHI had been accidentally introduced).

The gBlock, received as a dried DNA sample (500 ng), was hydrated and digested at 37ºC for 3 hours with BamHI and BshT1 enzymes (ThermoFisher Scientific, Waltham, MA, USA). Afterwards, digested DNA was purified using the QIAgen® PCR purification kit according to manufacturer´s instructions. On the other hand, the plasmid encoding the Rev(1.4)-GFP reporter (a gift from Dr. Beric Henderson, University of Sydney, Australia) was digested using the same enzymes and used as cloning vector. The integrity of the insert (digested gBlock) and vector (digested Rev(1.4)-GFP plasmid) DNAs was analyzed using agarose gel electrophoresis. A 1.5 % agarose gel was used and electrophoresis was carried out at 90 V for 20 minutes.

After confirming the integrity of the DNAs, a ligation reaction using T4 DNA ligase (ThermoFisher Scientific, Waltham, MA, USA) was carried out overnight at 4ºC in a 10 µL volume. For that purpose, the ligation buffer and ATP solution were mixed with both the restricted gBlock and vector. As a negative control, a ligation reaction without the insert was included.

Ligation reactions were used to transform chemically-competent *E.coli* DH5-α strain bacteria. The insert-containing and negative control ligations were each mixed with 50 µL of bacteria. After 1 hour incubation on ice, transformation mixes were given a heat shock for 30 seconds at 37ºC and later on, kept on ice for another 2 minutes. Subsequently, transformation mixes were transferred to 15 mL Falcon tubes containing 500 µL of LB medium, and were incubated for 1h at 37ºC with shaking at 190 rpm. Finally, 300 µL from each sample were plated in kanamycin(+) (30 µg/mL) LB-agar plates and incubated at 37ºC for 24 hours to allow for the growth of bacterial colonies. 8 colonies were picked randomly from the insert-containing plate. Each picked colony was placed in a 50 mL Falcon tube containing 10 ml LB medium and kanamycin (30 µg/mL) and incubated overnight at 37ºC with shaking at 190 rpm.

In order to identify colonies containing correctly inserted cNESs, a PCR amplification and electrophoresis screening approach was used. For that purpose, a 500 µL aliquot of bacterial suspension was taken from each 50 mL tube and transferred to a 1.5 mL eppendorff tube. Eppendorff tubes were centrifuged at 13.000 rpm for 3 minutes, the supernatant was removed and the bacterial pellet was

resuspended in water (50 µL). Next, samples were incubated for 5 minutes at 100ºC and centrifuged again at 13.000 rpm for 3 minutes. 1 µL of the supernatant was used as template in PCR reactions using taq DNA polymerase (Takara Bio, San Jose, CA, USA) and a pair of primers (UJAR3 and Revseq) specifically designed to bind upstream and downstream sequences flanking the cloning site of the vector. Thus, the size of the PCR product informs about the presence of one or more inserts. The empty (Rev(1.4)-GFP) vector and a previously validated NES-containing recombinant plasmid (Rev(1.4)-GFP-WN4) were used as negative and positive controls, respectively. PCR products were analyzed using agarose gel electrophoresis (1.5 % gel, run at 90 V for 20 minutes).

After PCR screening, the remaining bacteria of selected clones containing a single cNES motif inserted were used for plasmid DNA purification using the QIAgen® miniprep kit according to manufacturer´s instructions. DNA concentrations were adjusted to 100 ng/µL and samples were sent for sequencing (StabVida, Caparica, Portugal) in order to identify and validate the cloned cNES motifs.

2.4. *Cell transfection and nuclear export assay*

HeLa cells (obtained from ECACC, Salisbury, UK) were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100 µg/mL streptomycin (all from ThermoFisher Scientific, Waltham, MA, USA) at 37ºC in a humidified atmosphere containing 5% CO_2. 24 h before transfection, cells were seeded in 12-well plates with glass coverslips. To carry out the nuclear export assay, recombinant Rev(1.4)-GFP plasmids containing the different cNESs (confirmed by sequencing) were transfected into the cells using X-tremeGENE 9 DNA transfection reagent (Roche Diagnostics, Basel, Switzerland) following manufacturer's instructions. The plasmid Rev(1.4)-GFP-WN4, containing a previously demonstrated functional NES (Sendino et al., 2020), was used as positive control and the empty Rev(1.4)-GFP vector was used as negative control.

Each plasmid was transfected in two wells. 24 h after transfection, 5 µg/mL actinomycin D (AD) and 10 µg/mL cycloheximide (CHX) (both drugs from Sigma-Aldrich, Saint Louis, Missouri, USA) were added to one of the wells (referred to as AD+ treatment). To the second well (referred to as AD- treatment) only CHX was added. As detailed in the original report (Henderson & Eleftheriou, 2000), AD and CHX are essential components of the assay. On the one hand, CHX inhibits protein translation in eukaryotic cells, this way it can be assumed that any fluorescence detected in the cytoplasm of treated cells corresponds to exported Rev(1.4)-GFP and not to newly synthesized GFP-tagged proteins. On the other hand, AD acts as an inhibitor of Rev NLS-mediated nuclear import, which facilitates the detection of weaker NESs that might be unable to overcome the activity of Rev NLS.

After 3h of drug treatment, cells were fixed with 3.7% formaldehyde (Sigma-Aldrich) in phosphate-buffered saline (PBS) for 30 minutes, washed with PBS, and directly mounted onto microscope slides using Vectashield mounting medium containing DAPI (Vector Laboratories, Burlingame, CA, USA). DAPI is a DNA-staining dye that was used to visualize the nuclei.

Samples were analyzed using a Zeiss Axioskop fluorescence microscope. To ensure unbiased assessment, the identity of the samples was masked before the analysis. At least 200 transfected cells per sample were examined to establish the proportion of cells. The localization of the reporter was classified as nuclear (N), nuclear and cytoplasmic (NC) or cytoplasmic (C). Based on this proportion, each of the tested c-CBL motifs was assigned a nuclear export activity score between 0 (no export activity, inactive) and 9 (maximal export activity) using the assay scoring system described in the original report (Henderson & Eleftheriou, 2000). Transfection and fluorescence microscope analyses were performed twice in order to verify the reproducibility of the results.

3. RESULTS

3.1. *In silico prediction of potential nucleocytoplasmic transport signals in c-CBL*

No potential NLS motifs in c-CBL were predicted using either Wregex or PSortII softwares. On the other hand, 2 putative NES sequences were predicted by Wregex, and 13 by LocNES. The 2 potential NESs predicted by Wregex were also predicted by LocNES and were selected for experimental testing, as we have previously found that candidate NESs predicted by independent softwares are more likely to be functional (García-Santisteban et al., 2012). One more candidate NES motif, predicted only by LocNES, but reaching a very high score (according to this software) was also selected for testing. Thus, three candidate NES (cNES) motifs predicted by this *in silico* analysis, located in the protein domains shown in Figure 5, were cloned and experimentally evaluated.

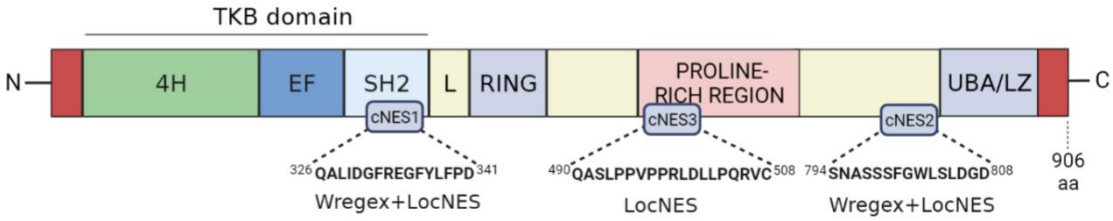

Figure 5. **Schematic representation of the c-CBL protein showing the position of the predicted candidate NESs**. The amino acid sequence of each cNES motif and the bioinformatics tools that predicted each motif are indicated.

3.2. C*loning candidate c-CBL NES motifs into the pRev(1.4)-GFP nuclear export reporter*

In order to functionally compare motifs of the same length, neighboring residues were added to the first two candidate NES sequences, to obtain a standard 19 amino acid fragment. To clone the selected candidate motifs, their amino acid sequences were reverse-translated into cDNA (Table 1).

Table 1. **Candidate NES amino acid and cDNA sequences**. The position of each cNES in c-CBL amino acid sequence is indicated. Neighboring amino acids, added to obtain a 19 amino acid fragment, appear underlined. The cDNA sequence shown was obtained by reverse translation using Sequence Manipulation Suite (Stothard, 2000).

Name	Position	Tested amino acid sequence	cDNA sequence
cNES 1	326-341	PLFQALIDGFREGFYLFPD	cct ctc ttc caa gca ctg att gat ggc ttc agg gaa ggc ttc tat ttg ttt cct gat
cNES 2	794-808	DISNASSSFGWLSLDGDPT	gat atc tct aat gcc agc tcc tcc ttt ggc tgg ttg tct ctg gat ggt gat cct aca
cNES 3	490-508	QASLPPVPPRLDLLPQRVC	caa gct tcc ctt ccc ccg gtg cca cca cga ctt gac ctt ctg ccg cag cga gta tgt

As shown in Figure 6, a gBlock containing the DNA sequences of the 3 candidate NESs in tandem was designed. Flanking each NES sequence, several nucleotides were added to introduce restriction sites for BshTI (AgeI) and BamHI enzymes, as well as spacers to allow for optimal enzyme activity. Care was taken to keep the reading frame of each motif. Before ordering, the sequence of the gBlock was assessed using NEBcutter and it was confirmed that no additional restriction sites for AgeI and BamHI had been accidentally introduced.

agc tag gat cca cct ctc ttc caa gca ctg att gat ggc ttc agg gaa ggc ttc tat ttg ttt cct gat ata **ccg gta cta gta ctag gat cc**a gat atc tct aat gcc agc tcc tcc ttt ggc tgg ttg tct ctg gat ggt gat cct aca ata **ccg gtg att aca gatg gat cc**a caa gct tcc ctt ccc ccg gtg cca cca cga ctt gac ctt ctg ccg cag cga gta tgt ata **ccg gtc atc a**

Restriction sites Spacers

Figure 6. DNA sequence of the gBlock containing the 3 selected cNESs. Restriction sites for BamHI (5´-ggatcc-3´) and BshTI (5´-accggt-3´) are highlighted in bold and spacing sequences are coloured in red.

The digested Rev(1.4)-GFP plasmid (vector) was already available at the laboratory. To generate the inserts, the gBlock was digested with BamHI and BshTI enzymes. We used electrophoresis in a 1.5% agarose gel, to check the migration of DNA in order to verify that the restriction reactions had occurred properly and to ensure that DNAs were not degraded. As expected, single bands of different size were observed in this analysis (Fig. 7). Of note, no band corresponding to the non-digested gBlock was visible in the gel, which may be explained by the low amount of DNA loaded. These results indicate that both vector and insert were correctly digested and that no degradation of the DNA had occurred. Therefore, these insert and vector DNAs were used for ligation and subsequent bacterial transformation.

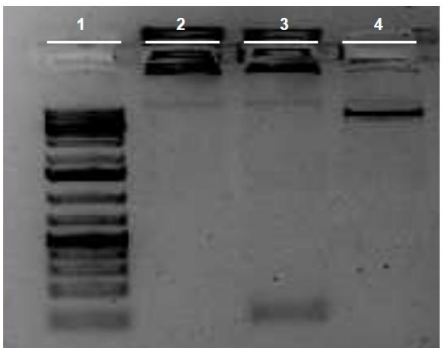

Figure 7. Results of agarose electrophoresis analysis to verify insert and vector prior to ligation. Lane 1: DNA ladder; lane 2: undigested gBlock; lane 3: BamHI/BshTI-digested gBlock; lane 4: digested Rev(1.4)-GFP vector. Single bands of different size were observed in lanes 3 (approx. 100 bp) and 4 (approx. 5 Kbp). No band was visible in lane 2 (undigested gBlock), most likely due to low amount of DNA loaded.

After transformation, *E.coli* DH5-α bacteria were plated in kanamycin(+) plates, and incubated overnight at 37ºC. As shown in Figure 8, no colonies could be seen in the negative control (C^-) plate (Fig. 8A). These bacteria had been transformed with a negative control ligation mix without insert, where the vector could not be circularized and thus, was unable to confer kanamycin resistance to the bacteria. In contrast, many colonies appeared in the plate with bacteria transformed with the ligation mix containing the insert (Fig. 8B). In this case, the circularized, recombinant plasmid conferred resistance to kanamycin.

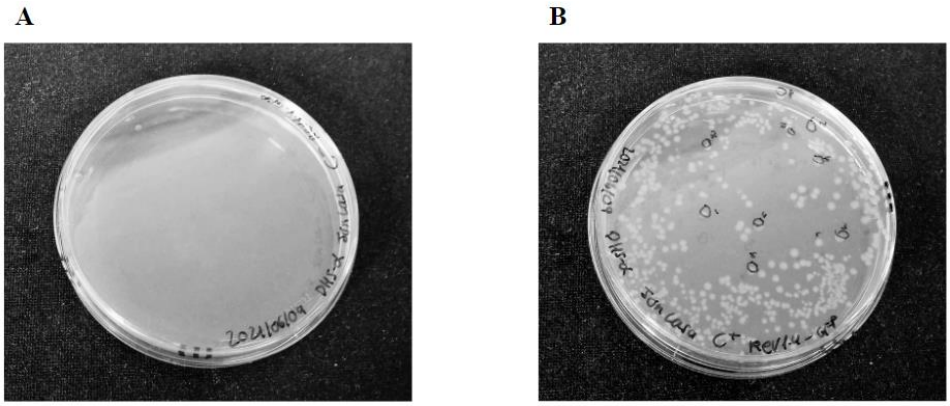

Figure 8. Kanamycin(+) plates with DH5-α *E.coli* after overnight incubation at 37ºC. (A) Negative control plate (C^-) where no colonies were observed. (B) Plate with bacteria transformed with the ligation mix containing the insert, where over a hundred colonies were observed.

8 colonies were randomly picked from the plate shown in Figure 8B, transferred to tubes with kanamycin-containing LB medium and incubated overnight. In order to determine whether insertion of cNESs had actually happened, DNA was extracted from each colony by boiling, and subjected to PCR amplification with specific primers (UJAR3 and Revseq). As illustrated in Figure 9, these primers have

been designed to hybridize with sequences flanking upstream and downstream the cloning site of the vector. That way, the insertion of cNESs could be detected based on the amplicon size, as amplicons of empty vectors would be about 70 bp smaller than those of cNES-bearing vectors.

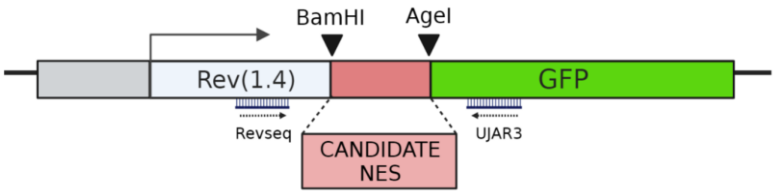

Figure 9. Primer position. UJAR3 and Revseq primers anneal specifically to flanking sequences, upstream and downstrem of the Rev(1.4)-GFP vector NES cloning site.

As shown in Figure 10, electrophoresis analysis of the PCR products indicated that the 8 selected colonies (C_1-C_8) carried a recombinant Rev(1.4)-GFP plasmid containing at least one cNES inserted. The migration pattern of 7 out of the 8 picked colonies was identical to that of the positive control, meaning that they contained one single cNES insert. However, one sample (C_1, in lane 3) showed 2 different bands, implying that this plasmid contained, most likely, 2 inserted cNESs.

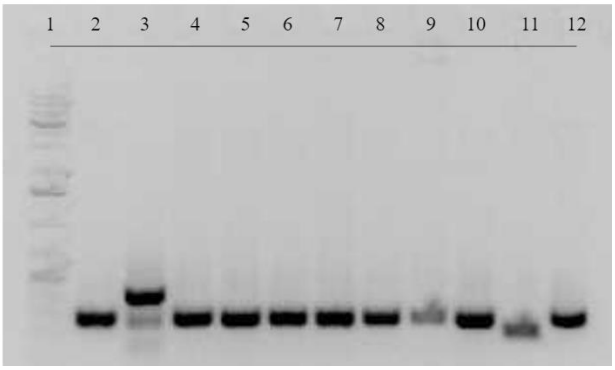

Figure 10. **Results of agarose gel electrophoresis of the PCR products amplified from picked colonies.** Lane 1: DNA ladder; lanes 2 and 12: positive control (Rev(1.4)-GFP.WN4-NES plasmid) containing one single inserted NES; lanes 3-10: 8 samples corresponding to picked colonies (C_1-C_8); lane 11: negative control (empty Rev(1.4)-GFP plasmid). The result in lane 3 (sample C_1) suggests the presence of more than one NES insert.

Among the colonies verified by PCR to contain one single insert, 6 were chosen for plasmid DNA purification and sequencing. Sequencing results revealed that just 2 different candidate NESs were inserted among the sequenced colonies: cNES 1 (Fig. 11A), present in colony C_2 and 4 other colonies, and cNES 2 (Fig. 11B) present in colony C_6. No colony with cNES 3 insertion was found.

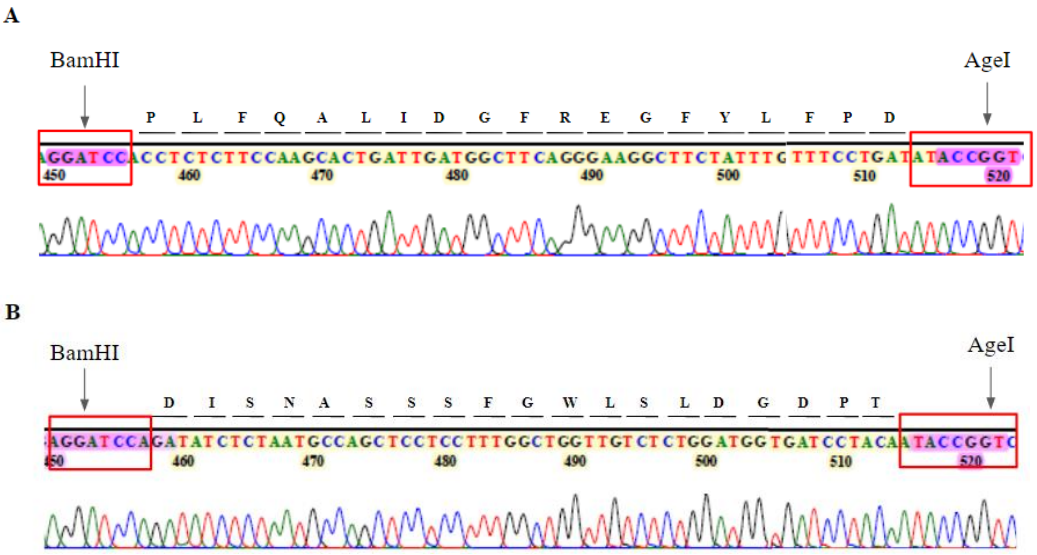

Figure 11. **Electropherograms of sequenced colonies**. Codons and the corresponding amino acids are represented. The sequences matched with cNES1 and cNES2 sequences, and no cNES3 sequence was found among the sequenced colonies. (A) Most colonies (5 out of 6) carried the cNES1 insertion. (B) Only one colony carried the cNES2 insertion.

Therefore, nuclear export assays were carried out to know if the cloned cNESs (cNES 1 and cNES 2) were functional.

3.3. Results of Rev(1.4)-GFP nuclear export assays

Rev(1.4)-GFP.cNES1 and Rev(1.4)-GFP.cNES2 plasmids were transfected into HeLa cells to assess the ability of these motifs to revert Rev NLS-mediated nuclear import and induce cytoplasmic localization of the reporter. As a positive control, Rev(1.4)-GFP.WN4-NES, a plasmid containing a previously demonstrated functional NES (Sendino et al., 2020) was used. The empty vector (Rev(1.4)-GFP) was used as the negative control. As detailed in the "Material and methods" section, some of the transfected cells were subjected to AD treatment in order to allow the identification of potentially weak NESs.

Using a fluorescence microscope, the localization of the reporter was evaluated in at least 200 individual cells from each of the 8 samples (negative control +/- AD, positive control +/- AD, cNES 1 +/- AD and cNES 2 +/- AD). The localization of the reporter, in each cell, was classified as nuclear (N), nuclear and cytoplasmic (NC) or cytoplasmic (C). Transfection and fluorescence microscope analysis were performed twice in order to verify reproducibility of the results.

Representative examples of the localization of the reporters in each sample are shown in Figure 12, and a graph with the percentage of cells with different localizations of the reporter in each sample is shown in Figure 13.

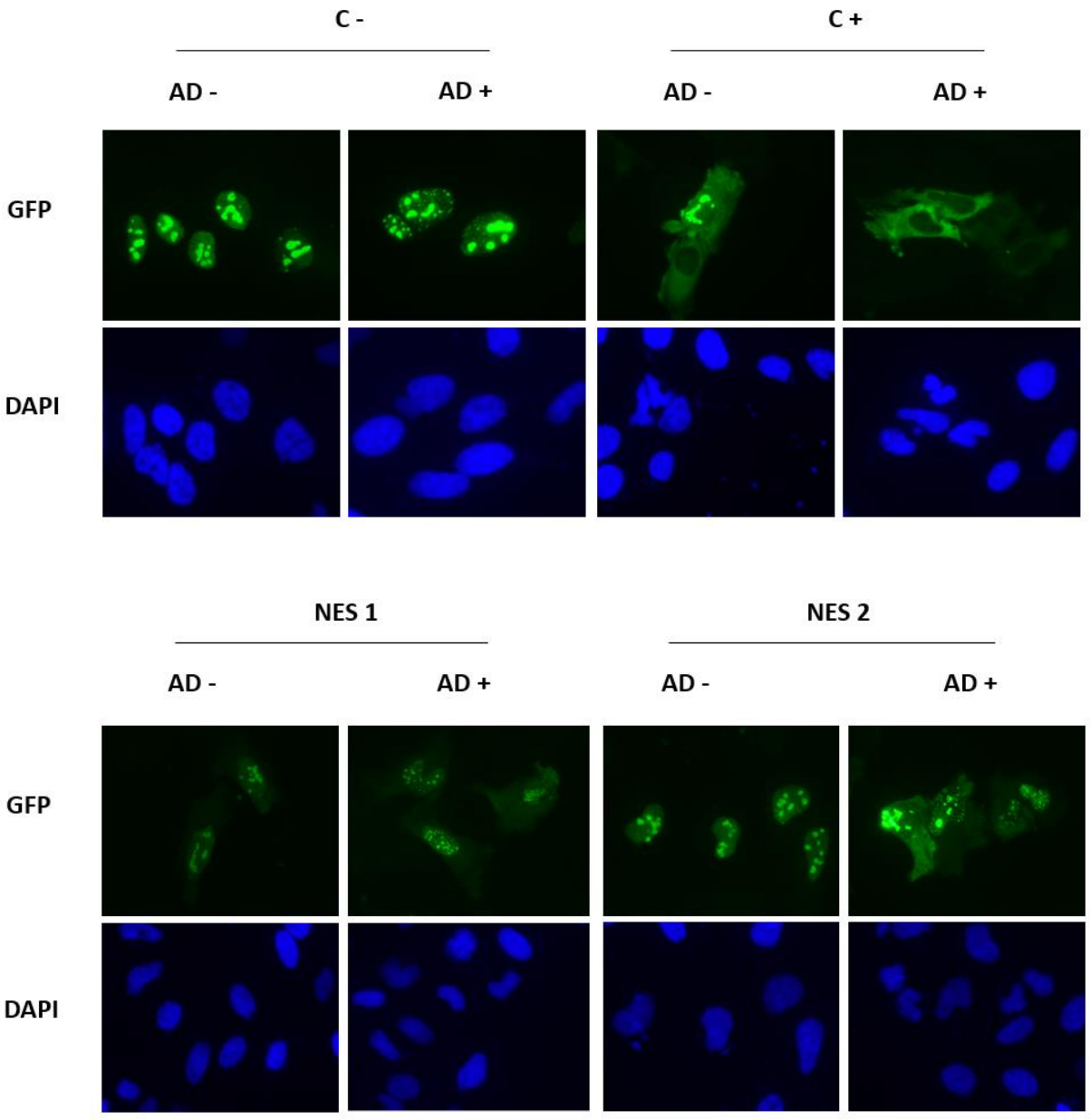

Figure 12. Fluorescence microscope images of transfected HeLa cells showing representative examples of the results of the nuclear export assay. C^- (negative control): empty Rev(1.4)-GFP reporter. C^+ (positive control): Rev(1.4)-GFP.WN4 plasmid, containing a proven functional NES. NES 1 and NES 2: plasmids containing the 2 candidate NESs of c-CBL identified in our bioinformatic search. Cycloheximide and actinomycin D were added to one of the 2 wells of each sample (AD + treatment). Only cycloheximide was added to the second well (AD - treatment). DAPI staining allows to visualize the nucleus of each cell.

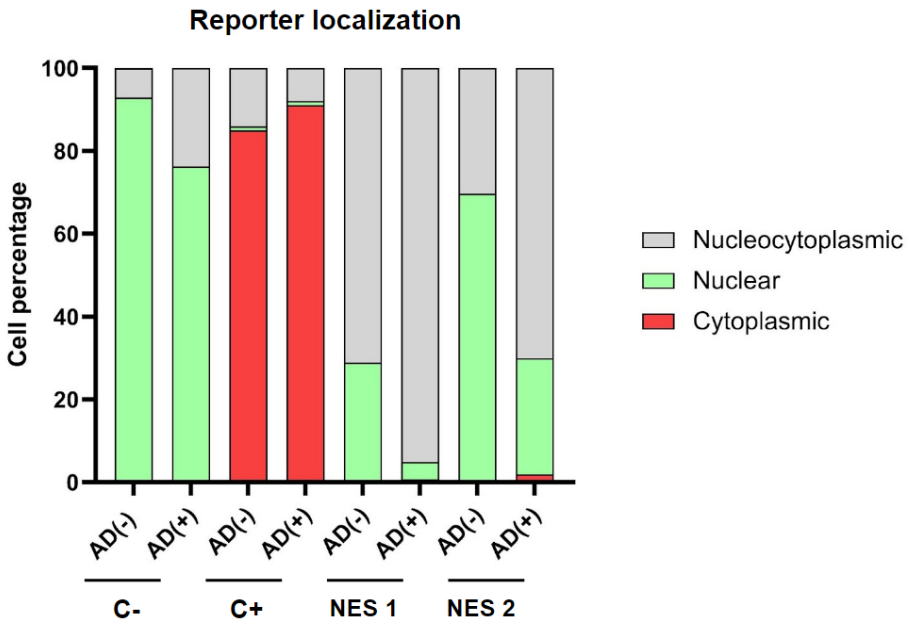

Figure 13. Graph representing the percentage of cells showing differential localization of the reporter. The localization of the reporter was exclusively nuclear in most cells transfected with the empty reporter (C^-). In the positive control sample (C^+) the reporter was cytoplasmic in most cells. In cells transfected with plasmids containing NES 1 and, to a lesser extent NES2, the percentage of cells with nucleocytoplasmic fluorescence was higher than in the negative control. In all cases, the percentage of cells with nuclear fluorescence decreased upon AD treatment. Results shown are the average from two separate export assays, except in the case of C^+ (AD -) and NES1 (AD +/-) samples. In these three samples the results are based on a single experiment, due to technical issues during the second transfection.

As expected, in the negative control cells (transfected with empty the Rev(1.4)-GFP plasmid), the localization of the reporter was almost fully nuclear in either AD(-) and AD(+) treatments. Contrary to the negative control, in the positive control cells (transfected with Rev(1.4)-GFP.WN4-NES plasmid), the localization of the reporter was predominantly cytoplasmic both in the presence or absence of AD, clearly showing that the functional NES used as a positive control was strong enough to fully overcome Rev-NLS-mediated nuclear import of the reporter.

Cells transfected with the reporter containing the candidate c-CBL cNES 1 showed mostly nucleocytoplasmic localization of the reporter, even in the absence of AD. Upon AD treatment the percentage of cells with fully nuclear localization of the reporter was negligible. Finally, cells transfected with the reporter containing c-CBL cNES 2 showed also a partial nucleocytoplasmic localization of the reporter, which clearly increased under AD(+) treatment, when reimport was prevented by the drug.

These results indicate that both tested candidate c-CBL NESs are functional, as they enabled cytoplasmic relocation of the reporter to some extent. Our data show that NES 2 is less active than NES 1.

Based on the percentage of cells showing N, NC or C localization of the reporter, and according to the NES activity scoring system (between +1 and +9) described in the original report (Henderson and Eleftheriou, 2000), a nuclear export score of +3 was assigned to c-CBL NES 1 motif while a score of +2 was assigned to NES 2.

4. DISCUSSION

Characterizing the subcellular localization of proteins is crucial to help understand not only their function and related pathogenesis, but also to unravel the organization of the cell as a functional unit. Protein localization to the cytoplasm and/or the nucleus is often dependent on their interaction with nuclear import and export receptors. The nuclear export receptor CRM1 has a major role in protein localization, as it mediates NES-dependent nuclear export of several hundred proteins (Fung et al., 2021).

Despite its relevance, it is still poorly detailed how broad the CRM1 cargo spectrum really is. In this regard, even though a list of putative CRM1 cargo proteins (the so-called XPO1/CRM1 exportome) has been reported (Kirli et al., 2015), validation of most of the putative cargoes in the list remains to be carried out to date.

A useful approach to further validate putative CRM1 cargoes is the identification of functional NES motifs in these proteins. To this end, bioinformatics NES predictors, such as Wregex (Prieto et al., 2014) and LocNES (Xu et al., 2015), can be combined with functional assays, such as the Rev(1.4)-GFP nuclear export assay (Henderson & Eleftheriou, 2000). As an example, this approach was applied by Prieto and colleagues to identify pathogenic mutations that may alter NES motifs and thus compromise protein function in cancer cells (Prieto et al., 2016).

A similar strategy was used in the present work, to further study the poorly detailed nucleocytoplasmic localization and transport of c-CBL, a protein listed as potential CRM1 cargo in a global XPO1/CRM1 exportome analysis. Based on previous works, it was already known that, besides its association to the plasma membrane, cilia and Golgi apparatus (Scaife et al., 2000), the location of c-CBL is mainly cytoplasmic (Thul et al., 2017). However, some studies imply that it may locate to the nucleus in certain occasions (Denis et al., 1999), suggesting that c-CBL could have specific nucleocytoplasmic transport signals -either NESs or NLSs- that mediate its proper, regulated, localization to the nucleus or cytoplasm. However, no such nuclear transport signals have been yet described in c-CBL.

13 different candidate c-CBL NESs were predicted *in silico*. It is not feasible to functionally test all candidates and thus, we carried out a selection. On the one hand, we selected those motifs predicted by

the two programs, as it was described in previous reports that candidate NESs predicted by independent softwares are more likely to be functional (García-Santisteban et al., 2012). On the other hand, we selected a candidate NES predicted only by one program (LocNES), but with the highest score. Thus, 3 candidates were selected to be cloned for experimental assay. Among six randomly picked insert-containing colonies, we did not identify one of the candidates (cNES 3). Therefore, two c-CBL cNESs (cNES 1: PLFQALIDGFREGFYLFPD and cNES 2: DISNASSSFGWLSLDGDPT) were finally experimentally tested in the nuclear export assay. The results of this assay indicate that both motifs are functional NESs (Fig.14).

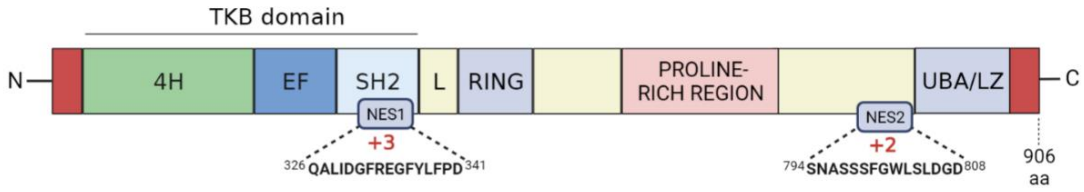

Figure 14. Schematic representation of the c-CBL protein showing the newly identified NES motifs. The position, sequence and nuclear export assay score of each motif (NES 1 and NES 2) are indicated.

Our finding that c-CBL bears functional NES motifs provides further support to the previously proposed interaction between c-CBL and CRM1 (Kirli et al., 2015). The identified NESs are both weak, but they could function in an additive manner to mediate more efficient nuclear export of c-CBL. This possibility could be tested inactivating one, the other, or both in the full-length protein, which would also provide evidence for the physiological relevance of the identified transport motifs. In the context of full-length c-CBL, the function of the identified NESs could depend on protein structure and also on protein-protein interactions. In this regard, intriguingly, one of the NESs locates in the SH2 domain, which recognizes and binds tyrosine-phosphorylated sequences of interacting proteins. Thus, this NES might be masked by interacting partners. As reported in previous works (Rodriguez et al., 2003), NES masking can be a way of regulating the nuclear export activity. In this case, we speculate that NES 1 masking could enable nuclear retention or lower protein export of c-CBL when interacting with specific proteins in the context of its different cellular functions.

Besides the two functional NESs validated here, c-CBL, may bear additional functional nucleocytoplasmic transport motifs (for example, the predicted but not tested cNES 3). Remarkably, no NLSs were predicted in our *in silico* analysis. Interestingly, a study reported that v-CBL, an oncogenic variant of c-CBL, bears a NLS motif in the TKB domain (Langdon et al., 1989), raising the possibility that, although not identified in our analysis, c-CBL may also bear a NLS motif.

It is also possible that c-CBL is not actively imported into the nucleus, raising the question of what the role of the NESs could be. In this regard, it has to be taken into account that several studies have shown

that a considerably large number of NLS-lacking proteins (previously thought to remain in the cytoplasm) can leak into the nucleus, which indicates that the border control at NPCs is less perfect than previously believed (Mohr et al., 2009; Kirli et al., 2015; Timney et al., 2016). In this scenario, a potential role of the NESs could be to prevent nuclear mislocalization of c-CBL through CRM1-mediated export. Since all the c-CBL substrates described to date locate out of the nucleus and there is no conclusive evidence on nuclear c-CBL's substrates, keeping c-CBL out of the nucleus could be crucial to maintain its proper function. Accordingly, alterations in c-CBL's localization could compromise its cytoplasmic functions and lead to various illnesses, including immune disorders, a Noonan syndrome-like condition, and a great variety of malignancies (Katzav et al., 2015; Martinelli et al., 2010).

In the case of cancer, many tumor samples from the Catalogue Of Somatic Mutations In Cancer (COSMIC) database (Tate et al., 2019) have been reported to bear mutations in c-CBL, including 5 missense substitutions in NES 1 and 6 missense substitutions in NES 2. While the functional consequences of these substitutions remain to be experimentally determined, it is possible that, as described for NES mutations in other proteins (Prieto et al., 2016), these mutations might affect NES-mediated nucleocytoplasmic transport of c-CBL. Therefore, oncogenic mutations in the identified NESs may point to a link between c-CBL's aberrant nucleocytoplasmic transport and cancer. Accordingly, future research should clarify the role of NES 1 and NES 2 motifs in c-CBL-related tumorigenesis, and the potential nature of mutations in these motifs as cancer drivers.

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