A functional study of nucleocytoplasmic transport signals of the EhNCABP166 protein from *Entamoeba histolytica*

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SUMMARY

EhNCABP166 is an *Entamoeba histolytica* actin-binding protein that localizes to the nucleus and cytoplasm. Bioinformatic analysis of the EhNCABP166 amino acid sequence shows the presence of 3 bipartite nuclear localization signals (NLS) and a nuclear export signal (NES). The present study aimed to investigate the functionality of these signals in 3 ways. First, we fused each potential NLS to a cytoplasmic domain of ehFLN to determine whether the localization of this domain could be altered by the presence of the NLSs. Furthermore, the localization of each domain of EhNCABP166 was determined. Similarly, we generated mutations in the first block of bipartite signals from the domains that contained these signals. Additionally, we added an NES to 2 constructs that were then evaluated. We confirmed the intranuclear localization of EhNCABP166 using transmission electron microscopy. Fusion of each NLS resulted in shuttling of the cytoplasmic domain to the nucleus. With the exception of 2 domains, all of the evaluated domains localized within the nucleus. A mutation in the first block of bipartite signals affected the localization of the domains containing an NLS. The addition of an NES shifted the localization of these domains to the cytoplasm. The results presented here establish EhNCABP166 as a protein containing functional nuclear localization signals and a nuclear export signal.

Key words: *Entamoeba histolytica*, nuclear localization signal (NLS), nuclear export signal (NES), actin-binding protein, nucleocytoplasmic transport system.

INTRODUCTION

The enteric protozoan parasite *Entamoeba histolytica* is the aetiological agent of human amoebiasis, which is responsible for more than 50 million clinical cases and 50 000–100 000 deaths worldwide per year. The parasite can be found worldwide, but it is most common in tropical and subtropical areas of the world. (WHO/PAHO/UNESCO, 1997; Stanley, 2003; Pritt and Clark, 2008). *Entamoeba histolytica* trophozoites are highly motile and undergo complex, dynamic actin cytoskeletal rearrangements (Guillén, 1996). The complex properties of the actin cytoskeletal dynamics in *E. histolytica* are essential for cellular functions involved in pathogenicity, the interaction between the amoebic cell and its target cell (Singh *et al*. 2004), phagocytosis of intestinal cells and human erythrocytes (Marion, 2005), cytolysis of target cells (Leippe and Herbst, 2004), cell migration (Blazquez *et al*. 2008) and host immune system evasion through tissues, which are all processes that are regulated by actin-binding proteins (ABPs). ABPs are defined as proteins that are able to bind actin monomers, polymers or both and can control the dynamics of actin through distinct mechanisms. One important characteristic of some ABPs is the presence of a calponin-homology (CH) domain; tandem pairs of this domain ranging from 2 (e.g., a-actinin and filamin) to 4 copies (e.g., fimbrin) are required for its function. A number of cytoskeletal ABPs have been identified and characterized in *E. histolytica* (Binder *et al*. 1995; Vázquez *et al*. 1995; Vargas *et al*. 1996; Ebert *et al*. 2000; Marion *et al*. 2004; Sahoo *et al*. 2004; Coudrier *et al*. 2005; Díaz-Valencia *et al*. 2005; Virel and Backman, 2004, 2006).

A phylogenic analysis of *Entamoeba* proteins that contain a CH domain suggested the existence of at least 5 phylogenetically distinct families of potential ABPs (Hon *et al*. 2010). One of these proteins (Group D, NCBI Accession number: EHI_093850) is a nucleocytoplasmic ABP that was recently described by our group. EhNCABP166 consists of 1387 amino

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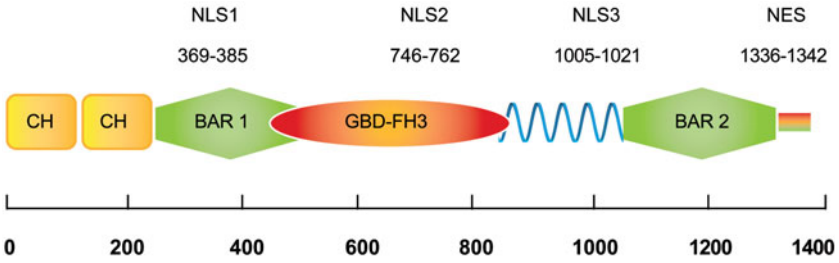

Fig. 1. Schematic representation of the EhNCABP166 protein. EhNCABP166 contains an actin-binding domain comprised of 2 calponin homologue domains (CH) (ABD 1-233); 2 BAR domains, BAR1 (248-512) and BAR2 (1057-1314); a GTPase-binding domain (GBD/FH3 438-852); and an undefined alpha helix region (853-1056). *In silico* analysis showed that EhNCABP166 contains 3 bipartite nuclear localization signals (NLSs), which are located in the EhNCBAR1-166 domain (NLS1: 369-KKIEELEKLVSVMKEKKE-385), EhNCGBD/FH3-166 (NLS2: 746-KKQIEIIKNDNEKERKN-762) and the alpha helix region (NLS3: 1005-KKQLENENEIIKKENKK-1021). A nuclear export signal (NES 1336-LFNLSLAL-1342), which is located in the C-terminal region of the protein, is also described.

acids and contains 2 CH domains with high homology, 2 BAR domains and a GTPase-binding domain/formin 3 homology domain (GBD/FH3) (Fig. 1). *In vitro*, EhNCABP166 is able to bind diverse phosphoinositides, GTPases and filamentous actin (F-actin). *In vivo*, EhNCABP166 has the ability to bind F-actin (Campos-Parra *et al*. 2010). The presence of ABPs has recently been detected in nuclei of eukaryotic cells with specific functions (Uribe and Jay, 2009; Castano *et al*. 2010). In higher eukaryotes, it has been reported that diverse ABPs, including N-WASP, Flightless I and c-Abl, contain nuclear and export and localization signals (NES and NLS, respectively) and have been found in both the cytoplasm and nucleus (Van Etten *et al*. 1989; Taagepera *et al*. 1998; Lee *et al*. 2004; Wu *et al*. 2006). These NLSs and NESs mediate the transport of macromolecules between the nucleus and the cytoplasm. Proteins targeted to enter the nucleus due to containing a functional NLS could be carried in by importins (karyopherin) (Imamoto *et al*. 1995). There are 2 types of importins, α and β. Importin α proteins recognize the NLS on cargo proteins. Once the cargo is bound by importin α, the complex is recognized and bound by importin β, which subsequently binds to the fibrils of the nuclear pore complex (NPC) and is responsible for translocation of the cargo. Importin β is able to bind directly to some NLSs present in different proteins and transport them to the nucleus (Marfori *et al*. 2011). Proteins that transfer cargo outside of the nucleus are called exportins (one of the most studied exportins is the protein CRM1). These proteins mediate the export of numerous proteins that carry an NES from the nucleus to the cytoplasm. To transport cargo to the cytoplasm, CRM1 must be associated with RanGTP (Boulo *et al*. 2007). In this regard, O'Reilly *et al*. (2011) reported an *in silico* analysis of 36 predicted proteomes. They scanned the proteomes for candidate karyopherin-β (KAP-β) family members. KAP-βs are functionally classified as importins or exportins depending on the direction of transport they have been shown to mediate. Candidate members of each KAP-β subfamily have been found in all eukaryotic super groups (O'Reilly *et al*. 2011). *Entamoeba histolytica* was examined in this analysis, and the obtained data suggest the presence of KAP-βs in this parasite. Some KAP-βs mediate nuclear import through recognition of nuclear localization signals in cargo proteins (Chook and Süel, 2011).

NLS motifs consist of clusters of positively charged amino acids (Arg and Lys) and can be mono- or bipartite (Xu *et al*. 2010). A wide variety of functional NES motifs have been identified, and their diversity is relatively high (Kutay and Güttinger, 2005). The presence of regularly spaced hydrophobic residues is a characteristic of these signals. There are few reports of the presence of NLSs or NESs in *E. histolytica*. However, the possible existence of an NLS and an NES in the protein Ehp53 has been reported. This protein is located in both the nucleus and cytoplasm (Mendoza *et al*. 2003). Furthermore, it has been reported that fusion of the SV40 large T antigen monopartite NLS to *E. histolytica* enolase results in increased levels of this protein in the nucleus (Tovy *et al*. 2010), which suggests the existence of nuclear transport components in this parasite that allow the migration of proteins that contain NLSs to the nucleus. A preliminary *in silico* analysis showed that EhNCABP166 contains 3 NLSs and an NES. The location of each potential NLS within EhNCABP166 was determined *via* fusion of NLS1, 2 and 3 to a cytoplasmic domain of ehFLN, which is localized exclusively in the cytoplasm of *E. histolytica* trophozoites (Díaz-Valencia *et al*. 2007). Furthermore, due to the large size of EhNCABP166, we decided to evaluate the localization of the domains containing NLSs and the domains without predicted NLSs to evaluate the functionality of the NLSs in EhNCABP166. Similarly, we evaluated the effect of mutations in the first block of the NLSs.

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Furthermore, to determine the functionality of the NES, we fused this signal to a couple of constructs that localize in the nucleus. In this report, we provide evidence, for the first time, that EhNCABP166 contains functional NLSs and an NES that could allow transport between the nucleus and cytoplasm.

MATERIALS AND METHODS

Strain and culture conditions

Trophozoites of the pathogenic strain *E. histolytica* HM1:IMSS were axenically cultured in TYI-S-33 medium (Diamond *et al*. 1978). The bacterial strain *Escherichia coli* DH5*α* was used in the recombinant DNA applications. This bacterial strain was cultured in Luria-Bertani medium at 37 °C in the presence of ampicillin (100 *μ*g/ml).

Detection of EhNCABP166 and transfected constructs using transmission electron microscopy

To determine the specific location of EhNCABP166 and the 3 transfected constructs (NLS3Ac^{-}d100^{-}, EhNCR1C-166 and EhNCR1CK1005A/K1006A-166) in the nucleus of *Entamoeba histolytica*, trophozoites were fixed with 0·1% glutaraldehyde and 4% paraformaldehyde for 1 h at 25 °C, embedded in LR White, and then polymerized under UV at 4 °C overnight. Thin (60 nm) sections were obtained, mounted on Formvar-covered nickel grids and then they were incubated with an anti-EhNCABP166 polyclonal antibody (1:40 dilution) or an anti-HSV monoclonal antibody (1:100) (Novagen, Wisconsin, USA) for 1 h at 25 °C. Goat anti-mouse IgG conjugated to 20-nm gold particles (Ted Pella, Cardiff, UK) was used as the secondary antibody (1:40 dilution for the polyclonal antibody and 1:60 for the monoclonal antibody); samples were incubated for 1 h at 25 °C. Thin sections were observed with a Morgagni 268D Philips transmission electron microscope (FEI company, Eindhoven, The Netherlands).

DNA constructs and transfection assays

To determine the subcellular localization of the domains constituting the protein, the following 7 constructs were generated: EhNCABD-166 (1-233), EhNCBAR1-166 (248-512), EhNCGBD/FH3-166 (438–852), EhNCR1C-166 (853-1314), EhNCR1C + NES (853-1387), EhNCBAR2ΔNES-166 (1057-1314), and EhNCBAR2 + NES-166 (1057-1387) (Fig. 4). To generate fusions of the different regions of the EhNCABP166 gene to an HSV tag sequence, plasmid DNA containing the EhNCABP166 gene was amplified *via* PCR using specific primers for each construct that were modified at the 5′ and 3′ ends to incorporate KpnI and BamHI restriction sites (see Supplementary Table S1, online only).

To fuse the EhNCABP166 NLSs to Ac^{-}d100^{-} to obtain NLS1 Ac^{-}d100^{-}-HSV, NLS2 Ac^{-}d100^{-}-HSV and NLS3 Ac^{-}d100^{-}-HSV, the construct pExEhNeo/HSV-tagged Ac^{-}d100^{-} was used as a template, which was previously generated by our research team (Díaz-Valencia *et al*. 2007). Plasmid DNA containing the Ac^{-}d100^{-} domain was amplified *via* PCR using a specific primer modified at the 5′ end to incorporate each NLS and a *Kpn*1 restriction site, together with a specific primer modified at the 3′ end to incorporate a *BamH*I restriction site (see Supplementary Table S1 online version only). In all cases, the reverse primer possessed a 3′ sequence that encoded an HSV (herpes simplex virus) tag (SQPELAPEDPED). The obtained PCR products were purified and digested with the *Kpn*I and *BamH*I endonucleases and subsequently ligated into a pExEhNEO vector that had been cleaved at the same restriction sites. These plasmids were replicated in the *E. coli* DH5*α* strain. Plasmid DNA was purified using a Plasmid Maxi kit (Qiagen). *Entamoeba histolytica* trophozoites of the HM1:IMSS strain were transfected by electroporation with 200 *μ*g DNA/10^7 cells (Hamann *et al*. 1995). Transfected cells grown for 48 h after electroporation were selected based on their resistance to 10 *μ*g/ml G418 added to the culture. Positive clones were isolated and further subjected to higher concentrations of G418 (up to 30 *μ*g/ml).

Directed mutagenesis of nuclear localization signals

To generate mutants of EhNCGBD/FH3-166 and EhNCR1C-166, both domains were cloned into the Pcr2.1 vector. Once they were cloned into this vector, mutants of EhNCGBD/FH3-166 (EhNCGBD/FH3K746A/K747A-166) and EhNCR1C-166 (EhNCR1CK1005A/K1006A-166) were generated *via in vitro* site-directed mutagenesis using the QuikChange© kit (Stratagene, Cedar Creek, Texas, USA) according to the manufacturer's suggested protocol and using specific primers (see Supplementary Table S1 online version only). All mutations were confirmed by automatic sequencing. Then, the mutated domains were cloned into the pExEhNeo vector. These plasmids were replicated in the *E. coli* DH5*α* strain. Plasmid DNA was purified using a Plasmid Maxi kit (Qiagen). *Entamoeba histolytica* trophozoites of the HM1:IMSS strain were transfected *via* electroporation with 200 *μ*g DNA/10^7 cells (Hamann *et al*. 1995). Transfected cells that were grown for 48 h after electroporation were selected based on their resistance to 10 *μ*g/ml G418 added to the culture. Positive clones were isolated and further subjected to higher concentrations of G418 (up to 30 *μ*g/ml).

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Confocal microscopy

The cellular localization of the constructs within transfected *E. histolytica* trophozoites was determined. Briefly, $2{\cdot}4 \times 10^5$ trophozoites were pelleted and washed with 0·89% NaCl. Then, 1 ml of 4% paraformaldehyde, pre-warmed to 37 °C, was added, and the cells were incubated for 45 min at 37 °C. Following fixation, the cells were permeabilized with 0·1% Triton-PBS for 1 min. The fixed and permeabilized cells were washed with PBS 3 times, quenched using 50 mM NH_4Cl in PBS for 30 min at room temperature, blocked with 1% BSA-PBS for 1 h at 37 °C and incubated with the anti-HSV Tag monoclonal antibody (Novagen, Wisconsin, USA) at a 1:150 dilution, in 1% BSA-PBS at 37 °C for 1 h. Then, the cells were washed 3 times with 1% BSA-PBS and incubated with a goat anti-mouse IgG Alexa Fluor® 488 secondary antibody (Molecular Probes® 1:900) at 37 °C for 1 h. The cells were subsequently washed again with PBS and mounted using 5 μl of Vectashield© with DAPI.

Also, cellular localization of PI(3,4) P_2 within wild type trophozoites was determined. To determine cellular location of this lipid in the wild type, a purified anti-PI(3,4) P_2 antibody mouse monoclonal (Echelon Biosciences®, Salt Lake City, USA) at a 1:50 dilution was used. As secondary antibody, a goat anti-mouse IgG Alexa Fluor® 488 (Molecular Probes® 1:800) was used. To determine the cellular location of PI(3,4) P_2 and HSV tagged constructions within transfected cells (pExEhNeo/HSV tagged-EhNCBAR1-166 or pExEhNeo/HSV tagged-NLS1Ac$^-$d100$^-$), a purified anti-PI(3,4) P_2 mouse monoclonal antibody (Echelon Biosciences®, Salt Lake City, USA) at a 1:50 dilution was used; a rabbit anti-HSV (GenScript® NJ, USA) at a 1:100 dilution was used. As secondary antibodies, a goat anti-mouse IgG TRITC (Zymed) and a goat anti-rabbit IgG FITC (Zymed) were used, both at a 1:800 dilution. For these samples the same microscopy protocol mentioned above was used.

The samples were analysed *via* confocal microscopy (FluoView 1000 Olympus and Leica SP5).

RESULTS

Nuclear location of EhNCABP166 under TEM

Through an immunostaining and confocal analysis of *E. histolytica*, we had previously demonstrated the presence of EhNCABP166 in both the nucleus and cytoplasm (Campos-Parra *et al*. 2010). To determine whether the association of EhNCABP166 with the nucleus corresponds to intranuclear localization, we decided to analyse this protein by transmission electronic microscopy (TEM), which is a very precise technique that allows determination of the position of the protein relative to the nuclear envelope. A polyclonal antibody that recognizes EhNCABP166 was used (Campos-Parra *et al*. 2010). As depicted in Fig. 2, the TEM micrograph showed that EhNCABP166 is located at the inner perinuclear zone and throughout the entire intranuclear space.

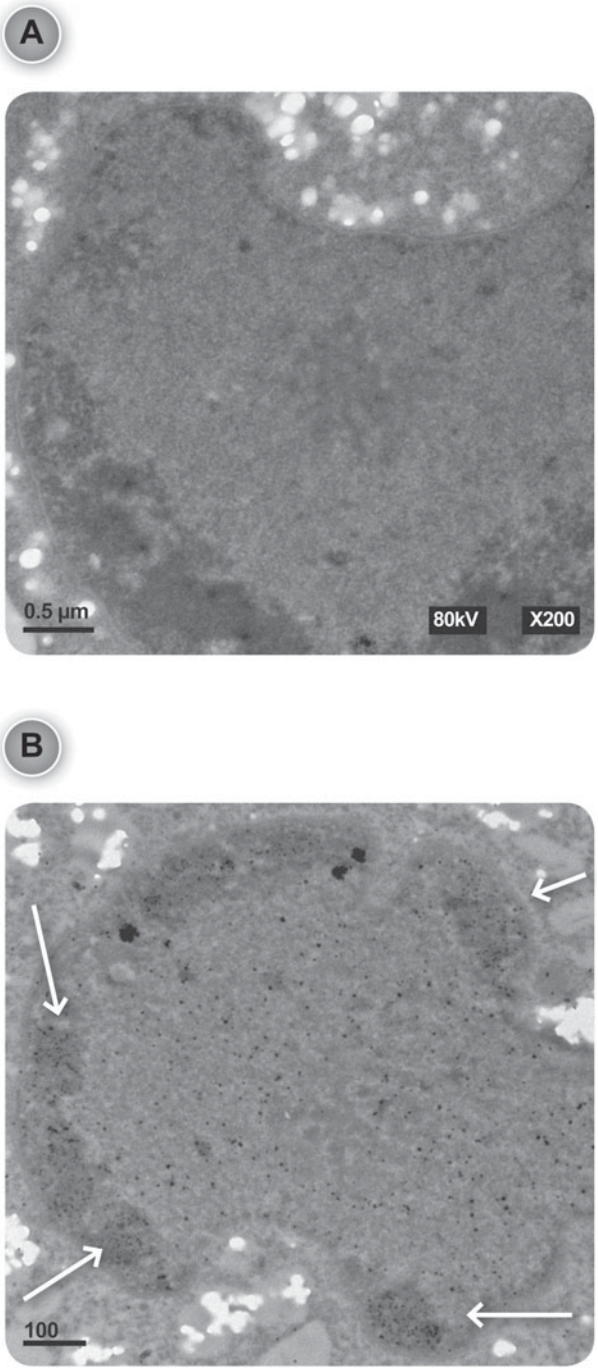

Fig. 2. Transmission electron microscopy analysis of *Entamoeba histolytica* trophozoites. (A) Secondary antibody control. As depicted in the figure, the secondary antibody did not recognize any epitope. (B) Staining with the EhNCABP166 polyclonal antibody followed by the secondary antibody conjugated to gold particles. EhNCABP166 is localized in the nucleus in the perinuclear zone. Arrows indicate some areas of EhNCABP166 enrichment. Both images show the nuclei of wild-type trophozoites. Scale Bars = 0·5 μm (A) and 1 μm (B).

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Subcellular location of NLS1-Ac-d100⁻, NLS2-Ac-d100⁻ and NLS3 Ac-d100⁻

To determine whether NLS1, NLS2 and NLS3, which were found through an *in silico* analysis of EhNCABP166, were functional signals, each NLS was fused to the Ac⁻d100⁻ domain (Fig. 3A), which is a cytoplasmic domain of ehFLN (Díaz-Valencia *et al*. 2007). The Ac⁻d100⁻ domain has a molecular mass of 38·3 kDa and is comprised of 4 quasi-repetitive units, with repeats 1, 2 and 3 mainly forming β sheets and repeat 4 exhibiting a low content of β sheets. The Ac⁻d100⁻ domain does not contain a predicted NLS and, as shown in Fig. 3B, it is located in the cytoplasm and at the cytoplasmic membrane. Fusion of each of the EhNCABP166 NLSs to Ac⁻d100⁻ (Fig. 3C, D and E) altered the localization of Ac⁻d100⁻. The addition of these signals resulted in the migration of Ac⁻d100⁻ toward the nucleus.

These data suggest that NLS1 (369-KKIEE-LEKLVSVMKEKK-385), NLS2 (746-KKQIE-IIKNDNEKERKN-762) and NLS3 (1005-KKQLENENEIIKKENKK-1021) are functional NLSs.

Subcellular localization of EhNCABP166 domains

To determine the subcellular localization of the diverse domains that make up EhNCABP166, every domain was cloned, fused with an HSV epitope tag and transfected into *E. histolytica* trophozoites. Seven constructs (Fig. 4) were produced, as described in the Materials and Methods section. The subcellular localization of these constructs was determined *via* confocal microscopy using an anti-HSV monoclonal antibody.

Analysis of the transfected cells using confocal microscopy showed that the intact EhNCABP166 protein localized to the inner perinuclear zone, cytoplasm and plasma membrane (Fig. 5A), as expected. However, the perinuclear staining appeared to be more intense. The actin-binding domain EhNCABD-166 is a protein fragment containing 2 CH domains but no NLS, and this fragment was clearly located on membrane extensions (Fig. 5B). Additionally, some trophozoites expressing EhNCABD-166 exhibited a different structure and shape, which suggested that EhNCABD-166 might be competing for F-actin with other ABPs present in *E. histolytica*. The EhNCBAR1-166 domain (31·8 kDa) was mainly localized to the cytoplasm (Fig. 5C). EhNCGBD/FH3-166 (50·7 kDa) and EhNCR1C-166 (55·7 kDa) were located in the inner perinuclear zone and in the cytoplasm, similar to intact EhNCABP166 (Fig. 5D and E). Additionally, EhNCR1C-166 was also found to be highly enriched in the inner perinuclear area. EhNCGBD/FH3-166 contains NLS2 (746-KKQIEIIKNDNEKERKN-762), and EhNCR1C-166 contains NLS3 (1005-KKQLENENEIIKKENKK-1021), which suggests that the NLSs present in these domains are responsible for the perinuclear location of EhNCABP166. Furthermore, EhNCR1C + NES-166 (64·4 kDa), which is a construct comprised of the alpha region and the EhNCBAR2 + NES-166 domain was analysed. This construct localized to the inner perinuclear area, cytoplasm and protrusions (Fig 5F). This domain contains NLS3 and the NES; therefore, as expected, this construct was found in both the nucleus and cytoplasm.

EhNCBAR2ΔNES-166, which is a 31-kDa domain, localized to the inner perinuclear zone and cytoplasm (Fig. 5G). However, *in silico* analysis of this domain did not indicate the presence of an NLS. We added the last 73 amino acids of EhNCABP166, which includes an export signal (1336-LFNSLAL-1342), to this domain to obtain the construct EhNCBAR2 + NES-166. This construct was located mainly in the cytoplasm and, to a lesser extent compared to EhNCBAR2ΔNES-166, in the perinuclear zone (Fig. 5H). This result suggests that the decreased presence of EhNCBAR2 in the perinuclear area is due to the NES, which promotes export to the cytoplasm.

Mutation of EhNCABP166 NLSs

The classical bipartite NLS consists of 2 sets of 2–3 basic amino acids separated by 10–12 residues. The EhNCABP166 NLSs represent this type of signal. The first block in the 3 NLSs is conserved (Table 1). To ascertain whether the amino acid components of the first block of NLS2 and NLS3 are critical for the transport of EhNCABP166 to the perinuclear area, lysines 746 and 747 (NLS2) and lysines 1005 and 1006 (NLS3) were mutated to alanine (Fig. 6A). A mutated NLS1 was not generated because EhNCBAR1-166 did not localize to the inner perinuclear area as was observed for EhNCGBD/FH3-166 and EhNCRC1-166. The results show a diminished presence of the EhNCGBD/FH3K746A/K747A construct in the inner perinuclear area, mainly located close to the plasma membrane. Mutation of the lysines within the first block of NLS2 affected the shuttling of EhNCGBD/FH3-166 toward the perinuclear zone (Fig. 6B). EhNCR1CK1005A/K1006A-166 was located in the cytoplasm and in the cytoplasmic membrane, whereas its presence in the nucleus was diminished (Fig. 6C). These results suggest that the presence of lysines within the first block of the NLSs is critical to specify migration to the nucleus.

Nuclear location of the transfected constructs under TEM

To confirm the results obtained using confocal microscopy concerning whether the location of the

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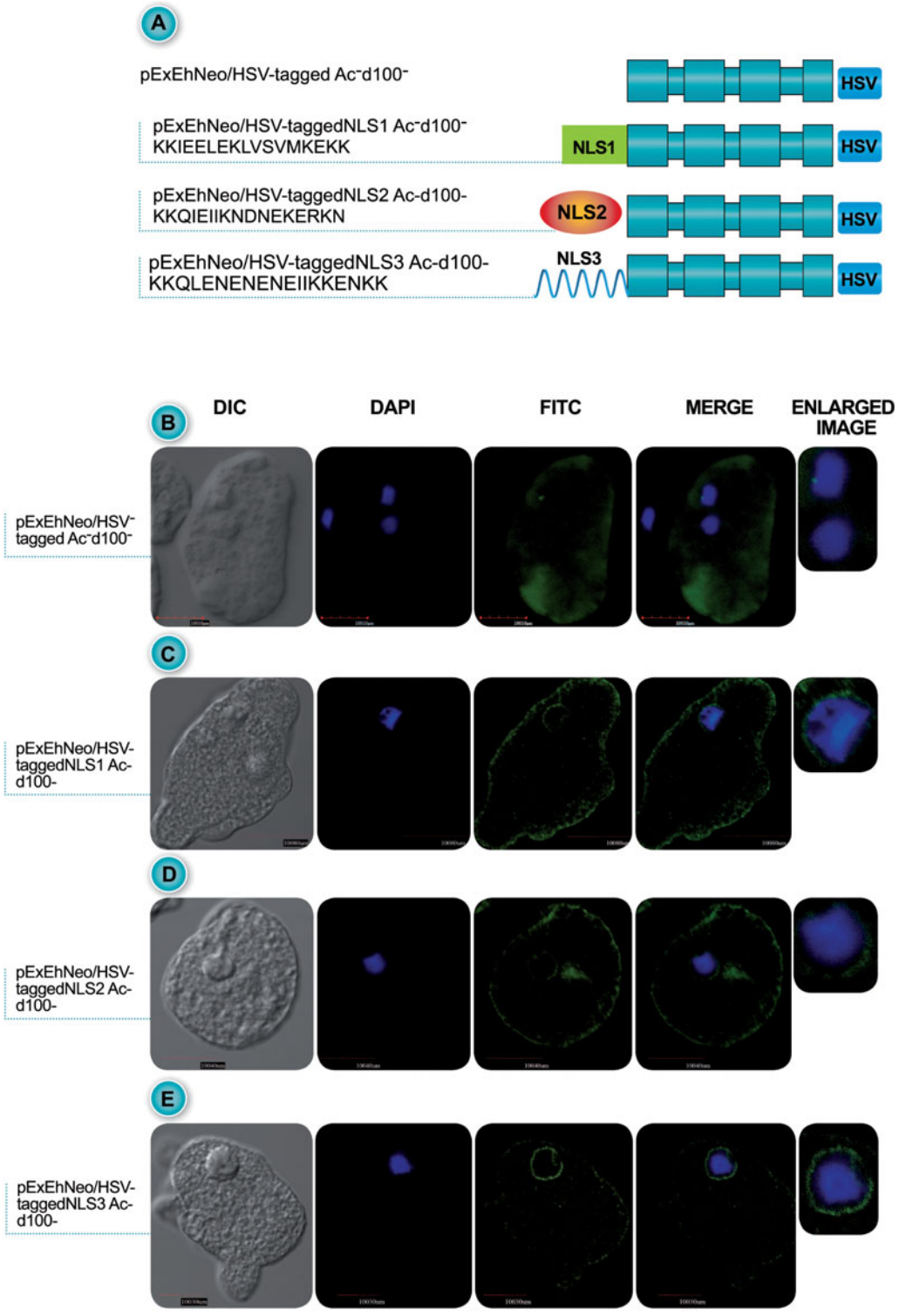

Fig. 3. Cloning, expression and cellular analysis of the NLSs fused to Ac-d100- in transfected trophozoites. (A) Schematic diagram of NLS1, NLS2 and NLS3 fused to Ac-d100-, which was overexpressed in *Entamoeba histolytica* trophozoites. (B) The subcellular localization of Ac-d100- was analysed using confocal microscopy. Ac-d100- is distributed in the cytoplasm; this domain was used as a cytoplasmic control. Fusion of NLS1(C), NLS2 (D) and NLS3 (E) to Ac-d100- resulted in transport of this domain to the perinuclear zone. These results suggest that NLS1, 2 and 3 are functional signals. The localization of these constructs was determined using a monoclonal anti-HSV antibody and an FITC-labelled secondary antibody. Cells were stained with DAPI to visualize the nuclei. All sequences were fused to an HSV tag at their 3′ end (blue box). All transfected cells were grown under 30 μg ml^{-1} of G418. Scale Bar = 1000 μm.

different constructs (NLS3Ac-d100-, EhNCR1C-166 and EhNCR1CK1005/1006) in the perinuclear zone corresponded to intranuclear localization, we sought to analyse the constructs *via* TEM. A monoclonal antibody that recognizes the HSV epitope was used. As depicted in Fig. 7B, C and D,

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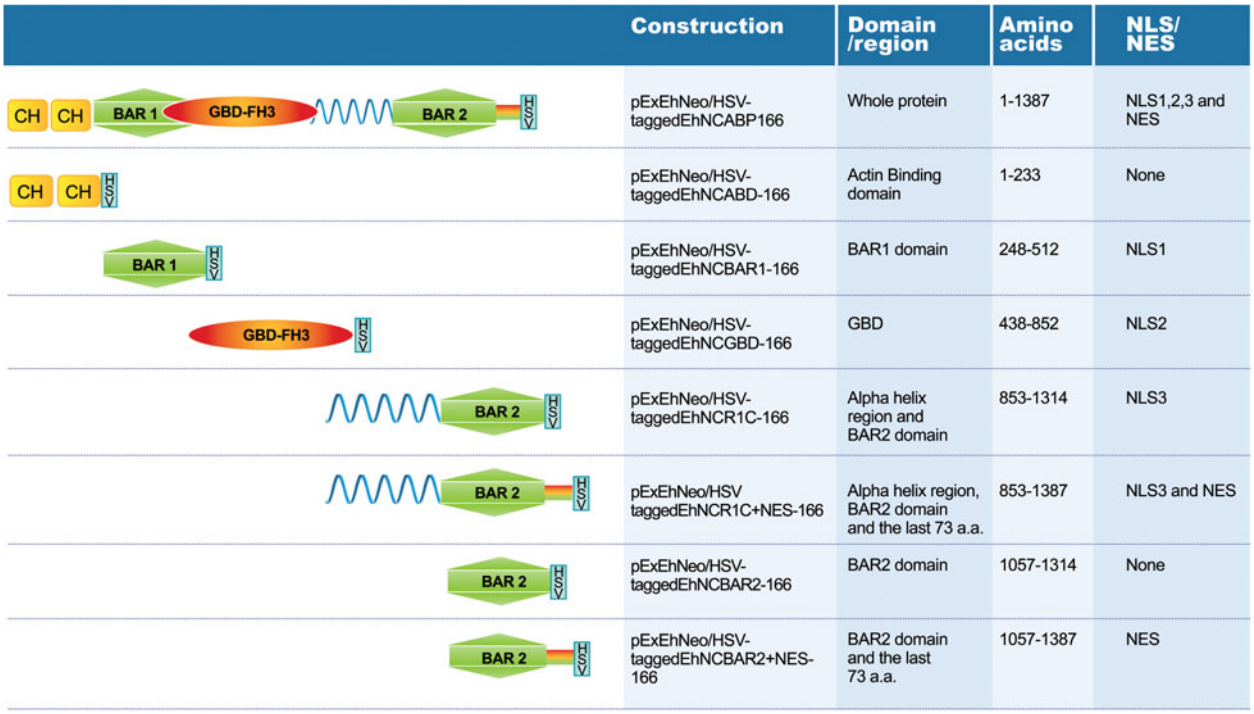

Fig. 4. Schematic diagram of full-length EhNCABP166 and different EhNCABP166 constructs that were overexpressed in *Entamoeba histolytica* trophozoites. The diagram shows the name of the construction, the region and/or domain, and the amino acids that cover every construction. Also the diagram shows the presence of signals from every construction.

TEM micrographs showed that all of the constructs were located in the nucleus in the inner perinuclear zone and throughout the entire intranuclear space. Mutation of lysines 1005 and 1006 resulted in a diminished presence of EhNCR1C-166 in the nucleus.

Location of PI(3,4)P_2 within wild type and transfected cells

Transfected EhNCBAR1-166 domain was located outside the nucleus even when this domain contained the NLS1 (Fig. 5D), despite the fact that NLS1 is a functional signal that allows Ac$^-$d100$^-$ to migrate to the nucleus upon fusion with this NLS (Fig. 3A). The EhNCBAR1-166 domain contains 2 consensus binding sites for phosphoinositide (3,4)P_2 that overlaps with the NLS sequence. To determine the possible interaction of PI(3,4)P_2 with the EhNCBAR1-166 domain within transfected trophozoites, the subcellular localization of both components was determined *via* confocal microscopy using an anti-PI(3,4)P_2 monoclonal antibody and a rabbit anti-HSV antibody. First, the location of PI (3,4)P_2 was determined within wild type cells. The result showed that the phosphoinositide localized to cytoplasm, plasma membrane and nucleus (Fig. 8A). In transfected cells with the EhNCBAR1-166 domain, PI(3,4)P_2 and EhNCBAR1-166 domain were localized mainly to the cytoplasm. The result showed a co-localization of EhNCBAR1-166 domain with PI (3,4)P_2 (Fig. 8B).The arrows show some orange dots, suggesting an interaction between these molecules within *E. histolytica*-transfected trophozoites. Also, we determined the location of PI(3,4)P_2 within transfected cells with NLS1Ac$^-$d100$^-$; this construction only contains the NLS1 sequence attached to the Ac$^-$d100$^-$ cytoplasmic domain. The results showed that PI(3,4)P_2 and NLS1Ac$^-$d100$^-$ were localized to cytoplasm and nucleus (Fig 8C). These results will be discussed below.

DISCUSSION

Here, we report the nuclear presence of EhNCABP166, a nucleocytoplasmic ABP in *E. histolytica*. In the nucleus, this protein is located both in the inner perinuclear area and throughout this organelle. Additionally, we report the existence of NLSs and an NES in EhNCABP166. To determine the functionality of the signals present in EhNCABP166, we applied different strategies. We demonstrated that the individual NLSs 1, 2 and 3 are functional signals that can specify the migration of Ac$^-$d100$^-$ to the nucleus. The Ac$^-$d100$^-$ domain alone is not shuttled to the nucleus, but fusion with the bipartite signals results in its being shuttled to this organelle. Additionally, cloning of the different domains and regions comprising EhNCABP166 allowed us to determine the functionality of the signals in each domain or construct. The results showed that EhNCABD-166 and EhNCBAR1-166 were located in the cytoplasm. Similar to other ABDs

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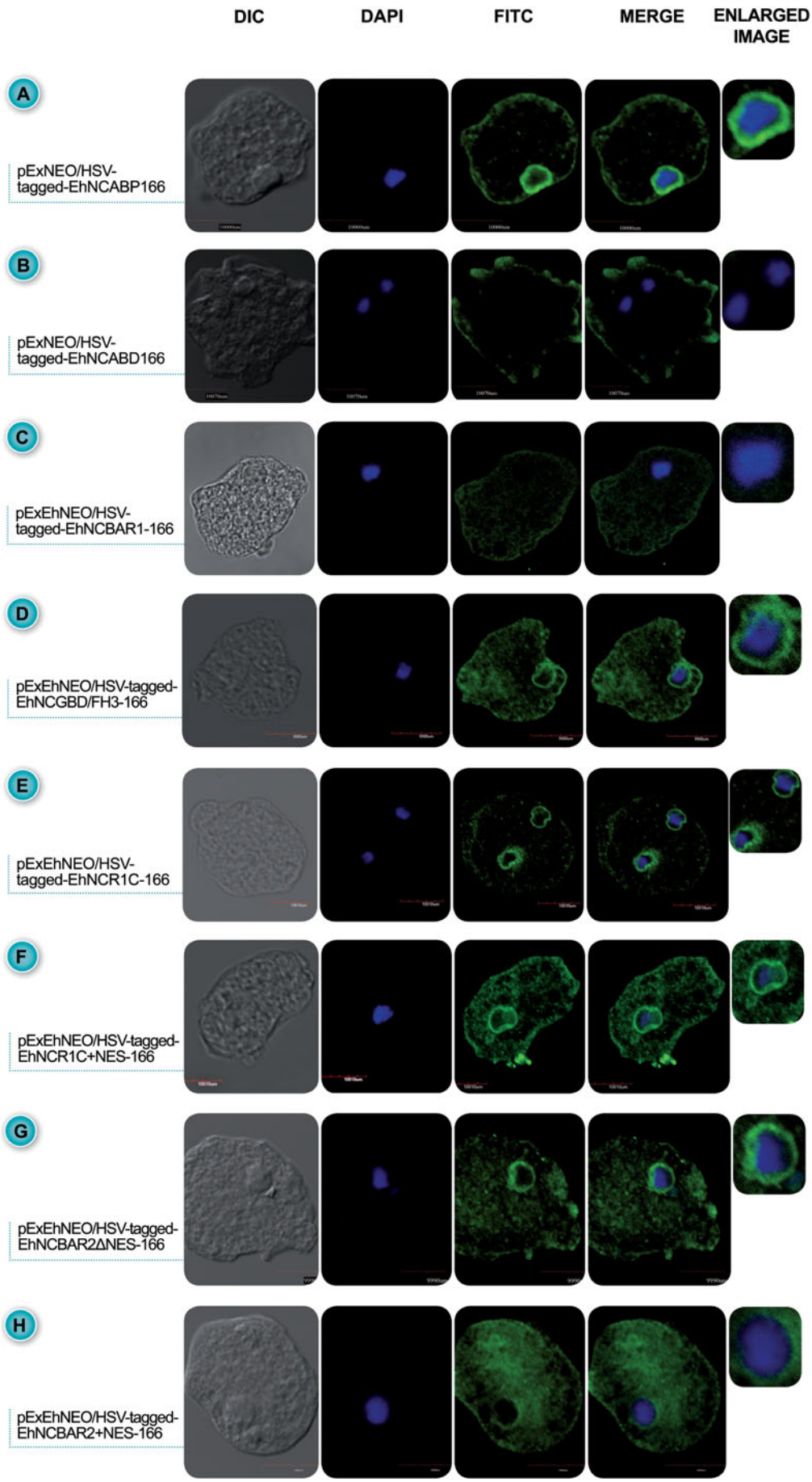

Fig. 5. See the following page for figure legend.

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Table 1. Bipartite NLS and NES of EhNCABP166

((A) The amino acid residues constituting representative bipartite nuclear localization signals (NLS) are indicated with the single letter code. Bold letters indicate the residues that have been shown to be particularly important for recognition by importins. Examples of other NLSs reported in other organisms are shown. (B) The amino acid residues constituting short nuclear export signals (NES) are indicated with the single letter code. Bold letters indicate the residues that have been shown to be particularly important for recognition by exportin 1. Examples of other NESs reported in other organisms are shown.)

A

Protein	NLS amino acid sequence
EhNCABP166 (NLS1)	**KK**IEELEKLVSMKE**KK**
EhNCABP166 (NLS2)	**KK**QIEIIKNDNE**KERK**
EhNCABP166 (NLS3)	**KK**QLENENEIIKKEN**KKK**
Nucleoplasmin	**KR**PAATKAGQA**KKKK**
Human IL5	**KK**YIDGQKKKCGEE**RRR**
MAPKAP Kinase 2	**KK**IEDASNPLLL**KRRKK**

B

Protein	NES amino acid sequence
EhNCABP166	**L**FNS**L**A**L**
HsDcpS	**L**RQD**L**R**L**
HTLV-1rex	**L**YSS**L**S**L**
Rad24	**L**RDN**L**T**L**

belonging to ABPs like filamin, EhNCABD-166 is located in the cortical region (Washington and Knecht, 2008), which is an area that is presumed to be rich in F-actin. EhNCABD-166 has a relatively small molecular mass of 27·7 kDa, which could allow it to be shuttled to the nucleus *via* diffusion, as has been observed for certain proteins (Fried and Kutay, 2003). The nuclear pore complex (NPC) permits the passive diffusion of small molecules, but this diffusion becomes inefficient when the molecular weight of the diffusive species approaches 20–40 kDa. The unique localization of EhNCABD-166 to areas where cell membrane ruffling occurs suggests the functionality of this domain, which is supported by the lack of an NLS and the absence of diffusion of this domain toward the nucleus. EhNCBAR1-166, which contains NLS1, was located mainly in the cytoplasm, despite the fact that NLS1 is a functional signal that allows $Ac^{-}d100^{-}$ to migrate to the nucleus upon fusion with this NLS. These findings can be explained by the fact that the EhNCBAR1-166 domain contains 2 consensus binding sites for $PI(3,4)P_2$ located at 359-ELRVQIKQKIKK-370 and 376-KLVSVMKEKK-385. The NLS1 located within 369-KKIEELEKLVSMKEKK-385 overlaps with the phosphoinositide-binding sites. A previous *in vitro* result showed that the EhNCBAR1 domain has an affinity for this phosphoinositide (Campos-Parra *et al*. 2010). To test the possible interaction of $PI(3,4)P_2$ with EhNCBAR1-166 domain within transfected trophozoites, subcellular localization of the PI and the EhNCBAR1-166 domain was determined. Results showed that EhNCBAR1-166 co-localizes with $PI(3,4)P_2$ in these cells. *In vitro* and *in vivo* results suggest the interaction between this PI and EhNCBAR1-166 domain. Furthermore, we determined the location of $PI(3,4)P_2$ within transfected cells with $NLS1Ac^{-}d100^{-}$ construction. The results showed that $PI(3,4)P_2$ and $NLS1Ac^{-}d100^{-}$ were localized in the cytoplasm and nucleus. The location of $PI(3,4)P_2$ and $NLS1Ac^{-}d100^{-}$ in these cells is similar to the location of this PI in wild type cells and similar to the location of this construction within transfected cells with $NLS1Ac^{-}d100^{-}$, so, the location of both components is not modified. Comparing the $PI(3,4)P_2$ location among the images, a reduction in the presence of $PI(3,4)P_2$ can be seen in the nucleus of transfected cells with EhNCBAR1-166. This suggests again the interaction between $PI(3,4)P_2$ and EhNCBAR1-166 in the cytoplasm. The

Fig. 5. Cloning, expression and cellular analysis of EhNCABP166 and different constructs in *Entamoeba histolytica* trophozoites. (A–H) The distribution of all HSV-tagged domains and regions of EhNCABP166 in transfected trophozoites, analysed by confocal microscopy. (A) EhNCABP166 is located in the inner perinuclear area, cytoplasm and cytoplasmic membrane region; (B) EhNCABD-166 is located on membrane extensions; (C) EhNCBAR1-166 is located in the cytoplasm and the cytoplasmic membrane; (D) EhNCGBD-166 is located in the inner perinuclear area, cytoplasm and cytoplasmic membrane region; (E) EhNCR1C-166 is located in the inner perinuclear zone and cytoplasm; (F) EhNCR1C + NES-166 is located in the inner perinuclear zone, cytoplasm and membrane region; (G) EhNCBAR2ΔNES-166 is located in the inner perinuclear zone and cytoplasm; and (H) EhNCBAR2 + NES-166 is located in the cytoplasm and in the nucleus. Localization to these regions was determined using a monoclonal anti-HSV antibody and a FITC-labelled secondary antibody. Cells were stained with DAPI to visualize the nuclei. All sequences were fused to an HSV tag at their 3′ end (blue box). All of the transfected cells were grown under 30 μg/ml of G418. Scale Bar = 1000 μm.

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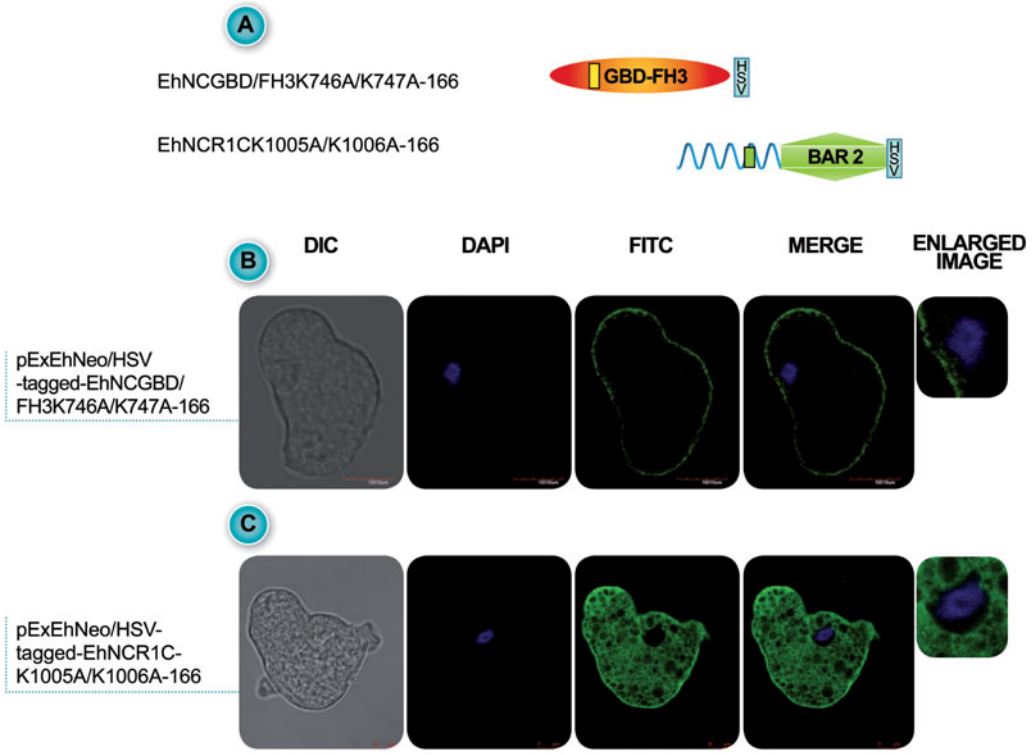

Fig. 6. Cloning, expression and cellular analysis of mutated domains of EhNCABP166 in transfected trophozoites. (A) A schematic diagram of the mutated domains of EhNCABP166 that were overexpressed in *Entamoeba histolytica* trophozoites. Double mutations were generated in the EhNCABP166 NLSs involving an exchange of the lysines in the first block of the bipartite nuclear localization signals for alanines. (B) EhNCGBD/FH3K746A/K747A-166 shows a diminished presence in the perinuclear zone, while the mutated domain is located mainly at the cytoplasmic membrane. (C) EhNCR1CK1005A/K1006A-166 is located in the cytoplasm and at the cytoplasmic membrane and shows a diminished presence in the perinuclear zone. These results suggest that lysines in the first block of NLS2 and NLS3 are necessary for EhNCGBD/FH3-166 and EhNCR1C-166 to be transported to the perinuclear zone. All sequences were fused to a HSV tag at their 3′ end (blue box). All of the transfected cells were grown under 30 μl/ml of G418. Scale Bar = 1000 μm.

construct consisting of NLS1 fused to Ac$^-$d100$^-$ only contained the NLS1 sequence and not the amino acids that surround the NLS, which are apparently important for the interaction with PI(3,4)P_2. It has been proposed that the cellular location of some proteins is regulated by different phosphoinositides (Liu *et al*. 2005; Saarikangas *et al*. 2010). These results suggest that the interaction of PI(3,4)P_2 with consensus binding sites for PI(3,4)P_2 could block the recognition site for importin and preclude the migration of EhNCBAR1-166 toward the nucleus.

EhNCGBD/FH3-166, which contains NLS2, was located at the nucleus, cytoplasm and plasma membrane. Fusion of NLS2 to Ac$^-$d100$^-$ resulted in the localization of this domain in the nucleus, which depended on lysines 746 and 747, as mutation of these residues reduced the presence of EhNCGBD/FH3-166 in the inner perinuclear zone. Therefore, NLS2 is a functional signal that is partially responsible for the migration of EhNCABP166 to the nucleus. EhNCR1C-166, which contains NLS3, was located mainly in the inner perinuclear zone, similar to EhNCABP166, and to a lesser extent, in the cytoplasm and at the plasma membrane. Fusion of NLS3 to Ac$^-$d100$^-$ resulted in this domain localizing to the nucleus, which depended on lysines 1005 and 1006 because mutation of these residues reduced the presence of EhNCR1C-166 in the nucleus. The results obtained using TEM corroborate the intranuclear location of these constructs. These results suggest that NLS3 is a functional signal that is responsible for the migration of EhNCABP166 to the nucleus. Addition of the nuclear export signal, which is within the last 73 amino acids of EhNCABP166, to EhNCR1C-166 (EhNCR1C + NES-166) resulted in a mainly cytoplasmic localization pattern, suggesting that the NES was responsible for the transport of EhNCR1C-166 towards the cytoplasm. EhNCBAR2ΔNES-166 was located at the inner perinuclear zone, cytoplasm and at the plasma membrane (Fig. 5g), but there was no indication of a predicted NLS in this domain; therefore, the nuclear location of EhNCBAR2ΔNES-166 was not expected. EhNCR1C-166 includes the EhNCBAR2ΔNES-166 domain and, as mentioned above, EhNCR1C-166 (containing NLS3) was located in the nucleus. Additionally, the EhNCR1CK1005A/1006A mutations within NLS3 led to a decrease in the nuclear

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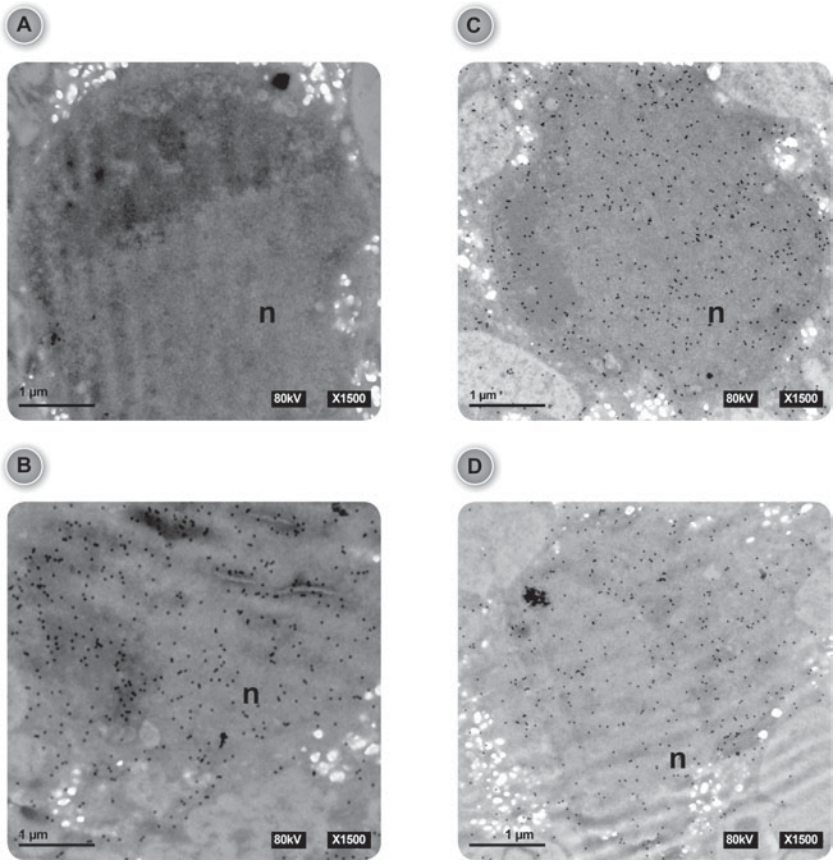

Fig. 7. Transmission electron microscopy analysis of transfected *Entamoeba histolytica* trophozoites. (A) Secondary antibody control. As depicted in the figure, the secondary antibody does not recognize any epitope. (B) Staining with an anti-HSV monoclonal antibody was followed by a secondary antibody conjugated to gold particles. NLS3Ac^{-}d100^{-} is located in the nucleus. (C) Staining with an anti-HSV monoclonal antibody was followed by a secondary antibody conjugated to gold particles. EhNCR1C-166 was localized in the nucleus. (D) Staining with an anti-HSV monoclonal antibody was followed by a secondary antibody conjugated to gold particles. The degree of EhNCR1CK1005A/K1006A-166 localization to the nucleus is less than that of EhR1C-166. All images show the nuclei of transfected trophozoites. Scale Bar = 1 μm.

localization of this sequence. However, EhNCBAR2ΔNES-166 was also located at the nucleus, which indicated that this domain contains a hypothetical NLS that is not functional (or is masked) in EhNCRIC-166. It is also possible that EhNCBAR2ΔNES-166 could interact with other proteins that allow its transportation toward the nucleus. For both EhNCR1C-166 and EhNCBAR2ΔNES-166, the addition of the last 73 amino acids of EhNCABP166 (containing the NES 1336-**L**FNS**L**A**L**-1342) shifted the localization of both constructs toward the cytoplasm. This type of export signal is present in proteins such as the human scavenger decapping enzyme (DcpS) and the RAD24 protein (Shen *et al*. 2008; Lopez-Girona *et al*. 1999).

These results suggest that the nuclear export signal present in EhNCABP166 is a functional signal and could be recognized by an importin β responsible for transporting proteins containing an NES to the cytoplasm (such as the CRM1 protein). Analysis *in silico* performed by O'Reilly *et al*. (2011) showed the possible presence of CRM1 (also known as exportin 1) in *E. histolytica*. Exportins shuttle between the nucleus and cytoplasm, by binding cargo molecules under high RanGTP levels inside the nucleus, and release their cargo upon hydrolysis of Ran-bound GTP in the cytoplasm (Güttler *et al*. 2010). Additionally, *in silico* analysis revealed the presence of a Ran protein in *E. histolytica*. The putative Ran protein is designated by the access code EHI_148190, shares 73% identity and 85% homology with *Homo sapiens* Ran (data not shown) and is conserved in diverse organisms (Gorlich and Kutay, 1999; Mosammaparast and Pemberton, 2004). In light of these results, we suggest the existence of essential components belonging to a functional nucleocytoplasmic transport system, such as karyopherin α and β, exportins and Ran, in *E. histolytica*. Further studies will confirm the presence of all of the components of this system and its characteristics in this parasite. Moreover, some ABPs with specific functions have been reported in the nucleus (Uribe and Jay, 2009; Castano *et al*. 2010). ABPs are multifunctional proteins with different functions in specific compartments. Some ABPs contain an NLS that allows them be translocated to the nucleus, and

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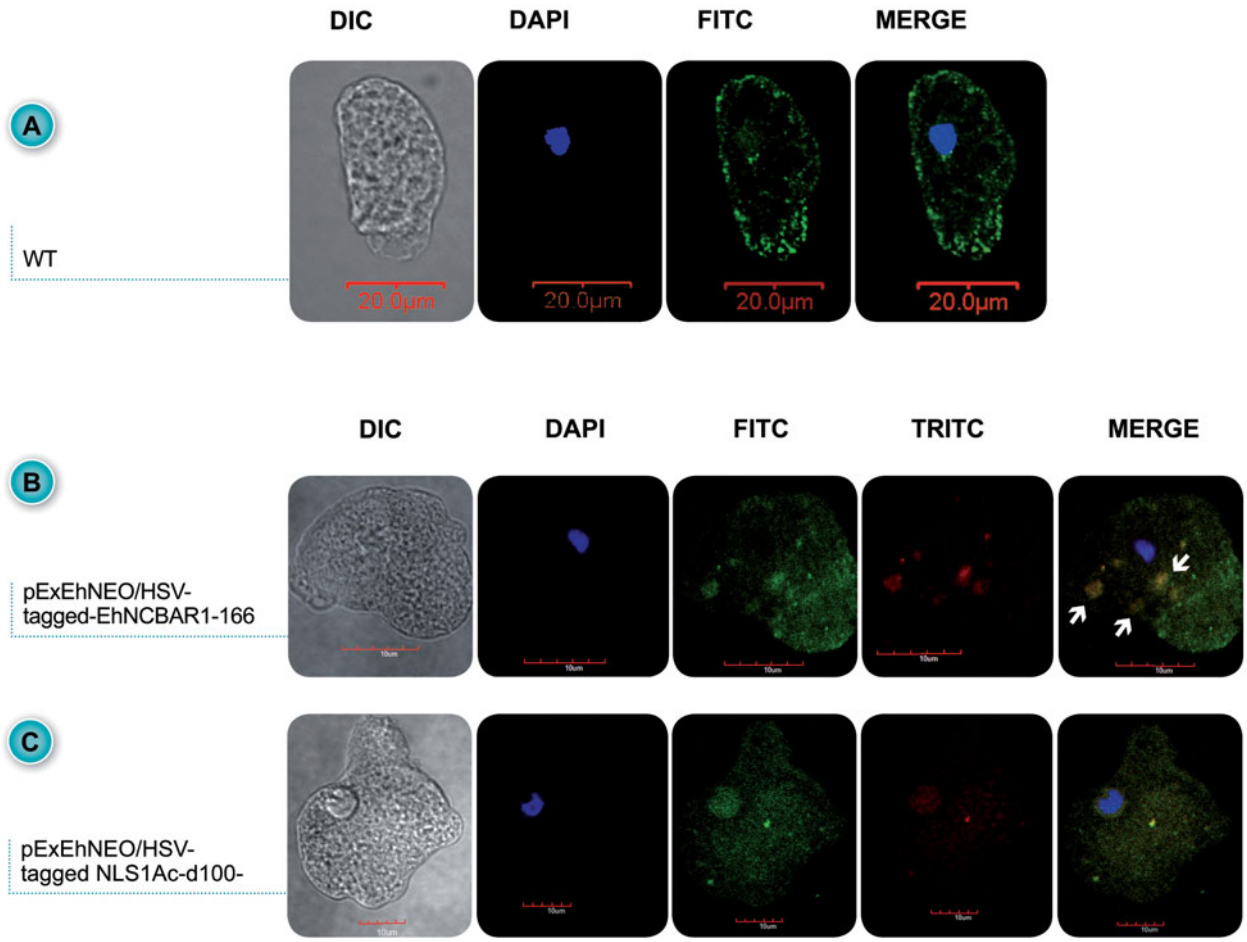

Fig. 8. Cellular analysis of PI(3,4)P_2 and EhNCBAR1-166 domain in wild type and transfected trophozoites. (A) PI(3,4)P_2 was localized to cytoplasm (FITC), plasma membrane and nucleus within wild type trophozoites. (B) EhNCBAR1-166 domain was localized to the cytoplasm (FITC) and PI(3,4)P_2 was localized mainly to the cytoplasm (TRITC) within transfected trophozoites with EhNCBAR1-166 construction. Arrows indicate co-localization of PI(3,4)P_2 with EhNCBAR1-166 domain (C) NLS1Ac$^-$d100$^-$ was localized to the cytoplasm and nucleus (FITC) and PI(3,4)P_2 was localized to the cytoplasm and nucleus (TRITC) within transfected trophozoites with NLS1Ac$^-$d100$^-$ construction. All of the transfected cells were grown under 30 μl/ml of G418. Scale Bars = 20 and 10 μm as indicated.

similarly, some ABPs contain an NES. Certain proteins containing both signals have been reported to be components of signalling pathways, protein carriers or regulators of cellular processes (Malki *et al*. 2010; Rodríguez *et al*. 2010; Magico and Bell, 2011). Following this idea, we can think about multi-functionality of several proteins from *E. histolytica*. The presence of NLSs or NES in proteins of *E. histolytica* suggests a role for these proteins in the nucleus besides having another function in the cytoplasm. We performes an *in silico* analysis to determine the existence of proteins containing NLSs or NESs in proteins from *E. histolytica*, and found that many proteins exist which possibly contain this kind of signal (Suppl. Table 2, online version only). The existence of these signals is logical in some proteins due their know function, but in other proteins the presence of these signals is not very obvious. So now it is important to study all the possible functions that a single protein with this kind of signal could have in this parasite. Therefore, our group is carrying out a variety of experiments to determine the function of the EhNCABP166 protein in the nucleus.

Taking all of our results together, we report for first time, the presence of functional NLSs and an NES in an ABP in *E. histolytica*, and we suggest that this protein could be shuttled between the nucleus and cytoplasm by a nucleocytoplasmic transport system in this parasite.

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REFERENCES

Binder, M., Ortner, S., Erben, H., Scheiner, O., Wiedermann, G., Valenta, R. and Duchêne, M. (1995). The basic isoform of profilin in pathogenic *Entamoeba histolytica*. *European Journal of Biochemistry* **233**, 976–81. http://dx.doi.org/10.1111/j.1432-1033.1995.976_3.x.

Blazquez, S., Guigon, G., Weber, C., Syan, S., Sismero, O., Coppée, JY., Labruyère, E. and Guillén, N. (2008). Chemotaxis of *Entamoeba histolytica* towards the pro-inflammatory cytokine TNF is based on PI3 K signalling, cytoskeleton reorganization and the Galactose/N-acetylgalactosamine lectin activity. *Cellular Microbiology* **10**, 1676–1686. http://dx.doi.org/10.1111/j.1462-5822.2008.01158.x.

Boulo, S., Akarsu, H., Ruigrok, R. W. and Baudin, F. (2007). Nuclear traffic of influenza virus proteins and ribonucleoprotein complexes. *Virus Research* **124**, 12–21. http://dx.doi.org/10.1016/j.virusres.2006.09.013.

Campos-Parra, A. D., Hernández-Cuevas, N. A., Hernandez-Rivas, R. and Vargas, M. (2010). EhNCABP166: A nucleocytoplasmic actin-binding protein from *Entamoeba histolytica*. *Molecular & Biochemical Parasitology* **172**, 19–30. http://dx.doi.org/10.1016/j.molbiopara.2010.03.010.

Castano, E., Philimonenko, V. V., Kahle, M., Fukalová, J., Kalendová, A., Yildirim, S., Dzijak, R., Dingová-Krásna, H. and Hozák, P. (2010). Actin complexes in the cell nucleus: new stones in an old field. *Histochemistry and Cell Biology* **133**, 607–626. http://dx.doi.org/10.1007/s00418-010-0701-2.

Chook, Y. M. and Süel, K. E. (2011). Nuclear import by karyopherin-βs: recognition and inhibition. *Biochimica et Biophysica Acta* **1813**, 1593–1606. http://dx.doi.org/10.1016/j.bbamcr.2010.10.014.

Coudrier, E., Amblard, F., Zimmer, C., Roux, P., Olivo-Marin, J. C. and Guillén, N. (2005). Myosin II and the Gal-GalNAc lectin play a crucial role in tissue invasion by *Entamoeba histolytica*. *Cellular Microbiology* **7**, 19–27. http://dx.doi.org/10.1111/j.1462-5822.2004.00426.x.

Diamond, L. S., Harlow, D. R. and Cunnick, C. C. (1978). A new medium for the axigenic cultivation of *Entamoeba histolytica* and other *Entamoeba*. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **72**, 431–432.

Díaz-Valencia, J. D., Almaraz-Barrera, M. de J., Arias-Romero, L. E., Hernandez-Rivas, R., Rojo-Domínguez, A., Guillén, N. and Vargas, M. (2005). The ABP-120 C-end region from *Entamoeba histolytica* interacts with sulfatide, a new lipid target. *Biochemical and Biophysical Research Communications* **338**, 1527–1536. http://dx.doi.org/10.1016/j.bbrc.2005.10.119.

Díaz-Valencia, J. D., Almaraz-Barrera, M. de J., Jay, D., Hernández-Cuevas, N. A., García, E., González-De la Rosa, C. H., Arias-Romero, L. E., Hernandez-Rivas, R., Rojo-Domínguez, A., Guillén, N. and Vargas, M. (2007). Novel structural and functional findings of the ehFLN protein from *Entamoeba histolytica*. *Cell Motility and the Cytoskeleton* **64**, 880–896. http://dx.doi.org/10.1002/cm.20232.

Ebert, F., Guillén, N., Leippe, M. and Tannich, E. (2000). Molecular cloning and cellular localization of an unusual bipartite *Entamoeba histolytica* polypeptide with similarity to actin binding proteins. *Molecular and Biochemical Parasitology* **111**, 459–464. http://dx.doi.org/10.1016/S0166-6851(00)00331-5.

Fried, H. and Kutay, U. (2003). Nucleocytoplasmic transport: taking an inventory. *Cellular and Molecular Life Sciences* **60**, 1659–1688. doi: 10.1007/s00018-003-3070-3.

Görlich, D. and Kutay, U. (1999). Transport between the cell nucleus and the cytoplasm. *Annual Review of Cell and Developmental Biology* **15**, 607–660. http://dx.doi.org/10.1146/annurev.cellbio.15.1.607.

Guillén, N. (1996). Role of signaling and cytoskeletal rearrangements in the pathogenesis of *Entamoeba histolytica*. *Trends in Microbiology* **4**, 191–197. http://dx.doi.org/10.1016/0966-842X(96)10033-0.

Güttler, T., Madl, T., Neumann, P., Deichsel, D., Corsini, L., Monecke., T., Ficner, R., Sattler, M. and Görlich, D. (2010). NES consensus redefined by structures of PKI-type and Rev-type nuclear export signals bound to CRM1. *Nature Structural & Molecular Biology* **17**, 1367–1376. http://dx.doi.org/10.1038/nsmb.1931.

Hamann, L., Nickel, R. and Tannich, E. (1995). Transfection and continuous expression of heterologous genes in the protozoan parasite *Entamoeba histolytica*. *Proceedings of the National Academy of Sciences, USA* **92**, 8975–8979.

Hon, C. C., Nakada-Tsukui, K., Nozaki, T. and Guillén, N. (2010). Dissecting the actin cytoskeleton of *Entamoeba histolytica* from a genomic perspective. In *Anaerobic Parasitic Protozoa, Genomics and Molecular Biology* (ed. Graham Clark, C., Johnson, , Patricia, J. and Adam, Rodney D.), p. 81–118. Caister Academic Press, Norfolk, UK.

Imamoto, N., Shimamoto, T., Takao, T., Tachibana, T., Kose, S., Matsubae, M., Sekimoto, T., Shimonishi, Y. and Yoneda, Y. (1995). In vivo evidence for involvement of a 58 kDa component of nuclear pore-targeting complex in nuclear protein import. *The EMBO Journal* **14**, 3617–3626.

Kutay, U. and Güttinger, S. (2005). Leucine-rich nuclear-export signals: born to be weak. *Trends in Cell Biology* **15**, 121–124. http://dx.doi.org/10.1016/j.tcb.2005.01.005.

Lee, Y. H., Campbell, H. D. and Stallcup, M. R. (2004). Developmentally essential protein flightless I is a nuclear receptor coactivator with actin binding activity. *Molecular and Cellular Biology* **24**, 2103–2117. http://dx.doi.org/10.1128/MCB.24.5.2103-2117.2004.

Leippe, M. and Herbst, R. (2004). Ancient weapons for attack and defense: the pore forming polypeptides of pathogenic enteric and free-living amoeboid protozoa. *The Journal of Eukaryotic Microbiology* **51**, 516–521. http://dx.doi.org/10.1111/j.1550-7408.2004.tb00286.x.

Liu, F., Wagner, S., Campbell, R. B., Nickerson, J. A., Schiffer, C. A. and Ross, A. H. (2005). PTEN enters the nucleus by diffusion. *Journal of Cellular Biochemistry* **96**, 221–234. http://dx.doi.org/10.1002/jcb.20525.

Lopez-Girona, A., Furnari, B., Mondesert, O. and Rusell, P. (1999). Nuclear localization of Cdc25 is regulated by DNA damage and a 14-3-3 protein. *Nature, London* **397**, 172–175. http://dx.doi.org/10.1038/16488.

Magico, A. C. and Bell, J. B. (2011). Identification of a classical bipartite nuclear localization signal in the Drosophila TEA/ATTS protein scalloped. *PLoS One* **6**, e21431. http://dx.plos.org/10.1371/journal.pone.0021431.

Malki, S., Boizet-Bonhoure, B. and Poulat, F. (2010). Shuttling of SOX proteins. *The International Journal of Biochemistry & Cell Biology* **42**, 411–416. http://dx.doi.org/10.1016/j.biocel.2009.09.020.

Marfori, M., Mynott, A., Ellis, J. J., Mehdi, A. M., Saunders, N. F., Curmi, P. M., Forwood, J. K., Bodén, M. and Kobe, B. (2011). Molecular basis for specificity of nuclear import and prediction of nuclear localization. *Biochimica et Biophysica Acta* **1813**, 1562–1577. http://dx.doi.org/10.1016/j.bbamcr.2010.10.013.

Marion, S., Tavares, P., Arhets, P. and Guillén, N. (2004). Signal transduction through the Gal-GalNAc lectin of *Entamoeba histolytica* involves a spectrin-like protein. *Molecular and Biochemical Parasitology* **135**, 31–38. http://dx.doi.org/10.1111/j.1462-5822.2005.00487.x.

Marion, S., Laurent, C. and Guillén, N. (2005). Signalization and cytoskeleton activity through myosin IB during the early steps of phagocytosis in *Entamoeba histolytica*: a proteomic approach. *Cellular Microbiology* **7**, 1504–1518. http://dx.doi.org/10.1111/j.1462-5822.2005.00573.x.

Mendoza, L., Orozco, E., Rodríguez, M. A., García-Rivera, G., Sánchez, T., García, E. and Gariglio, P. (2003). Ehp53, an *Entamoeba histolytica* protein, antecesor of the mammalian tumor suppressor p53. *Microbiology* **149**, 885–893. http://dx.doi.org/10.1099/mic.0.25892-0.

Mosammaparast, N. and Pemberton, L. F. (2004). Karyopherins: from nuclear-transport mediators to nuclear-function regulators. *Trends in Cell Biology* **14**, 547–556. http://dx.doi.org/10.1016/j.tcb.2004.09.004.

O'Reilly, A. J., Dacks, J. B. and Field, M. C. (2011). Evolution of the karyopherin-β family of nucleocytoplasmic transport factors; ancient origins and continued specialization. *PLoS One* **6**, e19308. http://dx.plos.org/10.1371/journal.pone.0019308.

Pritt, B. S. and Clark, C. G. (2008). Amebiasis. *Mayo Clinic Proceedings* **83**, 1154–1160. http://dx.doi.org/10.4065/83.10.1154.

Rodríguez, E., Aburjania, N., Priedigkeit, N. M., DiFeo, A. and Martignetti, J. A. (2010). Nucleo-cytoplasmic localization domains regulate Krüppel-like factor 6 (KLF6) protein stability and tumor supresor function. *PLoS One* **5**, e12639. http://dx.plos.org/10.1371/journal.pone.0012639.

Saarikangas, J., Zhao, H. and Lappalainen, P. (2010). Regulation of the actin cytoskeleton-plasma membrane interplay by phosphoinositides. *Physiological Reviews* **90**, 259–289. http://dx.doi.org/10.1152/physrev.00036.2009.

Sahoo, N., Labruyère, E., Bhattacharya, S., Sen, P., Guillén, N. and Bhattacharya, A. (2004). Calcium binding protein 1 of the protozoan parasite *Entamoeba histolytica* interacts with actin and is involved in cytoskeleton dynamics. *Journal of Cell Science* **117**, 3625–3634. http://dx.doi.org/10.1242/jcs.01198.

Shen, V., Liu, H., Liu, S. W., Jiao, X. and Kiledjan, M. (2008). DcpS scavenger decapping enzyme can modulate pre-mRNA splicing. *RNA* **14**, 1132–1142. http://dx.doi.org/10.1261/rna.1008208.

Singh, D., Naik, S. and Naik, S. (2004). Role of cysteine proteinase of *Entamoeba histolytica in* target cell death. *Parasitology* **129**, 127–135. http://dx.doi.org/10.1017/S0031182004005451.

Stanley, S. L., Jr. (2003). Amoebiasis. *Lancet* **361**, 1025–1034. http://dx.doi.org/10.1016/S0140-6736(03)12830-9.

Taagepera, S., McDonald, D., Loeb, J. E., Whitaker, L. L., McElroy, A. K., Wang, J. Y. and Hope, T. J. (1998). Nuclear-cytoplasmic shuttling of C-ABL tyrosine kinase. *Proceedings of the National Academy of Sciences, USA* **95**, 7457–7462.

Tovy, A., Siman, Tov. R., Gaentzsch, R., Helm, M. and Ankri, S. (2010). A new nuclear function of the *Entamoeba histolytica* glycolytic enzyme enolase: the metabolic regulation of cytosine-5 methyltransferase 2 (Dnmt2) activity. *PloS Pathogens* **6**, e1000775. http://dx.plos.org/10.1371/journal.ppat.1000775.

Uribe, R. and Jay, D. (2009). A review of actin binding proteins: new perspectives. *Molecular Biology Reports* **36**, 121–125. http://dx.doi.org/10.1007/s11033-007-9159-2.

Van Etten, R. A., Jackson, P. and Baltimore, D. (1989). The mouse type IV c-abl gene product is a nuclear protein, and activation of transforming ability is associated with cytoplasmic localization. *Cell* **58**, 669–678. http://dx.doi.org/10.1016/0092-8674(89)90102-5.

Vargas, M., Sansonetti, P. and Guillén, N. (1996). Identification and cellular localization of the actin-binding protein ABP-120 from *Entamoeba histolytica*. *Molecular Microbiology* **22**, 849–857. http://dx.doi.org/10.1046/j.1365-2958.1996.01535.x.

Vázquez, J., Franco, E., Reyes, G. and Meza, I. (1995). Characterization of adhesion plates induced by the interaction of *Entamoeba histolytica* trophozoites with fibronectin. *Cell Motility and the Cytoskeleton* **32**, 37–45. http://dx.doi.org/10.1002/cm.970320105.

Virel, A. and Backman, L. (2004). Molecular evolution and structure of alpha-actinin. *Molecular biology and evolution* **21**, 1024–1031. http://dx.doi.org/10.1093/molbev/msh094.

Virel, A. and Backman, L. (2006). Characterization of *Entamoeba histolytica* alpha-actinin. *Molecular and Biochemical Parasitology* **145**, 11–17. http://dx.doi.org/10.1016/j.molbiopara.2005.09.003.

Washington, R. W. and Knecht, D. A. (2008). Actin binding domains direct actin-binding proteins to different cytoskeletal locations. *BMC Cell Biology*. **9**, 10. doi:10.1186/1471-2121-9-10.

WHO/PAHO/UNESCO. (1997). *A Consultation with Experts on Amoebiasis*. Epidemiological Bulletin No. 18, Mexico City, Mexico.

Wu, X., Yoo, Y., Okuhama, N. N., Tucker, P. W., Liu, G. and Guan, J. L. (2006). Regulation of RNA-polymerase-II-dependent transcription by N-WASP and its nuclear-binding partners. *Nature Cell Biology* **8**, 756–763. http://dx.doi.org/10.1038/ncb1433.

Xu, D., Farmer, A. and Chook, Y. M. (2010). Recognition of nuclear targeting signals by Karyopherin-β proteins. *Current Opinion in Structural Biology* **20** 782–790. http://dx.doi.org/10.1016/j.sbi.2010.09.008.