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Nuclear transport in *Entamoeba histolytica*: knowledge gap and therapeutic potential

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Review Article

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Abstract

Entamoeba histolytica is the protozoan parasite that causes human amoebiasis. It is one of the leading parasitic disease burdens in tropical regions and developing countries, with spread to developed countries through migrants from and travellers to endemic regions.

Understanding *E. histolytica*'s invasion mechanisms requires an understanding of how it interacts with external cell components and how it engulfs and kills cells (phagocytosis). Recent research suggests that optimal phagocytosis requires signalling events from the cell surface to the nucleus *via* the cytoplasm, and the induction of several factors that are transported to the plasma membrane. Current research in other protozoans suggests the presence of proteins with nuclear localization signals, nuclear export signals and Ran proteins; however, there is limited literature on their functionality and their functional similarity to higher eukaryotes.

Based on learnings from the development of antivirals, nuclear transport elements in *E. histolytica* may present viable, specific, therapeutic targets.

In this review, we aim to summarize our limited knowledge of the eukaryotic nuclear transport mechanisms that are conserved and may function in *E. histolytica*.

Introduction

The parasite *Entamoeba histolytica* is distributed throughout the world, being most prevalent in tropical and subtropical areas (Haque *et al.* 2003; Stanley, 2003; van Hal *et al.* 2007; Pritt and Clark, 2008; Al-Areeqi *et al.* 2017; Yimer *et al.* 2017). It is a leading parasitic burden in developing countries (Bercu *et al.* 2007; Peterson *et al.* 2011). In developed countries, amoebiasis primarily affects travellers to and migrants from *E. histolytica* endemic regions (Hailemariam *et al.* 2004; Moran *et al.* 2005; Pritt and Clark, 2008; Mukherjee *et al.* 2010) and is prevalent in certain population groups (Salit *et al.* 2009; Hung *et al.* 2012).

There are no licensed vaccines for *E. histolytica* (Quach *et al.* 2014). There are limited treatment options, the most common being metronidazole described >50 years ago (Gonzales *et al.* 2009), in combination with luminal agents such as iodoquinol and paromomycin (Marie and Petri, 2013). Metronidazole is highly effective, however, it has toxic side-effects and *E. histolytica* can gain resistance to it (Kimura *et al.* 2007; Debnath *et al.* 2012). Auranofin, an approved drug for rheumatoid arthritis, is a promising therapeutic option for *E. histolytica* (Debnath *et al.* 2012). The few, suboptimal, treatment options raise the very real possibility of resistance. Given its global reach and high incidence in certain population groups, it is important that new viable treatment options for *E. histolytica* disease be identified.

A better understanding of the intracellular pathways of *E. histolytica* would increase therapeutic options that target components specific to *E. histolytica*, including nuclear transport mechanisms (Aslam *et al.* 2012; Uribe *et al.* 2012). Interestingly, proteins involved in phagocytosis and trogocytosis are also present in the parasite nucleus (Aslam *et al.* 2012; Uribe *et al.* 2012; Ralston *et al.* 2014).

In this review, we provide a brief overview of *E. histolytica* life cycle, pathology and current knowledge of parasite nuclear transport mechanisms.

Life cycle

Entamoeba histolytica exists as an infectious cyst or an amoeboid trophozoite (Marie and Petri, 2013; Ralston, 2015) (Fig. 1). It is transmitted *via* person to person contact or indirectly *via* inadequate sanitation or consumption of contaminated food and water (van Hal *et al.* 2007). Infection begins with ingestion of cysts that move through the stomach, protected from the acidic environment by a chitin-containing cell wall. Excystation takes place in the small intestine and eight trophozoites emerge. Trophozoites are involved in development of lytic phagosomes (most common) or amoebiasis (less common) (Begum *et al.* 2015).

Lytic phagosome

Trophozoites move into and colonize the large intestine (Faust and Guillen, 2012) and can survive for extended periods feeding on intestinal bacteria through phagocytosis (described below, Subsection 'Phagocytosis'), forming a lytic phagosome where bacteria are lysed (Becker *et al.*

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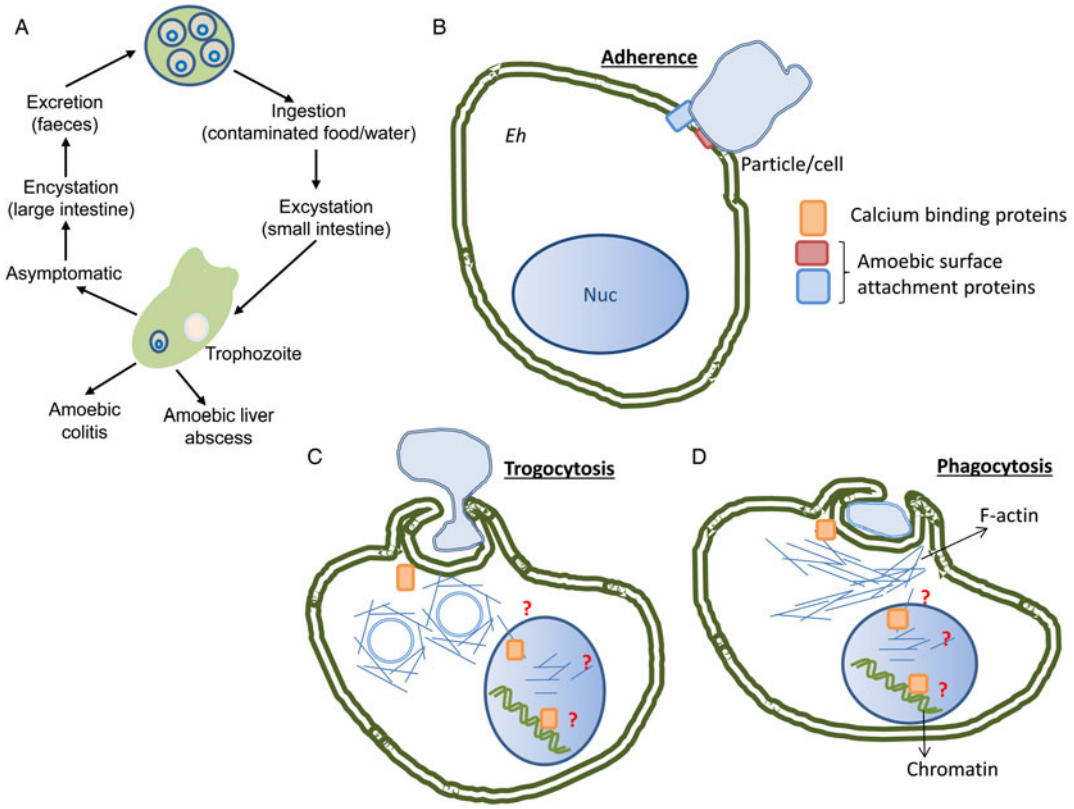

Fig. 1. The life cycle and pathogenic mechanisms of *Entamoeba histolytica*. (A) Infection occurs by ingestion of cysts (generally from fecally contaminated food or water). Excystation occurs in the ileum of the small intestine, releasing eight trophozoites. Trophozoites multiply by binary fission in the large intestine, colonizing it; mostly causing asymptomatic disease. Some trophozoites may invade the intestinal mucosa causing amoebic colitis, or enter the bloodstream, accessing liver and causing a liver abscess. Cyst formation is triggered by the dehydration of gut contents followed by excretion of cyst in feces. (B) Attachment to cells (live or apoptotic) or other particles is mediated by different amoebic cell surface molecules. (C) Larger or more deformable cells are more likely to be ingested by amoebic trogocytosis. (D) Smaller, less deformable or apoptotic cells are more likely to be ingested by phagocytosis. Signal transduction in the initiation of both processes includes amoebic kinases and calcium-binding proteins, all of which influence actin polymerization. Some calcium-binding proteins are found in the nucleus as well as cytoplasm; their nuclear transport mechanisms or nuclear functions are not known (indicated by question marks). Polymerized actin is present in the cytoplasm as F-actin and as shorter rods in the nucleus; their role is unclear.

2010). Trophozoites multiply by binary fission and some become encysted. Cysts can survive for months in the environment and reinfect another host (Eichinger, 2001; Aguilar-Diaz *et al.* 2011). In some disease, states trophozoites are excreted but these cannot survive outside of the host (Haque *et al.* 2003; Stanley, 2003; Pritt and Clark, 2008).

Amoebiasis

Trophozoites can invade the intestinal mucosa, where they feed on epithelial and red blood cells, causing amoebic colitis (Stanley, 2003; Pritt and Clark, 2008). Once the intestinal mucosal barrier has been breached, trophozoites can spread through the body causing a variety of systemic diseases.

Amoebic colitis

Clinical symptoms of amoebic colitis include cramping abdominal pain, weight loss, and watery or bloody diarrhoea for several weeks (Haque *et al.* 2003; Stanley, 2003; Pritt and Clark, 2008). If the mucosal surface is invaded, blood is present in feces, and in rare cases, patients present with fever (Haque *et al.* 2003; Stanley, 2003; Bercu *et al.* 2007; Pritt and Clark, 2008). Extensive fulminant necrotizing colitis is the most severe form of amoebic colitis and is often fatal.

Extra intestinal disease

A systemic infection of *E. histolytica* may occur months or years after the initial infection. The most common organ it invades is the liver, forming an amoebic liver abscess (Pritt and Clark, 2008). The patient can present with jaundice, fever (in 85–90% of cases), cough, rigor, cold sweats, enlarged liver and weight loss (Haque *et al.* 2003; Pritt and Clark, 2008).

Entamoeba histolytica can also invade other organs and systems. Colonization of the pleuro-pulmonary site results in cough, chest pain and difficulty breathing. Rarely, *E. histolytica* spreads to the central nervous system and presents with headaches, vomiting and change in mental status. Other, very rare, sites of infection include the heart, renal system, genitourinary tract and the skin (Haque *et al.* 2003; Stanley, 2003; Pritt and Clark, 2008).

Current treatment options

Asymptomatic intestinal colonization with *E. histolytica* is usually treated with luminal agents such as lodoquinol and paromomycin (Kimura *et al.* 2007) while tissue invasive disease is treated with metronidazole. Metronidazole is a non-specific antibiotic used to target anaerobic bacteria and protozoa infections (Stanley, 2003) and can cause cardiovascular and gastrointestinal adverse effects (Kimura *et al.* 2007). As the only mainstream treatment for invasive amoebiasis, there is rising concern that *E. histolytica* may become resistant to metronidazole (Debnath *et al.* 2012). There are limited studies investigating alternatives to metronidazole, e.g. a trial of a herbal preparation in patients with amoebiasis (Shah *et al.* 2016). Debnath *et al.* (2012) have found that auranofin (an approved drug used therapeutically for rheumatoid

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arthritis) is active against *E. histolytica* in culture raising the possibility of drug re-positioning. Auranofin targets the *E. histolytica* thioredoxin reductase, which prevents thioredoxin reduction and enhances the sensitivity of trophozoites to reactive oxygen-mediated killing (Debnath *et al.* 2012). In rare cases and if treatment fails to treat invasive amoebiasis, surgical intervention is required (Stanley, 2003).

Further understanding of the internal functioning of *E. histolytica* may open new doors for therapeutic interventions that target components specific to *E. histolytica*.

Pathogenicity

The mechanisms of *E. histolytica* pathogenicity include adherence, cytotoxicity and phagocytosis/trogocytosis.

Adherence

Adherence is essential for *E. histolytica* pathogenic infection. Thinning of colonic mucin by the secreted cysteine proteases allows binding of trophozoites to the mucin layer *via* Gal/GalNAc lectin. Subsequently, trophozoites are able to adhere directly to the host epithelial cells (Begum *et al.* 2015; Singh *et al.* 2016).

Cytotoxicity

Following adherence, *E. histolytica* can kill host cells and intestinal bacteria, *via* direct contact with the parasite and indirect exposure to secreted proteinases (Christy and Petri, 2011). Recent data suggest that direct contact leading to trogocytosis/phagocytosis may be the primary cytotoxic mechanism (Ralston, 2015). While the necrotic pathway may predominate (Berninghausen and Leippe, 1997) in some situations, *E. histolytica* often triggers apoptotic cell death at sites of invasion (Becker *et al.* 2010).

E. histolytica encoded amoebapores can form pores in lipid bilayers and are implicated in phagocytosis as they can mediate lysis of ingested content within phagosomes (Berninghausen and Leippe, 1997; Ralston, 2015). Work with mammalian cell lines has shown that *E. histolytica* virulence complex proteins can initiate epithelial damage by interaction with tight junction proteins followed by their degradation (Betanzos *et al.* 2013); it is not clear if this is indeed the case *in vivo*. Amoebapores may also contribute to contact-mediated target cell cytotoxicity.

Trogocytosis

Recent research suggests that trogocytosis is a major *E. histolytica* cytotoxic mechanism for engulfing/eating live cells (Ralston *et al.* 2014). Parasite binds to live cells followed by actin rearrangements and biting off of small portions of the ingested host cell (Fig. 1C). The host cell eventually dies and the trophozoite dissociates from it (Ralston *et al.* 2014; Ralston, 2015).

Phagocytosis

Phagocytosis is essential for acquiring nutrients, invading host tissues and causing pathogenicity (Christy and Petri, 2011). Phagocytosis is initiated when a particle binds to a cell surface receptor (Fig. 1D). This leads to rearrangement of parasite actin cytoskeleton providing the necessary force for phagosome formation (Christy and Petri, 2011). Early phagosomes are surrounded by a rim of filamentous (F) actin that is gradually depolymerized as the phagosome matures. Phagosome maturation is controlled by amoebic homologues of Ras superfamily members, the Rab proteins (Saito-Nakano *et al.* 2005; Avalos-Padilla *et al.* 2015; Verma *et al.* 2015; Hanadate *et al.* 2016; Verma *et al.* 2016; Verma and Datta, 2017), phosphatidylinositols and intracellular protein kinases (Somlata *et al.* 2011; Somlata *et al.* 2012). Several *E. histolytica* encoded Rab proteins have been shown to have roles in different stages of phagocytosis; exactly how the various proteins work together or synergize to enable phagocytosis is still not clear. Similar to higher eukaryotes, endocytosis in *E. histolytica* appears to be driven by ESCRT (endosomal sorting complexes required for transport). EhVps4 is suggested to be involved in phagocytosis and virulence based on the localization of a tagged, overexpressed, EhVps4 around ingested erythrocytes (Lopez-Reyes *et al.* 2010). EhVps32 has been shown to bind to EhADH112 (ALIX related protein) through the Bro1 domain in the latter; the exact functional outcome of this interaction is not yet defined (Banuelos *et al.* 2012) but may be related to multivesicular bodies formation. Actin-binding proteins (ABPs) are key controllers of phagocytosis and are involved in regulation of actin cytoskeleton dynamics at multiple levels. Somlata *et al.* (2011) and Somlata *et al.* (2012) showed that the initiation of erythrophagocytosis in *E. histolytica* depends on a C_2-containing domain kinase (EhC2PK), actin and calcium-binding proteins (EhCaBP1 and EhCaBP3).

The role of calcium-binding proteins in phagocytosis

Calcium (Ca^{2+}) is essential for many eukaryotic processes. In *E. histolytica* Ca^{2+} is involved in lysis of epithelial cells and in the parasite's developmental stages (cysts or trophozoites). Calcium-dependent processes are mediated by calcium-binding proteins and *E. histolytica* produces several of these (EhCaBPs), only some of which have been functionally characterized (Bhattacharya *et al.* 2006).

(Aslam *et al.* 2012) showed that phagocytic cups and newly formed phagosomes contain EhCaBP3, actin and myosin 1B. EhCaBP3 directly binds actin, affecting its polymerization and bundling activity and, with myosin 1B, assists closure of phagocytic cups to form phagosomes. Overall, calcium binding was shown to be essential for phagosome initiation and formation.

At least two calcium-binding proteins in *E. histolytica*, EhCaBP3 and EhCaBP1, are ABPs implicated in phagocytic processes and are localized in both the nucleus and cytoplasm of the parasite (Sahoo *et al.* 2004).

Nuclear transport in eukaryotes

There is limited literature on nuclear–cytoplasmic transport pathways in *E. histolytica*, or indeed, any protozoan parasite. We do know that these pathways are well conserved across eukaryotes, with yeast, mouse and human pathways being best characterized (Wente and Rout, 2010).

Nuclear transport

The eukaryotic genomic material is separated from the cytoplasm by the double membrane of the nuclear envelope (Rout and Aitchison, 2001) that contains numerous nuclear pore complexes (NPCs) that allow selective passage of molecules (Rout and Aitchison, 2001; Wente and Rout, 2010).

NPCs are large complex structures containing multiple copies of nucleoporins (Nups), forming a hollow central core, a nuclear basket, a luminal ring and cytoplasmic filaments (Fig. 2) (Rout and Aitchison, 2001; Wente and Rout, 2010). The central core Nups have numerous phenylalanine and glycine repeats (FG Nups) and mediate nucleocytoplasmic transport *via* interaction with transport components (Rout and Aitchison, 2001; Wente and Rout, 2010). Cytoplasmic filaments provide an initial docking site for active nuclear import. Nups or Nup-orthologues have been described in several protozoans, but only some of them have been shown to be *bona fide* NPC components. The

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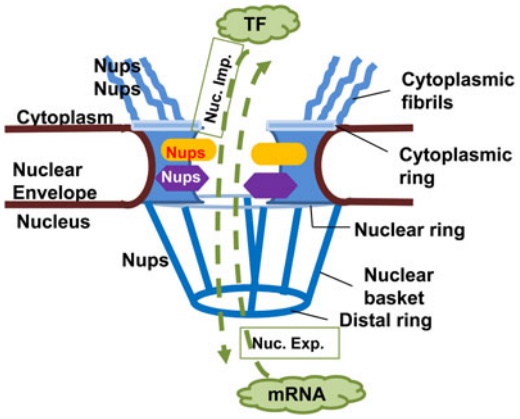

Fig. 2. Nuclear pore complex in Eukaryotes. The nuclear pore complex (NPC) is composed of a central channel, cytoplasmic fibrils, and a nuclear basket situated within a nuclear envelope. Nucleoporins (Nups) form the structure of the NPC, facilitating transport of macromolecules through it in both directions. Proteins, e.g. transcription factors (TF) must move from cytoplasm to the nucleus (nuclear import, Nuc. Imp.), while newly transcribed mRNA must move from the nucleus to the cytoplasm (nuclear export, Nuc. Exp.) to be translated into proteins.

delineation of Nups in the protozoan *Trypanosoma brucei* that localized to the nuclear envelope suggests a common origin from a complex NPC followed by extensive divergent evolution (Rout and Field, 2001; Degrasse and Devos, 2010).

Karyopherins (importins or exportins) bind to their cargoes by recognition of specific nuclear localization signals (NLSs) or nuclear export signals (NESs, Fig. 3) (Rout and Aitchison, 2001; Wente and Rout, 2010). The unidirectional nature of protein import and export through NPCs results from the participation of the small GTPase Ran that exists in different conformations when bound to GTP or GDP (Rout and Aitchison, 2001; Wente and Rout, 2010); nuclear Ran is largely GTP bound and cytoplasmic Ran is not. Ran is highly conserved across eukaryotes, highlighting its key function in eukaryotic biology (Feldherr *et al.* 2002). Importins (IMPs) and Exportins (EXPs) share an α-superhelical structure and their ability to interact with Ran (Wente and Rout, 2010). IMPs and EXPs have a cargo-binding domain, an NPC-binding domain(s) and an amino-terminal Ran-binding domain; numerous isoforms of IMPα, IMPβ and EXPs are known in human, mouse and yeast cells. To date, the number of karyopherins in protozoa is not known, and no studies have examined karyopherins experimentally in *E. histolytica*.

Nuclear import

The nuclear import cycle [Fig. 3(i)] is mediated by direct binding of IMPβ to cargo molecules or indirect binding *via* an adaptor protein (IMPα) (Wente and Rout, 2010). In the nucleus, RanGTP binds to the IMPβ complex which induces cargo displacement and IMPβ–RanGTP is transported back into the cytoplasm. Ran GTPase-activating protein (RanGAP) releases Ran from IMPβ through hydrolysis of RanGTP to GDP. The cycle starts over again when RanGDP is transported from the cytoplasm into the nucleus by nuclear transport factor 2 (NTF2). In the nucleus the exchange factor regulator of chromosome condensation 1 (RCC1) allows RanGDP to be exchanged to its GTP-bound form. Unlike Ran, functional RCC1 and RanGAP have not been identified in all protozoans analysed, raising the possibility of an evolutionarily divergent protozoa-specific Ran cycle (Frankel and Knoll, 2009).

Nuclear export

Nuclear export [Fig. 3(ii)] is similar to nuclear import, in the opposite direction. Several EXPs have been described (Guttler and Gorlich, 2011), with the best characterized being CRM1. CRM1 recognizes proteins with a leucine-rich NES and in

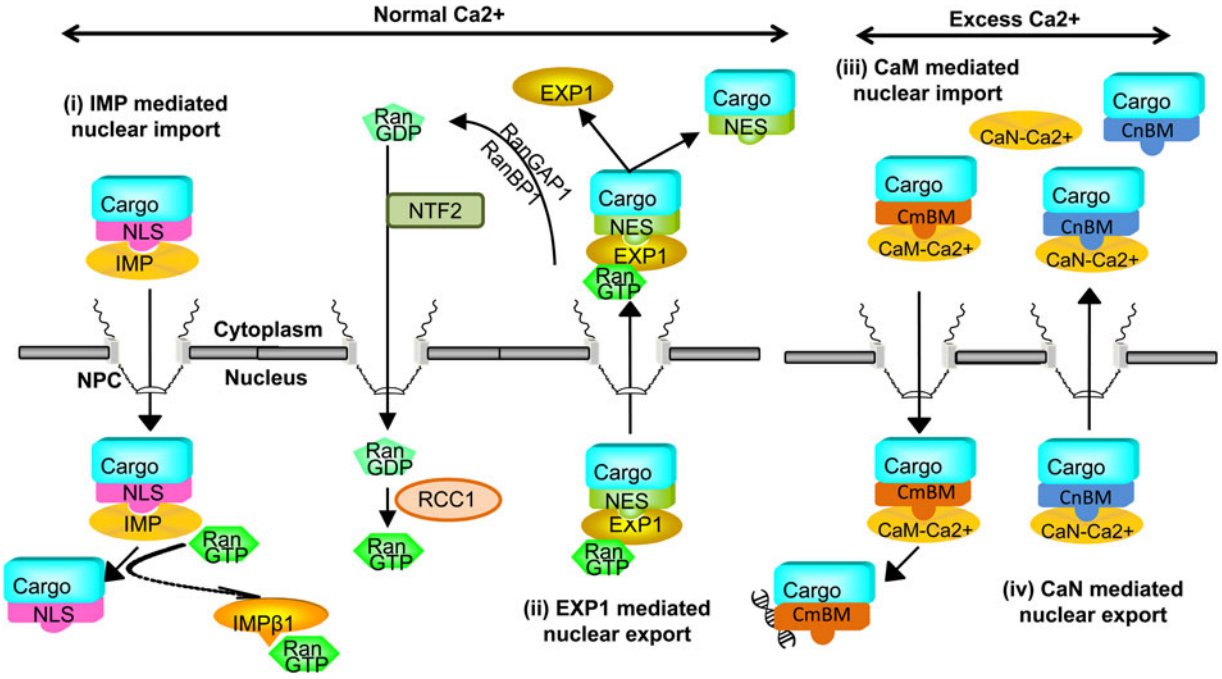

Fig. 3. Nuclear Import and Export of proteins. (i) Nuclear import usually initiates by the recognition of NLS containing cargo by importins (IMP), either IMPβ alone or *via* IMPα/β complex. The cargo-importin complex is transported into the nucleus where IMPβ is displaced by its binding to RanGTP and the cargo is released. (ii) Nuclear export usually initiates by the recognition of NES containing cargo by exportin (EXP) in its RanGTP bound state. The cargo-EXP-Ran complex is transported into the cytoplasm where hydrolysis of RanGTP to RanGDP (via the action of RanGAP1/RanBP1) leads to dissociation of the complex and release of cargo. RanGDP is recycled into the nucleus by its specific transporter NTF2, where it is changed to RanGTP *via* the action of RCC1. In the context of excess calcium (Ca^{2+}), e.g. due to release from intracellular stores, the IMP/EXP/Ran mediated transport may be inhibited. In this case, (iii) Calmodulin (CaM) can mediate nuclear import, while (iv) Calreticulin (CaN) can mediate nuclear export of cargo proteins that carry appropriate binding motifs (CmBM, CnBM).

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complex with RanGTP, translocates the complex to the cytoplasm through the NPC (Rout and Aitchison, 2001; Wente and Rout, 2010). In the cytoplasm, RanGTP is hydrolysed to cause dissociation of the CRM1–RanGTP–cargo complex. The unbound CRM1 and RanGDP are free to recycle back into the nucleus, to repeat the cycle. Limited data in protozoa suggest a minimalistic nuclear export system with CRM1 being the main EXP for all nuclear export (Fukuzawa *et al.* 2003; Cuevas *et al.* 2005; Mitra *et al.* 2016).

Calcium-dependent nuclear transport

Apart from the well-characterized nuclear transport pathways described above, there is at least one well-conserved IMP/EXP-independent transport pathway. Calmodulin-mediated nuclear import of proteins [Fig. 3(iii)] and calreticulin-mediated nuclear export [Fig. 3(iv)] are regulated by intracellular calcium mobilization. They are active under conditions of high intracellular calcium that inhibit IMP/EXP-Ran-mediated pathway (Wagstaff and Jans, 2009). Change in calcium levels may affect all nuclear transport within a cell as suggested by the observed conformational change in NPC on the release of calcium from cellular stores (Mooren *et al.* 2004; Erickson *et al.* 2006). Calcium-dependent signalling is well conserved across eukaryotes (Plattner and Verkhratsky, 2015), but calmodulin-dependent nuclear transport has not been investigated in protozoa to date.

Nuclear transport in *E. histolytica*

Limited literature suggests the existence of nuclear transport pathways in *E. histolytica* similar to those in higher eukaryotes, e.g. the canonical NLS of the SV40 large T antigen mediates nuclear localization in *E. histolytica* (Pemberton and Paschal, 2005). A few NLS and NES containing *E. histolytica* proteins have been described, including EhNCABP166 (Uribe *et al.* 2012) an ABP with a role in pathogenicity. The *in silico* predicted NLSs were able to mediate nuclear localization of a fusion partner that is normally cytoplasmic, confirming that they are functional in *E. histolytica*. Additionally, the predicted C-terminal NES was able to mediate cytoplasmic localization of a normally nuclear fusion partner. EhCaBP3, another ABP with a defined role in phagocytosis, is also localized to nucleus and cytoplasm of the parasite, but its transport mechanisms have not been delineated (Rout *et al.* 2011).

In silico analysis of the *E. histolytica* genome has predicted the presence of Ran (Uribe *et al.* 2012) as well as several IMP/EXP orthologues (Aurrecoechea *et al.* 2011); however, to date, no studies have confirmed their functionality.

Entamoeba histolytica has a robust calcium-dependent signalling mechanism, with effective communication from surface receptor engagement to induction of specific gene expression (Cruz-Vera *et al.* 2003). The identification of several *E. histolytica* calcium-binding proteins, including at least two that are defined as calmodulin-like, may suggest the existence of an active calcium-dependent nuclear transport pathway (Bhattacharya *et al.* 2006). Indeed, a recent study has shown that EhCaBP6 is a nuclear–cytoplasmic shuttling protein with calcium-dependent nuclear transport (Verma *et al.* 2017). Importantly, the study shows the existence of calcium-dependent nuclear transport in *E. histolytica*. This is consistent with the observation that increase in intracellular calcium induces changes in mRNA level of several *E. histolytica* genes, probably through a change in promoter occupancy (Debnath *et al.* 2005; Moreno *et al.* 2010). That calcium signalling cascades are well developed in *E. histolytica* is also evidenced by the identification of serco-endoplasmic reticulum and plasma membrane Ca^{2+}-ATPases (Martinez-Higuera *et al.* 2013).

The recent data on nucleocytoplasmic shuttling proteins in *E. histolytica*, in the context of existing literature on eukaryotic nuclear transport mechanisms, lead us to hypothesize that *E. histolytica* has nuclear transport mechanisms similar to higher eukaryotes, which may or may not be as extensive. When considered with the importance of calcium-mediated cytoskeleton-binding-dependent phagocytic mechanisms in *E. histolytica* pathogenesis, this leads us to further hypothesize that targeting the nuclear transport pathways will lead to inhibition or abrogation of *E. histolytica* pathogenicity. However, these hypotheses remain to be tested experimentally.

Despite recent literature on the subject, there are many questions that remain unanswered with respect to the nuclear transport mechanism within *E. histolytica*. For example, does *E. histolytica* have complex nuclear pores like higher eukaryotes with a similar array of nucleoporins? What known eukaryotic nuclear transport pathways are active in *E. histolytica*? There is only one study showing the presence of calcium-dependent nuclear transport (Verma *et al.* 2017). Are there NTFs and/or pathways specific to *E. histolytica*?

In silico predicted NTFs in E. histolytica

To determine whether karyopherin alpha, beta and Ran were present within the *E. histolytica* genome, we used sequences of the previously characterized karyopherin alpha (KPNA), beta (KPNB) and Ran proteins from higher eukaryotes, unicellular organisms, free-living and parasitic protozoans (Table 1) to explore the genome/proteome of *E. histolytica*. Genes for all three proteins were identified in the published *E. histolytica* genome; however, there were clear differences in the predicted proteins.

On searching the Amoeba database, only one putative importin α and Ran transcript (and hence protein) were identified and five transcripts for importin β. For the latter, we used the transcript with the highest homology with human and mouse importin β1.

Phylogenetic trees were constructed for KPNA, KPNB and Ran sequences (Fig. 4A, C and D). *Entamoeba histolytica* KPNA (Fig. 4A) did not cluster with any other sequence (group A). KPNA sequences from *Leishmania braziliensis*, *Trypanosoma cruzi* and *Naegleria gruberi* clustered together (group B) as did sequences from *Dictyostelium discoideum*, *Toxoplasma gondii* and *C. parvum* (group C). As expected, KPNA sequences from *Homo sapiens*, *Mus musculus* and *Saccharomyces cerevisiae* clustered together (group D). The eukaryotic KPNA is composed of an importin-β-binding (IBB) domain (KPNB is also termed importin-β) and armadillo (ARM) repeats (Fig. 4B). As expected, the IBB and ARM domains were almost identical in *H. sapiens*, *M. musculus* and *S. cerevisiae* proteins, with each protein having one IBB and three ARM repeats. Interestingly, *E. histolytica* protein lacks an IBB domain and has only two ARM repeats. This raises the possibility of a KPNA or KPNB only nuclear import mechanism in *E. histolytica* or an as yet unknown mode of interaction between KPNA and KPNB.

Similar to KPNA, *E. histolytica* KPNB did not cluster with KPNB from any other species (Fig. 4C, group A), as did *D. discoideum* (group B). *S. cerevisiae* and *T. gondii* clustered together (group C) as did *T. cruzi*, *L. braziliensis*, *N. gruberi*, *H. sapiens* and *M. musculus* (group D). Eukaryotic KPNB contains an Importin-beta N-terminal (IBN) domain and Huntingtin elongation factor 3 (HEAT) repeats. Unlike the well-studied KPNBs from humans, mice and yeast, KPNB from *E. histolytica* does not have IBN or HEAT repeats. Together with the bioinformatic analysis for KPNA, these limited analyses suggest that nuclear import pathways in *E. histolytica* evolved on a separate branch

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Table 1. List of sequences downloaded from the web

Organism & database URL	Sequence ID	Accession number	Sequence length
Homo sapiens http://www.ncbi.nlm.nih.gov	KPNA1	NM_002264.3	6,889 bp
	KPNB1	NM_002265.5	4,276 bp
	Ran	NM_001300797.1	2500 bp
Mus musculus http://www.ncbi.nlm.nih.gov	KPNA1	NM_008465.5	4022 bp
	KPNB1	NM_008379.3	5,909 bp
	Ran	NM_009391.3	2288 bp
Saccharomyces cerevisiae http://www.yeastgenome.org	KPNA1	YNL189W	542 bp
	KPNB1	YLR347C	2586 bp
	Ran	YLR293C	220 bp
Entamoeba histolytica http://amoebadb.org/amoeba	KPNA1	EHI_025350	1437 bp
	KPNB1	EHI_036520	2532 bp
	Ran	EHI_148190	686 bp
Cryptosporidium parvum Iowa II http://protozoadb.biowebdb.org	KPNA1	XP_627222.1	552 aa
	Ran	XP_001388364.1	212 aa
Leishmania braziliensis http://protozoadb.biowebdb.org	KPNA1	CAM40273.1	539 aa
	KPNB1	CAM38939.1	870 aa
Leishmania donovani http://protozoadb.biowebdb.org	Ran	ABZ91091.1	216 aa
Toxoplasma gondii http://protozoadb.biowebdb.org	KPNA1	AAP31033.1	537 aa
	KPNB1	XP_002370273.1	971 aa
	Ran	XP_002367181.1	216 aa
Trypanosoma cruzi http://protozoadb.biowebdb.org	KPNA1	XP_803276.1	533 aa
	KPNB1	XP_819701.1	866 aa
Dictyostelium discoideum http://www.dictybase.org	KPNA1	DDB_G0272318	516 aa
	KPNB1	DDB_G0269948	931 aa
	Ran	DDB_G0291235	212 aa
Naegleria gruberi http://genome.jgi-psf.org	KPNA1	C_90135	518 aa
	KPNB1	C_160092	854 aa
	Ran	C_30237	215 aa

from the free-living amoebas, yeast and higher eukaryotes and a support a novel functional mechanism of KPNA and KPNB homologues.

The Ran sequences (Fig. 4D) clustered into three groups. Unlike the KPNA and KPNB trees, Ran from *E. histolytica* clustered with *N. gruberi* and *D. discoideum* (free-living amoebas) (group A). *H. sapiens* and *M. musculus*, as expected, clustered together (group B), while the remaining four species (*C. parvum*, *T. gondii*, *L. donovani* and *S. cerevisiae*) clustered together in group C. Interestingly, the cluster of *E. histolytica* with *N. gruberi* (free-living amoebas) suggests that Ran in these two organisms has evolved from the same putative ancestor; however, this relationship may not be significant (low confidence, bootstrap = 35%).

Per cent identity, expectation values (evalue) and bit scores were calculated for *E. histolytica* KPNA, KPNB and Ran sequences relative to human and mouse proteins (Table 2) in NCBI-BLAST. *E. histolytica* KPNA and KPNB sequences have low per cent identity when aligned with human or mouse sequences that are below the accepted 30% threshold required to infer homologues. However, this is clearly offset by the highly significant evalues (evalue 10×10^{-10}) and bit alignment scores (score of $\geqslant 50$ is considered significant). Together, the above data suggest the presence of homologues of human and mouse KPNA and KPNB proteins in *E. histolytica*. The putative Ran protein of *E. histolytica* is clearly a homologue of the human and mouse proteins, as evidenced by the high per cent identities, evalues and bit scores.

Nuclear transport in other protozoans

Research findings in other single-celled protozoans may have implications for the nuclear transport system in *E. histolytica*. Two of the best-studied protozoans in this context are *D. discoideum* and *T. gondii*.

Nuclear transport system in D. discoideum

Dictyostelium discoideum is a free-living amoeba that like *Entamoeba*, is classified in the amoebozoa supergroup.

Investigation of an intact *D. discoideum* nucleus using cryo-electron tomography, showed an NPC with cytoplasmic, spoke and nuclear rings (see Fig. 2) (Beck *et al.* 2007). Snapshots of fluorescent cargo moving through the NPC showed that cytoplasmic filaments probably provide the initial docking sites for complexes that move through the central NPC channel. The nuclear basket region was less important for the transport process. Nuclear export of *D. discoideum* signal transduction and transcription protein (Dd-STATa) is regulated by phosphorylation, similar to that of several proteins in higher eukaryotes (Ginger *et al.* 2000).

Nuclear transport system in T. gondii

Toxoplasma gondii is the causative agent of toxoplasmosis and used as a model for analysis because it is amenable to genetic manipulation, and can be studied its natural host (Frankel *et al.* 2007; Frankel and Knoll, 2008; Frankel and Knoll, 2009).

A virulence gene in *T. gondii* is a divergent homologue of RCC1, named TgRCC1. An atypical *T. gondii* Ran orthologue (TgRan) was also identified, which localized in the nucleus and cytoplasm, whereas in higher eukaryotes Ran is localized predominantly in the nucleus (Frankel and Knoll, 2008). However, TgRan shared functional similarities with eukaryotic Ran: binding of GTP, GTPase activity and interaction with RCC1. The absence of RCC1 in both *T. gondii* and higher eukaryotes results in Ran being excluded from the nucleus.

The findings from *T. gondii* suggest that RanGTP and associated proteins in protozoans function similarly to higher eukaryotes.

Nuclear transport as a therapeutic target

Several inhibitors of nuclear import and export have been developed as anti-cancer therapeutic strategy (Bi *et al.* 2005; Parikh *et al.* 2014; Mahipal and Malafa, 2016; Ha *et al.* 2017), with at least two (Selinexor and SL-801) currently being in human clinical trials (NIH, 2017). Basic discovery research over the last two decades has shown that disruption of nuclear transport is a key aspect of several medically important cancers (Mahipal and Malafa, 2016). Once the specific NTFs had been identified, several high-throughput screening and *in silico* approaches were used to

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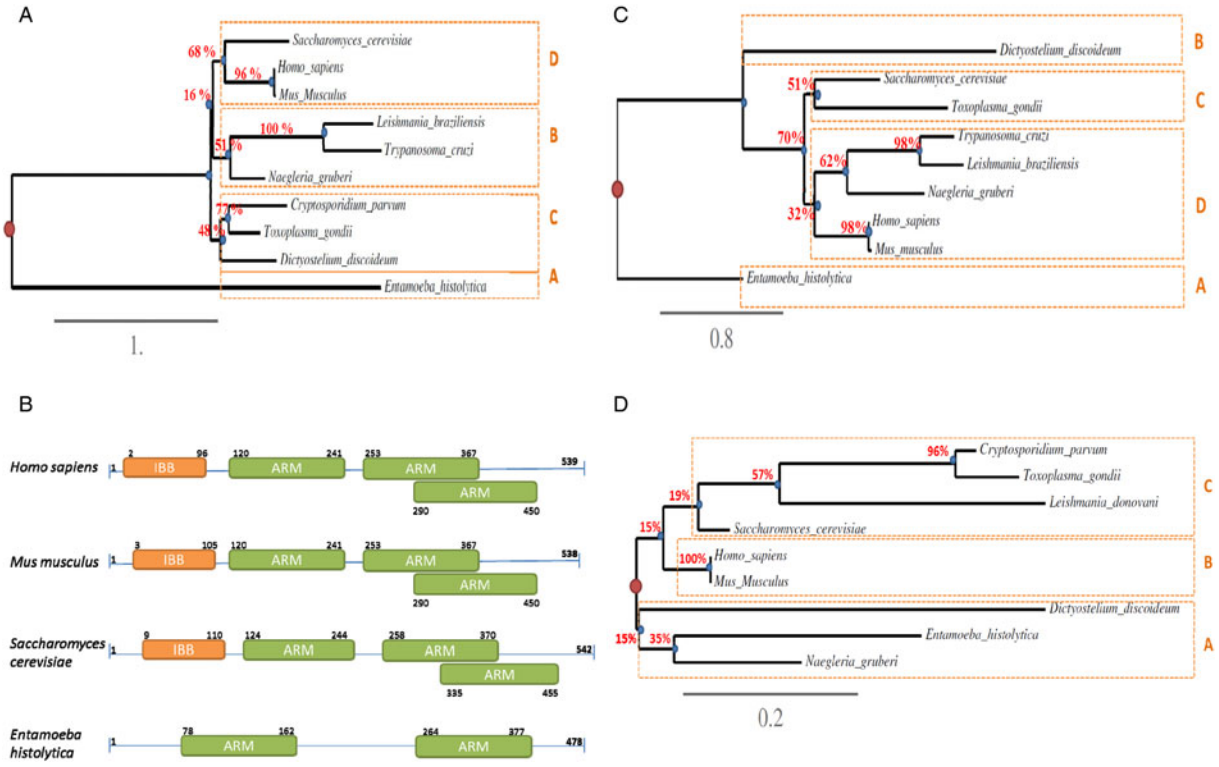

Fig. 4. Nuclear transport components in *Entamoeba histolytica*: *in silico* studyProtein sequences of KPNA1 (A), KPNB1 (C) and Ran (D) from human, mouse, yeast and selected protozoan species were entered into Phylogeny.fr and advance analysis input data box. The bootstrap tool was set to 400 iterations and the output depicted as a rooted tree. The red circle represents the ultimate hypothetical common ancestor of all the species sampled. The trees are composed of branches representing the lineages changing over time and nodes (blue circles) representing the putative ancestors for the sample species. The boxes shown in dashed orange lines represent groups (A–D) which define clusters of species whose sequences are more similar to each other within a group than to species in other groups. Numbers at nodes are bootstrap values, calculated in PhyML (Phylogeny.fr); these provide a statistical measure of significance and are represented as a percentage of 400 iterations. (B) The conserved domain database (CDD) on the NCBI website was used to investigate the presence of functional domains within KPNA1 for *Homo sapiens*, *Mus musculus* and *Saccharomyces cerevisiae*, and then compare them with predicted KPNA1 of *E. histolytica*, a comparative figure is shown. IBB, importin-β-binding domain; ARM, armadillo repeats.

identify and design small molecule inhibitors. Majority of the identified inhibitors are still in the discovery and animal testing stage, with a few that have progressed to human trials. Inhibitors of nuclear export are the best studied of these, and at least two are in human trials for various cancers (Senapedis *et al.* 2014). One such inhibitor has been approved for use in veterinary practice for canine cancers (London *et al.* 2014). Nuclear transport inhibitors with anti-cancer potential have recently been reviewed in depth (Mahipal and Malafa, 2016). A number of small molecule inhibitors of nuclear transport are currently under study by various groups for anti-viral efficacy [e.g. (Shechter *et al.* 2017; Wang *et al.* 2017)], however, there are none in clinical trials as yet.

Table 2. Homology of *Entamoeba histolytica* KPNA, KPNB and Ran sequences with their human and mouse counterparts

KPNA		
	Relative to human	Relative to mouse
% ID[a]	23	23
Evalue[b]	5×10^{-25}	1×10^{-25}
Bit score[c]	80–200	80–200
KPNB		
% ID	20	20
evalue	6×10^{-25}	6×10^{-21}
Bit score	80–200	80–200
Ran		
% ID	69	73
E value	2×10^{-56}	1×10^{-107}
Bit score	80–200	>200

[a]Per cent identity, threshold = 30%.
[b]Expectation value, threshold $\leqslant 10 \times 10^{-10}$.
[c]Alignment score, threshold $\geqslant 50$.

The main challenge to the use of inhibitors of nuclear transport as anti-amoebic drugs is the cytotoxic effects on host cells due to nuclear transport pathways being well conserved across eukaryotes (O'Reilly *et al.* 2011; Serpeloni *et al.* 2011). Thus, Leptomycin B, a highly effective inhibitor of CRM1 is not clinically viable due to high toxicity. This has been addressed to some extent by the recent development of synthetic CRM1 inhibitors with reduced toxicity and *in vivo* efficacy [Caly *et al.* (2015) and references therein]. Another strategy has been to target a specific interaction rather than a function of the transport factors, e.g. inhibition of the interaction of a dengue virus non-structural protein with IMPα/β protects against severe disease *in vivo* (Fraser *et al.* 2014). In the context of current knowledge of nuclear transport pathways in protozoan parasites, and the accumulating evidence that any mechanisms present are likely to be closely related to mechanisms in higher eukaryotes albeit of less complexity, the latter option of targeting specific interactions probably presents the best chances of developing successful anti-amoebic therapeutic strategies. Targeting interactions of the calmodulin-like proteins of *E. histolytica* (EhCaBP3, EhCaBP6, Eh-α-actinin2) may be feasible. Given their low homology with human calmodulin, targeting these proteins and their functions should result in low toxicity (Bhattacharya *et al.* 2006).

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Summary

Entamoeba histolytica, the causative agent of human amoebiasis, is a protozoan parasite capable of invading the colonic mucosa and reaching extra-intestinal organs. Understanding its invasion mechanisms requires knowledge of how it interacts with external cell components and how it kills cells (trogocytosis/phagocytosis). Recent research suggests that optimal phagocytosis requires signalling events from the cell surface to the nucleus, and the induction of several factors that are transported to the plasma membrane. There is limited research elucidating the existence of nuclear transport pathways in *E. histolytica*. Bioinformatics and indirect evidence suggest the existence of at least some (rudimentary?) form of Ran-dependent and calcium-dependent nuclear transport pathways. To date, there is no information (direct or indirect) regarding the structure of the NPC in *E. histolytica*, and no Nups have been identified. Given that nuclear to cell surface signalling appears to be involved in trogocytosis/phagocytosis of *E. histolytica*, this is a significant gap in our understanding of the parasite's pathological mechanisms. A deeper understanding of the molecular mechanisms underlying *E. histolytica* pathology is needed to identify new, viable drug targets. The recent development of small molecule inhibitors of nuclear transport for treatment of other disorders provides an opportunity to re-position these inhibitors for the treatment of invasive amoebiasis but this requires considerable research effort to define the nuclear transport pathways in *E. histolytica*. Re-positioning of drugs with known bioavailability and safety for human use offers a shorter pathway to the clinic for new indications compared with the traditional protocols for drug discovery and translation that can take decades.

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