Functional diversity and bilobe targeting by the MORN Domain Proteins of *Leishmania major*

By

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Declaration

I declare that the content of this thesis was undertaken and completed by myself, unless otherwise acknowledged and has not been submitted in support of an application for another degree or qualification in this or any other university or institution.

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Abstract

Leishmania is a protozoan parasite responsible for a spectrum of diseases known as leishmaniasis. The cell body of *Leishmania* consists of single copies of several organelles whose faithful duplication are crucial for survival. Previously, a novel cytoskeletal organelle called the bilobe was discovered while studying Golgi biogenesis in *T. brucei*. TbMORN1 became the first identified structural component of the bilobe with exclusive localization. TbMORN1 is composed of 15 MORN-repeats which may imply that the MORN-repeats are responsible for its bilobe targeting. Given that MORN proteins have only been characterized in *T. brucei* among the kinetoplastids, I chose to study the MORN proteins of *L. major* an important experimental model. 22 uncharacterized proteins were annotated to contain MORN-repeats in *L. major*, 14 of which share the feature of exclusively containing MORN-repeats ranging from 3-15 repeats, while the remaining 7 carry additional functional domains. The strategy was to express two apparently varied LmaMORNs (LmaMORN5 and LmaMORN6) from each other and from MORN1 in *L. major* promastigotes, screen them for bilobe localization and investigate their possible function. I showed that both LmaMORN5 and LmaMORN6 target the bilobe and the basal bodies while LmaMORN6 additionally stained the flagellum indicating flagellar function. These localizations were true even after detergent extraction, implying intimate associations with the bilobe and other cytoskeletal structures, hinting at a structural role. LmaMORN5 contained two compartmentalized regions along its sequence under selective pressure, shown to be secreted into the cytoplasm of infected macrophages and seemed to increased parasites infectivity six-fold, suggesting a possible role as a virulence factor. Thus, I reported for the first time two other MORN proteins (LmaMORN5 and LmaMORN6) in *L. major* that targets the bilobe in a manner similar to TbMORN1 in *T. brucei*, while each appear to display a differential functional role in the parasite's biology.

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1 Introduction

1.1 General introduction

Leishmania is a genus of protozoan parasites belonging to the order Kinetoplastida and family Trypanosomatidae. The genus *Leishmania* includes about 30 different species, of which more than 20 are known to infect humans (WHO 2015). The classification scheme now adapted by the World Health Organization (WHO) is based on molecular techniques, which differ from previous classification approaches which were based on extrinsic criteria such as vectors, clinical, geographical and biological characteristics (Figure 1.1). This classification divides the genus *Leishmania* into two subgenera called *Leishmania* and *Viannia*. The different species of *Leishmania* are responsible for a spectrum of diseases known collectively as leishmaniasis, which affects the poorest of the poor, and is primarily a public health concern in developing countries
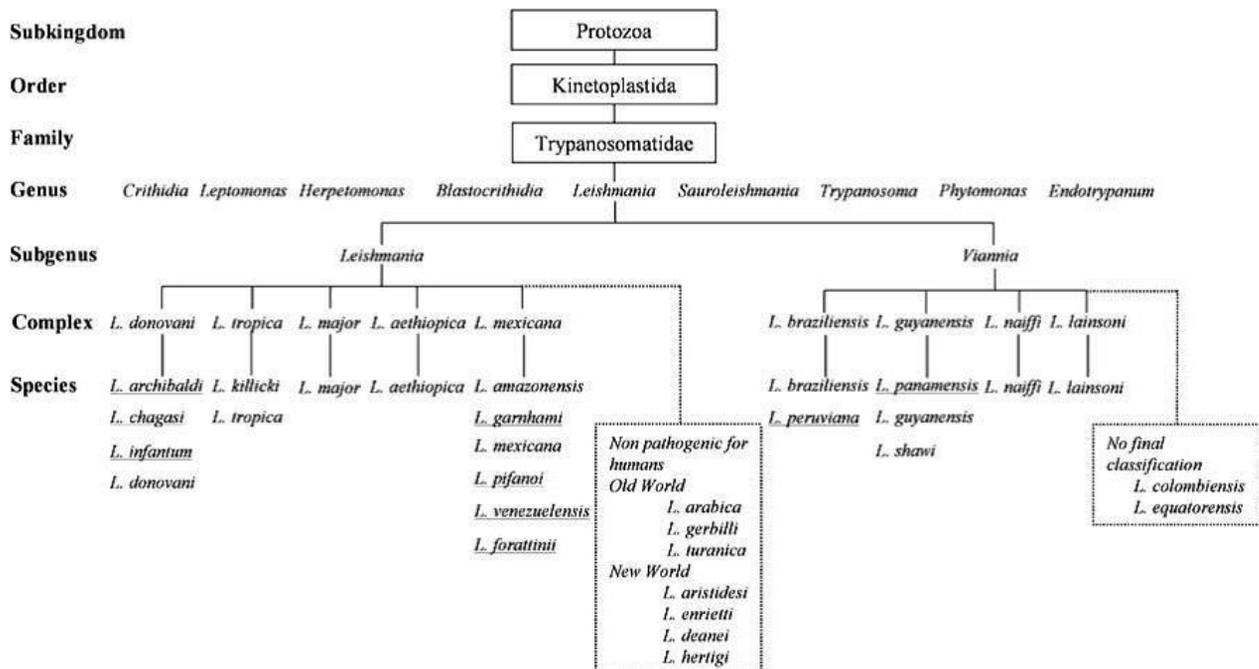
Figure 1.1 Taxonomy of *Leishmania* (reproduced from Advances in Parasitology Vol 64)

Leishmaniasis threatens the lives of approximately 350 million people in over 88 countries across five continents: Africa, Asia, Europe, North America and South America (Leifso et al., 2007). About 1.3 million new cases and between 20,000 and 30,000 deaths occur every year (WHO 2015).

Leishmania parasites are transmitted through the blood feeding habits of the female sandfly of the genus *Phlebotomus* (in the old world) or *Lutzomyia* (in the new world). Female sandflies need blood for their eggs to develop. They become infected with the *Leishmania* parasites when they suck blood from an infected person or animal. Over a period of 4 to 25 days, the parasites develop in the sandfly. When the infected female sandfly then feeds on a fresh source of blood, it inoculates the person or animal with the parasite, and the transmission cycle is completed.

Leishmaniasis manifests in three main clinical pathologies; cutaneous, mucocutaneous and visceral, with the pathology depending both on the *Leishmania* genotype/species and on host immunity (Figure 1.2). **Cutaneous leishmaniasis (CL),** the most common form of the disease, is caused by *L. major* or *L. tropica* infection. It usually leads to development of a skin ulcer which is generally self-healing but leaves deep scars. The majority of CL cases occur in the Americas, the Mediterranean basin, the Middle East and central Asia (WHO 2015). The species *L. braziliensis* causes **mucocutaneous leishmaniasis (ML)**, which results in lesions that can lead to destruction of the mucous membranes of the nose, mouth and throat cavities and surrounding tissues. Almost 90% of ML cases occur in Bolivia, Brazil and Peru (WHO 2015). **Visceral leishmaniasis (VL)** is caused by *L. donovani* and *L. infantum*, which is fatal if left untreated. Its symptoms include fever, anaemia, weight loss, and swelling

of the liver and spleen. VL is highly endemic in the Indian subcontinent and in east Africa (WHO 2015).

HIV and *Leishmania* coinfection is an emerging disease, and AIDS has been reported to increase the risk of visceral leishmaniasis by 100–1000 times in Southern Europe and South America (Desjeux and Alvar, 2003).

Visceral   Mucocutaneous   Cutaneous

Figure 1.2 Three clinical forms of leishmaniasis (http://globalhealthvet.com/page/3/).

There are no vaccines currently available against leishmaniasis. Present treatment is primarily limited to chemotherapy, but very few suitable drugs exist and their effectivity in reducing morbidity and disease progression has its shortcomings. The first line of treatment consists of two pentavalent antimonials, sodium stibogluconate (Pentostam) and meglumine antimoniate (Glucantime). These drugs are administered parenterally and the main shortcoming is widespread resistance. If these drugs are not effective, the second-line compounds pentamidine (Lomidine) and amphotericin B (Fungizone) are used, of which allergies and toxicity are a limiting factor. A more recent drug, miltefosine (an alkylphospholipid), was developed for the treatment of

cutaneous cancers and subsequently found effective for both cutaneous and visceral leishmaniasis through oral administration. However, its side effects excludes its use by pregnant women. This clearly shows the need for a vaccine or more effective drug treatments for leishmaniasis.

1.2 *Leishmania* Life cycle

The *Leishmania* life cycle generally consists of two distinct developmental stages: an extracellular flagellated promastigote stage that proliferates in the midgut of the sand fly vector, and an obligate intracellular non-motile amastigote stage that multiplies inside the infected cells of vertebrate hosts (Figure 1.3A). The life cycle is instigated by a sand fly vector obtaining a blood meal from an infected mammal, either a reservoir mammal such as rodents or dogs, or infected humans. The sand fly ingests amastigote-containing macrophages and monocytes in its blood meal. These amastigotes are released into the sand fly midgut, where they differentiate into flagellated, procyclic promastigotes and attach to the midgut epithelium. These procyclic promastigotes undergo metacyclogenesis to form the non-diving, infective metacyclic forms. There is evidence that several developmental stages exist between procyclic and metacyclic promastogotes, but little is known about their relationships and function. One study showed four major developmental forms of promastigotes: procyclic promastigotes, nectomonad promastigotes, leptomonad promastigotes and metacyclic promastigotes (Gossage et al., 2003). In addition, haptomonad promastigotes that possess an expanded flagellar tip and paramastigotes have been observed (Killick-Kendrick et al., 1974, Gossage et al., 2003).
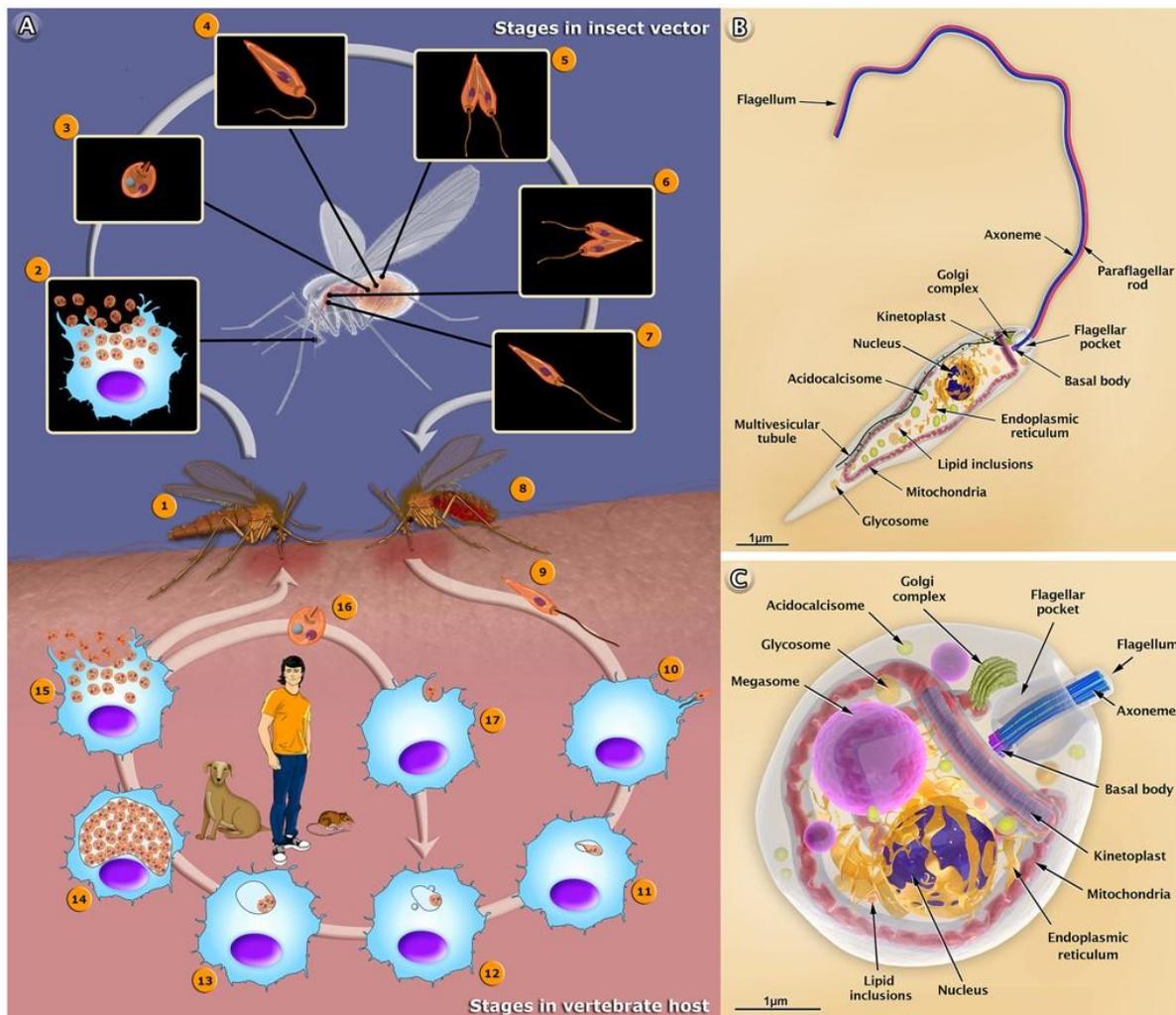
Figure 1.3 The life cycle of *Leishmania* (A), and structural organization of the promastigote (B) and amastigote (C) forms.

(A) The female sandfly (1) insect bites an infected mammal during the blood meal. Infected macrophages (2) with amastigote forms. (3) Amastigote form. (4) Amastigotes transform into procyclic promastigotes. (5) Procyclic promastigotes multiply in the midgut. (6) Promastigotes migrate toward the stomodeal valve in the anterior midgut and re-initiate cell division. (7) Promastigotes transform into infective metacyclic promastigotes. (8) The female sandfly releases the metacyclic promastigotes into a new mammalian host via regurgitation during the blood meal. (9) Metacyclic promastigotes. (10) Metacyclic promastigotes infect macrophages. (11) Metacyclic promastigotes transform into amastigotes. (12) Amastigotes attach to the membrane of the parasitophorous vacuole. (13) Amastigotes multiply in the vacuole. (14) Intense amastigote multiplication. (15) Amastigotes burst out of the cell. (16) Amastigote form. (17) An amastigote infects a macrophage. In the central portion of the figure, we added the most important reservoirs involved in the maintenance of the parasite. Schematic 3D representations of the organelles found in the *Leishmania* promastigote (B) and amastigote (C).

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Once the promastigotes become metacyclics, they detach from the mid-gut epithelium and migrate into the pharynx and buccal cavity. During the next blood meal, the infective metacylics are passed into the vertebrate host (Killick-Kendrick R, 1990). Once inside the host, metacyclics enter macrophages by receptor-mediated phagocytosis where they differentiate into the amastigote forms (Handman and Bullen, 2002). The amastigotes proliferate, eventually rupturing the infected macrophages, and are release to infect neighbouring macrophages, and the cycle begins again.

1.3 *Leishmania* cell structure

The cell body of *Leishmania* parasites changes between the amastigotes (Figure 1.3C) and promastigotes (Figure 1.3B) life stages during the course of its cell cycle. The amastigotes are small roughly spherical cells ranging from 2-4μm in diameter with a short remnant of a flagellum, while the Promastigotes are lance-like in shape and range in size from 5-14μm in length by 1.5-3.5μm in width with an emergent free flagellum. The cell body of *Leishmania*, like other kinetoplastids, are composed of a number of single copy organelles including a flagellum, basal body, Golgi, mitochondria, kinetoplast and its shape is maintain by a subpellicular corset of microtubules (MT) spanning the whole cell body by cross-linking to each other and to the plasma membrane (Gull, 1999).

The MTs are dynamic components of the cytoskeleton and play several important roles, including maintenance of cell shape, intracellular transport, cytoplasm and organelle organisation, mitosis and cell polarity (Daire and Pous, 2011). The cell shape

is dependent upon the subpellicular corset which involves more than a hundred microtubule profiles underlying the plasma membrane. The 24-nm-diameter microtubules form a helical pattern along the long axis of the cell, with regular intermicrotubule spacing (18–22 nm), and are cross-linked to each other and to the plasma membrane (Gull, 1999). MT stabilisation involves many proteins among which MT-associated motor proteins (kinesins and dyneins) play an important role. Kinesins and dyneins interact with microtubules by using energy derived from ATP hydrolysis to generate a force that can be used by the cell for various purposes, including transport of cargoes, segregation of organelles, destabilizing microtubules, alteration of morphology, or movement of the entire cell (Wickstead and Gull, 2006).

The flagellum of trypanosomatids are made up of an axoneme (a set of 9+2 microtubules) and a paraflagellar rod (PFR), a crystalline-like structure (Kohl and Bastin, 2005, Ralston and Hill, 2008). The *Leishmania* promastigote flagellum emerges from the flagellar pocket at the anterior end of the cell body. The flagellum provides motility to the promastogote stage and a means of attachment within the sand fly gut (Cuvillier et al., 2003). The length of the flagellum of *Leishmania* varies drastically during the parasite's life cycle, ranging from 20µm to 1µm (Rotureau et al., 2009). The dynamics of the flagellum are controlled by a process called intraflagellar transport (IFT). IFT is a bidirectional movement of particles between the flagellum membrane and the axoneme, operated by kinesin and dynein motors, and occurs through the motion of moving protein complexes referred to as IFT particles (Rosenbaum and Witman, 2002). Disruption of the IFT process by deletion of dynein or disruption of IFT particles in *T. brucei* and *L. mexicana* results in severe alteration of cell shape, with a direct correlation between cell body size and flagellum length in *Trypanosoma* (Kohl et al., 2003), and development towards an oval-shaped morphology in *Leishmania* (Adhiambo et al., 2005). Recent studies have suggested sensory and signaling

functions for *Leishmania* flagella (Gluenz et al., 2010). One study has reported the trypanosome flagellar membrane as being rich in lipid rafts (Tyler et al., 2009), which are microdomains within the lipid bilayer that have been implicated to act as a platform for signalling during infection at the surface of host cells (Smith and Helenius, 2004, Duncan et al., 2002, Lafont et al., 2004).

The flagellar pocket (FP) from which the flagellum emerge is an invagination of the membrane believed to serve as the exclusive site for endocytosis and exocytosis in *Leishmania*, and as such forms an important interface for parasite-host interactions (Bonhivers et al., 2008). Proteins bound for secretion are generally synthesized on the rough endoplasmic reticulum, thereafter passing to the Golgi apparatus and finally to the flagellar pocket. The Golgi lies at the heart of the secretory and biosynthetic pathways in eukaryotic cells. In sorting and glycosylating the protein output of the endoplasmic reticulum, it performs an indispensable role in cellular homeostasis.

The basal body complex forms the base of the flagellum where it nucleates the flagellar axoneme and is linked to the kinetoplast by filaments (Gull, 1999, Robinson and Gull, 1991). This complex is thought to play the role of microtubule-organising centre (MTOC). Initially cells contain one basal body and one pro-basal body. As the cell cycle progresses, the pro-basal body matures and initiates the growth of new flagella and two new basal bodies are formed, one next to each mature basal body.

The kinetoplast contains the mitochondrial DNA (kDNA), which is one of the defining feature of trypanosomatids. The kDNA is placed at a precise position in relation to the basal body during the life cycle stages. kDNA is arranged as a network of several thousand 1 kb minicircles that are heterogeneous in sequence and constructed as a few dozen 23 kb maxicircles. Maxicircles, like mitochondrial DNAs of higher

organisms, encode rRNA and proteins such as subunits of respiratory complexes. Minicircles encode guide RNAs involved in editing of maxicircle transcripts. The kDNA *in vivo* is condensed into a disk-shaped structure positioned in the mitochondrial matrix near the flagellar basal body.

Bilbo1 was the first identified protein component of the flagellar pocket collar (FPC) (Bonhivers et al., 2008). The flagellar pocket collar is positioned at the junction where the flagellum leaves the cell body in trypanosomes. Knockdown of Bilbo1 by RNA interference in cultured procyclic forms of *T. brucei* prevent FP biogenesis, leading to cells being unable to control endocytosis and exocytosis. As a result, the segregation of the Golgi was affected, positioning of the new flagellum was altered and there was cell cycle arrest leading to cell death. Furthermore, cultured bloodstream forms (BSF) were not viable when subjected to Bilbo1 RNAi in vitro (Bonhivers et al., 2008).

The **bilobe** structure was discovered in *T. brucei* while studying the mechanism of Golgi replication (He et al., 2005). Since then the Warren group have been trying to determine its shape, biogenesis and function. The bilobe was recently redefined as a hairpin shape that lies in the vicinity of the flagellar pocket (Esson et al., 2012). This bilobe structure was shown to be important for Golgi biogenesis, as depletion of TbCentrin2 (a bilobe component) using RNAi, leads to an inhibition of Golgi duplication (He et al., 2005). There are four known protein components of the bilobe: TbLRRP1 and TbMORN1, which are exclusively localized to it (Morriswood et al., 2009, Zhou et al., 2010) and Tbcentrin2 and Tbcentrin4, which are additionally present in the basal bodies (Shi et al., 2008, Wang et al., 2012). The bilobe is not an isolated structure but has close links with other components of the cytoskeleton. For example, the posterior end of TbMORN1 protein was shown to overlap with the Bilbo1 structure while the anterior end overlapped with the flagellar attachment zone

(Morriswood et al., 2009). TbMORN1, a MORN-repeat protein, was the first discovered protein to exclusively localize at the bilobe. Depletion of TbMORN1 by RNAi caused growth defects in procyclics and was lethal in bloodstream trypanosomes. This implies that TbMORN1 is an essential gene in the bloodstream form (Morriswood et al., 2009). Recently, the bilobe protein TbMORN1 was utilized as a probe for identification of seven new bilobe constituents and two new flagellum attachment zone proteins in *T. brucei* (Morriswood et al., 2013). These TbMORN1 binding partners and proximal neighbors could deepen our knowledge and understanding of the bilobe.

The precise function of MORN domains are yet to be establish but their localisation can be multifaceted in various organism. MORNs have been reported to bind the plasma membrane in plants (Ma et al., 2006), found in human testis (Choi et al., 2010), rhabdomeres (microvillar light gathering organelle) in *Drosophila* (Mecklenburg et al., 2010), basal complex in *Toxoplasma gondii* (Gubbels et al., 2006) and bilobe in *T. brucei* (Morriswood et al., 2009).

1.4 Membrane Orientation and Recognition Nexus (MORN)

MORNs were first discovered in junctophilin, a family of junctional membrane complex found in exitable cells of muscles and neurones (Landstrom et al., 2014). Junctophilins are characterized by eight amino-terminal MORN motifs that show an affinity to plasma membrane binding and a C-terminal transmembrane motif that anchors the protein to the endoplasmic reticulum or sarcoplasmic reticulum (Takeshima et al., 2000, Nishi et al., 2000). The MORNs have since been discovered

across the tree of life (Fig1.4), where they can be found within a variety of modular proteins with a wide range of enzymatic activities and protein-protein interacting domains. MORN-repeats are 23 amino acid long motifs, usually in multiple copies in several proteins with a general consensus sequence of YXGX(W/F)X6GXGX6GX2 (Im et al., 2007). The MORNs are conserved proteins with over 50,000 proteins annotated to contain MORN-repeats in over 3000 species (Pfam database).
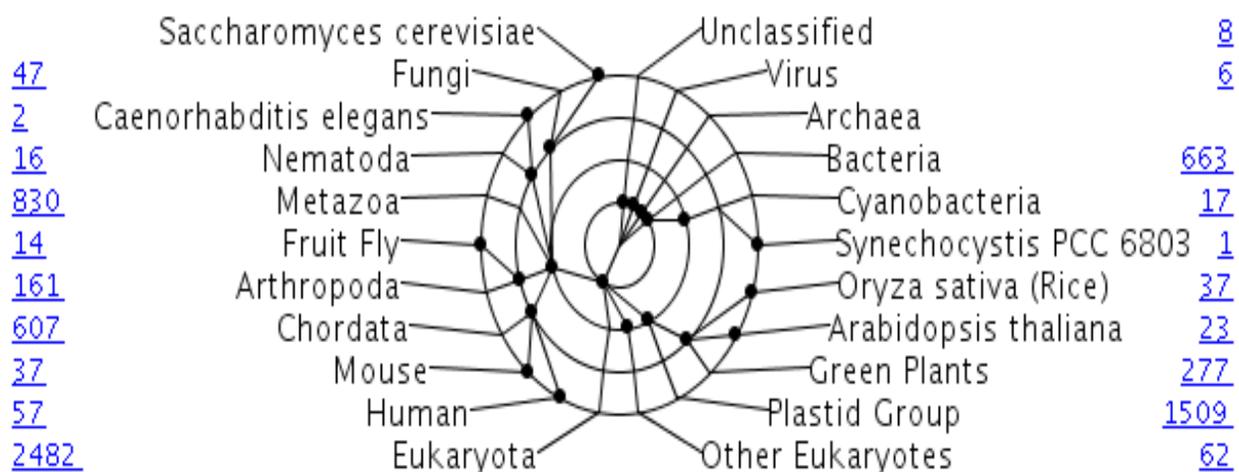
Fig 1.4. Evolutionary tree of MORN protein, adapted from Pfam database.

The use of MORN-repeats in variety of proteins with different function may suggest its role as a more universal/accessory domain. A similar example is the Pleckstrin homology (PH) domains, which are small modular domains that occur in a large variety of proteins. The domains can bind phosphatidylinositol within biological membranes and proteins such as the beta/gamma subunits of heterotrimeric G proteins and protein kinase C. Through these interactions, PH domains play a role in recruiting proteins to different membranes, thus targeting them to appropriate cellular compartments and enabling them to interact with other components of the signal transduction pathways (Interpro: http://www.ebi.ac.uk/interpro/entry/IPR001849).

1.4.1 Function of MORN domain

The function of MORN domains is not yet well-established, but it seems as though MORN proteins have evolved to play diverse roles in various organisms. The very first study that described MORN domain was performed in 2000 by Takeshima *et al*., who studied the role of junctophilin in junctional complex formation between the plasma membrane and the endoplasmic recticulum of excitable cells. The 8 MORN repeats present at the N-terminus of junctophilin were found to be essential for binding to the plasma membrane (Takeshima et al., 2000). Takeshima *et al*. proposed that the MORN motifs contribute to the of plasma membrane binding capacity of junctophilin.

Human amyotrophic lateral sclerosis 2 (ALS2) which contains 8 MORN motifs at its C-terminal domain, functions as a Rab5 guanine nucleotide exchange factor (GEF) involved in endosomal membrane trafficking (Hadano et al., 2006). The MORN domain was shown to be essential for the Rab5GEF function as its mutation leads to a rare juvenile form of amyotrophic lateral sclerosis (Kunita et al., 2004). A MORN protein called MOPT was identified in testis and skeletal muscles of mice and humans which localise in the proacrosomic vesicles of the early Golgi phase spermatids and was thought to play an important role in acrosome biogenesis during late spermiogenesis (Choi et al., 2010). MORN3 was shown to be abundant in mouse testis and was highly expressed in the spermiogenesis stage. By a way of knockout experiment, it was suggested that MORN3 may regulate spermatogenesis (Zhang et al., 2015). Human MORN4 is an ortholog of Drosophila RTP, was shown to conserved RTP features by binding myosin IIIs to control their behavior (Mecklenburg et al., 2015).

Retinophilin (RTP) is a light-regulated phosphoprotein in *Drosophila* photoreceptors and has been shown to localise at the rhabdomere, a microvillar light-gathering organelle. The 4 MORN-repeats at the N-terminus of RTP was shown to be essential for localisation to the rhabdomere (Mecklenburg et al., 2010). The stability of RTP was shown to be depended on its MORN domain binding NINACp174 (a myosin III protein) (Venkatachalam et al., 2010; Mecklenburg et al., 2015).

In rice, *Oryza sativa*, PIPK1 (OsPIPK1) contains eight MORN repeats at its N-terminus. By employing the modified yeast sequence of membrane-targeting (SMET) and Fat Western blot, it was shown that the MORN domain of OsPIPK1 contributes to membrane targeting and could bind phosphatidic acid (PA), Phosphatidylinositol 4-monophosphate (PI4P) and Phosphatidylinositol (4,5)-bisphosphate PI(4,5)P_2, suggesting a regulatory mechanism (Ma et al., 2006). Additionally, the MORN domain at the N-terminal of *Arabidopsis* phosphatidylinositol phosphate kinase 1(AtPIPK1) was shown to regulate the function and distribution of the enzyme in a lipid-dependent manner. By truncating the MORN domain, it was shown to have a binding affinity for PA and PI (4,5)P_2 and was essential for PA activation (Im et al., 2007). *Arabidopsis* Rab-E GTPases was also shown to interact with a plasma membrane phosphatidylinositol-4-phosphate 5-kinase via its MORN-repeat domain (Camacho et al., 2009). A MORN containing protein of *Arabidopsis*, ARC3, localized to the outer chloroplast envelope membrane. By a way of knockout, it was shown to be critical for chloroplast fission (Shimada et al., 2004). Similarly emryo-defective 1211 (EMB1211), consisting of three MORN repeats and a coiled coil region, was shown by way of knockout to be essential for normal embryonic development and chloroplast biogenenesis in *Arabidopsis* (Liang et al., 2010). These results showed that MORN

domain have binding affinity to phospholipids such as PA, PI4P and PI(4,5)P implying membrane targeting in plants.

In *Toxoplasma gondii,* TgMORN1 was shown to be associated with the centrocone and the inner membrane complex (IMC)/ basal complex; organelles involved in cell division (Gubbels et al., 2006). Disruption of TgMORN1 by overexpression or knockouts causes defects in the basal complex, which failed to efficiently constrict to complete daughter cell budding (Gubbels et al., 2006, Lorestani et al., 2010). In ciliate Tetrahymena, 129 proteins were annotated to contain MORN-repeats. A lipid binding assay was employed to show that the primary function of the MORN domains was to confer lipid binding affinity (Habicht et al., 2015). Particularly to cardiolipin and 1,2-diacylglycerol (DAG) but less so to phosphatidylinositol and phosphatidylserine.

All these results put together may indicate that MORN domain have a primary role of tethering proteins to membranes/lipid components but the fine localization specificity remains to be determined.

In the kinetoplastids, MORN1 is well characterized in *T. brucei*. RNAi on TbMORN1 in procyclic *T. brucei* causes defects in flagella motility (Broadhead et al., 2006). Morriswood *et al*. discovered TbMORN1 as a component of the bilobe structure in *T. brucei*, which is part of the flagella cytoskeleton and lies in the vicinity of the flagellar pocket. Knockdown of TbMORN1 in procyclics produced growth defects and a slight increase in the number of 2K2N cells, suggesting a delay in the initiation of cytokinesis. In contrast, loss of TbMORN1 in bloodstream forms was lethal implying essential gene (Morriswood et al., 2009).

There is no published characterization of MORN proteins in *Leishmania* but my initial survey of the SMART database showed 281 such proteins in the kinetoplastids of which 114 belong to *Leishmania* and 22 are found in *L. major* (Pfam database). Among the 22 MORN proteins of *L. major*, there are ones of apparent diverse functions like those with a kinase, kinesin, VPS9, FYVE, transmembrane or secretory peptides.

1.5 Project Aims

MORN-repeat proteins are complex, important family proteins found in a wide range of organisms with divergent localization and function. In the kinetoplastids, the best and most studied example is TbMORN1, which consists of 15 MORN-repeats and was found to exclusively localize at a novel cytoskeletal structure called the bilobe. TbMORN1 co-purifies with the flagellar cytoskeleton and was therefore thought to be a component of the bilobe. Depletion of TbMORN1 causes a slow growth phenotype in the *T. brucei* procyclics and was fatal in the bloodstream forms (Morriswood et al., 2009).

The aim of this project was to investigate two MORN proteins of *L. major* that were functionally distinct and with expected differences in localization to each other and to MORN1. To determine whether they too can localize to the bilobe structure and what their potential roles may be. The main strategy was to express GFP chimeras of these proteins in *L. major* promastigotes and screen them for possible bilobe localization. This would further provide more information about MORN-repeats and their possible function.

Given that all the kinetoplastid MORNs were only hitherto studied and characterized in *T. brucei*, a brief description of the 22 MORN protein identified in *L. major* will also be provided.

2 Materials and Methods

2.1 Materials

2.1.1 Stock solutions and reagents

2.1.2 Suppliers

Unless otherwise stated all chemicals were supplied by Sigma-Aldrich, UK and all tissue culture reagents were supplied by GIBCO, Invitrogen, UK.

2.1.3 Bacteria culture media

Luria-Broth (LB)

1% bacto-tryptone, 0.5% yeast extract, 1% NaCl; (pH 7.0).

LB agar

1% bacto-tryptone, 0.5% yeast extract, 1% NaCl, 1.8% Bacto-Agar (pH 7.0).

2.1.4 Antibodies

Name	Raised in	Target	supplier	Dilution
GFP	Rabbit (P)	Green fluorescent protein	Abcam	1:10000 (western)
YL1/2	Mouse (M)	Tyrosinated tubulin/ basal body	Gull Lab	Undiluted(fluorescent)
Bilbo1	Rabbit (P)	Bilbo structure	Robinson lab	1:1000 (fluorescent)

Table 2.1 List of primary antibodies

Antibody	Supplier	Dilution
Anti-rabbit HRP (IgG)	Jackson Labs, stratech	1:10000
AlexaFluor(R)633 rabbit	Molecular probes, invitrogen	1:500

Table 2.2 List of secondary antibodies

2.1.5 Solutions and Buffers

Buffers for protein separation and Western blot

• 1.5 M Tris-HCL pH 8.8 – 54.45 g Tris base, 150 ml distilled water, pH adjusted with HCL.

• 10% ammonium persulphate – 100 mg dissolved in 1 ml distilled water.

• 10% SDS – 50 g dissolved in 500 ml distilled water.

• 1 l of 10 x Gel running buffer pH 8.3 – 30.3 g Tris base, 144.0 g glycine, 10 g SDS.

• 1 l of 10 x Transfer Buffer pH 9.2 – 390 mM Glycine 29 g, 480 Mm Tris 58 g, 0.3% SDS 3.7 g.

• 1 l of 1 x transfer buffer working solution – 100 ml 10 x transfer buffer, 200 ml methanol, 700 ml distilled water.

Agarose gel electrophoresis buffer for DNA (1 l of 10 x TBE)

108 g Tris, 55 g boric acid, 40 ml 0.5 M Na_2EDTA (pH 8.0) in distilled water.

Transfection buffer (Cytomix)

120 mM KCl, 0.15mM $CaCl_2$, 10 mM K_2HPO_4, 25 mM Hepes, 2 mM EDTA and 5 mM $MgCl_2$, pH7.6 using KOH

Ampicillin (amp) stock solution

100 mg/ml ampicillin in distilled water, stored at -20°C.

Coomassie staining solution

0.2% Coomassie Blue, 7.5% Acetic Acid, 50% Ethanol

Coomassie destaining solution

20% methanol, 5% glacial acetic acid in water

4,6-diamidino-2-phenylindole (DAPI) 2000× stock solution

20 mg of DAPI (Sigma) was dissolved in 1 ml of distilled water and stored at -20ºC in a tube wrapped in foil.

Fixing agents

4% paraformaldehyde (PFA)/PBS - 16 g of PFA was added to 200 ml of just boiled water. NaOH was added until the liquid goes clear. 100 ml was added to 80 ml of H_2O and 20 ml of 10 × PBS then adjust to pH 7-8. Solution were aliquoted and frozen at -20°C.

Polyacrylamide gels for Protein Separation

Reagents	10% separation gel	4% Stacking gel
1.5 M Tris pH8.8	2.50 ml	
0.5 M Tris pH 6.8		1.25 ml
30% Acryl-bisacrylamide (BioRad)	3.35 ml	0.87 ml
10% SDS (Melford)	0.10 ml	0.05 ml
10% Ammonium Persulphate (BioRad)	0.10 ml	0.15 ml

TEMED (BioRad)	0.01 ml	0.005 ml
H2O	4.05 ml	2.78 ml

Table 2.3 Polyacrylamide gels Recipe for 2 gels

Agarose gel preparation

Agarose powder (0.7g) was dissolved in 100 ml of 1 x TBE by boiled in microwave. Then 2 µl of ethidium bromide was added before decanting content into a gel holder with a comb to form the wells and left for 20 min to set.

2.1.6 Leishmania culture media

Stock solution	voulme/500ml	Final concentration
5× M199	100 ml	1×
1 M Hepes, pH 7.4	20 ml	40 mM
10 mM adenine	0.5 ml	0.1 mM
1 mg/ml biotin (0.1%)	0.5 ml	1 µg/ml
2.5 mg/ml hemin (0.25%)	1 ml	5 µg/ml
100× pen-strep	5 ml	1×
100% serum	50 ml	10%
0.25 mg/ml biopterin	4 ml	2 µg/ml
Double distilled water	319 ml	

Table 2.4 Leishmania promastigotes culture media (M199) recipe

2.2 Methods

2.2.1 General cloning

2.2.1.1 Digestion and dephosphorylation of DNA

Digestion of DNA was performed using the restriction enzymes and reaction buffers following manufacturer's instructions. 1 µg of DNA were incubated with 5U of enzyme in total volume of 20µl, at 37°C for 2 h.

To prevent self-ligation of vector fragments, the 5'-phosphate groups of the vector DNA were removed by adding 0.5U of calf intestine alkaline phosphatase per µg of vector DNA and incubated at 37°C for 1 h.

2.2.1.2 DNA extraction from agarose gels

After restriction enzyme digests, the DNA was separated on an agarose gel and the desired fragments were excised from the gel. 'QIA quick gel extraction kit' was used for further purification following the manufacturer's instructions. The excised gel was weigh and dissolved in 300 µl buffer QG per 100 mg gel at 50°C and then applied to a qiaquick column. The column was centrifuged at 13000 ×g for 1min and the flow through was discarded. After one washing step with 500µl buffer PE, bound DNA was eluted in 30µl EB buffer.

2.2.1.3 DNA ligation

For ligation of DNA fragments, at least 40ng of digested insert with 3× dephosphorylated vector DNA were incubated with 1U of T4-DNA-Ligase in 10µl

ligation buffer. After incubation over night at 4°C the ligation was transformed into competent *E. coli* cells.

2.2.1.4 Preparation of competent *E. coli*

An aliquot of DH5α was streaked out onto LB agar containing no antibiotic and grown overnight. A 10ml culture of LB was inoculated with a single colony, picked with a sterile tip, and was incubated overnight at 37°C in a shaking incubator. 100ml of LB were inoculated with 0.5ml of the overnight culture and incubated for 3-4h in a shaking incubator at 37 ºC, until it reached a culture density of OD_{600} = 0.4-0.5. The cell cultures were transferred into two 50 ml falcon tubes and centrifuged at 4ºC for 5mins at 5000 *x* g, then re-suspended in 10 ml of ice-cold sterile 0.1M cacl$_2$ and allowed to stand on ice for 20 min before re-pelleting. The pellet was finally re-suspended in 10 ml of ice cold 0.1M cacl$_2$ with 10% (v/v) glycerol and incubate in ice for 30 min. Aliquots of 220 µl units were prepare and immediately frozen in dry ice with ethanol bath. Sample were then stored at -80ºC.

2.2.1.5 Transformation of *E. coli* strains

1 µl of DNA was added to an aliquot of *E. coli* strain DH5α and incubated on ice for 15 min. The bacteria was then heat shocked in a heating block at 42°C for 90s and put back on ice for 2 min. LB was added to the bacteria and allowed to grow at 37°C for 1 h. They were then plated on LB agar with 200 µg/ml ampicillin and incubate overnight at 37 °C. Individual colonies were picked and grown in 15 ml LB amps during the day and 1 ml of each suspension was added to 100 ml LB ampicillin and grown overnight

at 37°C. The resultants cell suspensions were centrifuged at 4000 rpm at 4°C and the DNA extracted by Qiagen MIDI prep.

2.2.1.6 Molecular Constructs

Lmjf32.0420 and Lmjf05.0390 full length open reading frame were generated by PCR (Kind gift Dr. Charles Brearley, UEA, UK), flanking BglII and BamHI restriction site in the forward and reverse primers respectively. The following oligonucleotide Primers were used; LmaMORN6 Bgl II fwd 5'-TCAAGATCTCCACCA TGAGCACTACCGATTCACCG -3' and LmaMORN6 BamH1 RV 5'-TACGGATCCCTCAATCTCCAGCCCATAGACG-3'; LmaMORN5 BglII fwd 5'-TCAAGATCTCCACCATGAACAGCACTATAAATAGC-3' and LmaMORN5 BamHI REV 5'- TACGGATCCCGCGACTCCCTCGGTTACCTTGGC-3'. The PCR products were digested with BglII and BamHI and then ligated into pXG-/GFP+ (strain B2863), (kind gift from Prof. Stephen M Beverley, University of Washington, St. Louis, USA), which was also digested with BamHI and dephosphorylated. The pXG-/GFP+ is a *Leishmania* specific vector for making C-terminal GFP fusion protein. BglII and BamHI restriction sites can ligate each other and therefore inserts can be in both possible orientations. The correct orientation was determined by a combination of restriction digest and sequencing. The GFP sequence in pXG-/GFP+ vector does not have a start codon; therefore the GFP is only expressed with an insert.

2.2.2 Cell culture and immunofluorescence

2.2.2.1 Transfection of *L. major*

The mid log phase promastigotes were harvested by centrifugation at 1300 × g for 10 min, washed once with cytomix buffer and finally resuspended in the same buffer at 2×10^8 cells/ml. Then 0.5 ml (1×10^8 cells) of cells was added into a 4 mm gap cuvette containing 10 µg of plasmid DNA, mixed by Pipetting up and down and then immediately electroporated in Genepulser II apparatus (Bio-Rad Laboratories, UK) twice with 1500 volts at 25 µF, waiting 10 seconds between electroporations. Cells were immediately transferred into fresh M199 medium and incubate overnight at 27°C. The following day, Geneticin disulfide (G418; Sigma) was added to the medium for the selection of transfected promastigote beginning with 4 µg/ml and increasing at every passage up to 100 µl/ml G418.

2.2.2.2 *Leishmania* Culture

The *L. major Friedlin (MHOM/IL/81/Friedlin)* cell line were maintained at 27°C in M199 media supplemented with haemin and 10% heat-inactivated, foetal calf serum (FCS) as described (Kapler et al., 1990). The cultures were routinely passaged every 3-4 days by pipeting 0.5ml of old culture into 4.5ml fresh M199 media (1:10 dilution) in a 25ml flask.

For long term storage, mid-long phase culture were pelleted and re-suspended in fresh M199 media with 10% glycerol. 1 ml was aliquoted into cryo-tubes and slowly frozen in freezing containers over night before transferring into -150 freezer. To revive *L. major* culture, an aliquot was quickly thawed at 37°C and transferred to a 5ml fresh M199 media containing appropriate antibiotics.

2.2.2.3 RAW264.7 culture

The murine macrophage cell line RAW264.7 were maintained at 37 °C with 5% CO_2 in RPMI 1640 medium with 10% FCS as previously described (Wheeler et al., 2011). Weekly passage was done by scraping cells to suspension and inoculating 10ml fresh media with 100 µl of cell suspension. Long term storage is a described above.

2.2.2.4 In vitro growth rate

In vitro cell growth was performed by inoculating 10^6 log phase promastigotes into a fresh 5ml M199 media, containing appropriate antibiotics. The cultures were counted every 24 h using an improved Neubauer haemocytometer. This experiment was done in triplicates and the mean value were then plotted on a graph.

2.2.2.5 Microscopy and Immunofluorescence

Localization of GFP fusion proteins were visualized by fluorescence microscopy. Approximately 200 µl of log phase promastigotes (4-5×10^6 cells/ml) were pelleted by centrifugation (1300 × g, 10 min) and washed with chilled PBS twice before re-suspended in 100 µl of PBS. Then 100µl of cells suspension was allowed to settle on the marked region of poly-L-lysine coated slides for 2 min at room temperature. The un-attached cells were removed by washing twice with PBS. The cells were fixed with 4% paraformaldehyde for 5 min and rinsed twice with PBS before permeabilizing with

cold methanol. The cells were then rehydrated in PBS, incubated with DAPI for 1 min to stain nuclear DNA and rinsed with distilled water before mounting in Fluoromount-G™ (SouthernBiotech, Cambridge BioScience Ltd., Cambridge, UK). Slides were sealed with clear nail vanish (boots, UK).

Immunolabelling was achieved by fixing cells as described above, and then blocking with 1% (w/v) bovine serum albumin (BSA) at room temperature for 30 min. The cells were incubated with primary antibodies for 1 h and then rinsed 3 times with PBS. This was followed by incubation with secondary antibodies for 30 min and then washed 3 times with PBS. The cells were then stained with DAPI for 1 min, wash twice with distilled water, mounted with fluoromount and sealed with nail vanish.

Epifluorescence images were obtained by the Zeiss Axioplan 2 upright microscope equipped with a zeiss AxioCam MRm digital camera. Unless otherwise state, all images were captured with a 63× objectives in z-stacks, which were then processed for deconvolution (Axiovision 4.7 software).

2.2.2.6 Detergent-resistant membrane (DRM) assays

Temperature-dependent resistance to detergent were tested as previously described (Tyler et al., 2009). 10^7 log phase promastigotes were pelleted by centrifugation at 1300 ×g, 4°C for 10 min and then washed two times in PBS at the same conditions.

For microscopy, pellets were re-suspended in 0.5 ml PBS and then 0.1 ml was settled on a poly-l lysine slide. Slide washed once with PBS to remove unattached cells and

then incubated with 1% Triton X-100 in PBS at 4°C or 37°C for 5 min (4 ºC incubation was on ice and 37°C was in incubator). Cells were washed in PBS, fixed with cold methanol, DAPI stained and mounted with flouromount. Images were obtained as described above (2.2.2.5).

For western blot, pellets were extracted in 0.5 ml of 1% Triton X-100 in PBS at 4°C or 37°C for 5 min before centrifugation. The supernatants were transferred into new tubes and SB was added to both supernatant and pellet before running 15 µl of each on a 10% gel for western blot analysis.

2.2.2.7 Protein Secretion Assay

L. major promastigotes were harvested from stationary phase cultures. Afterward, they were subcultured for 5 h in modified RPMI 1640 (a medium free of serum, tyrosine, and phosphate). The parasites were harvested by centrifugation at 1,500 ×g for 10 min at 4°C and the supernatant was again centrifuged at 13,500 ×g for 15 min at 4°C. Cell-free supernatant was concentrated tenfold by pressure ultrafiltration using an AMICON system with 30-kDa ultrafiltration regenerated cellulose membranes (Millipore Corp). The concentrated supernatant was analyzed by western blotting as described earlier.

2.2.2.8 Macrophage Infection Assay

RAW264.7 cells grown to near confluent were scraped to form a suspension. 10^6 cells from the cell suspension were used to seed sterile 13 mm diameter coverslips placed in 24-well plastic tissue culture plates in 1 ml of supplemented DMEM medium. The

cells were grown for 1-2 days. Late stationary phase promastigotes cultures were counted and then incubated with RAW264.7 monolayer in a parasite:macrophage ratio of 20:1 in supplemented DMEM. After 3 h of incubation, the cells were washed at least three times with PBS to remove any unbound parasites. 1ml of fresh supplemented DMEM was added in each well and incubated at 37°C with 5% CO_2 for different time points. At the desired time points, cell were fixed in cold methanol for at least 1 h and then rehydrated in PBS. The cells were then stained with DAPI for 1 min, wash twice with water and mounted with fluoromount.

For secretion, GFP chimera protein distribution were observed by microscopy. For infectivity, internalized parasites were counted in 100 macrophages and repeated three times. The standard errors were calculated by dividing the standard deviation by the square root of number of measurements, which is 3.

2.2.3 Western blot

2.2.3.1 Preparation of protein samples

10^7 log phase promastigotes were centrifuged at 1300 ×g for 10 min at 4 ºC and washed twice in cold PBS. The cells were lysed directly in 100 µl of boiling sample buffer and then sheared by passing the sample repeatedly through a 19 gauge needle. The sample were aliquoted and used immediately or kept in -20 ºC until used.

2.2.3.2 Sodium Dodecyl-Sulphate Polyacrylamide Gel Electrophoresis (SDSPAGE)

Proteins were separated by SDS PAGE (Laemmli, 1970). Samples were boiled for 5 min and 13 µl of each sample was loaded onto the gel alongside a protein standard molecular weight marker (Precision Plus Dual Colour Protein Standard, BioRad, UK).

The gels were electrophoresed in running buffer at 100 V for 1.5-2 h or until the protein standard indicated good separation at room temperature.

2.2.3.3 Coomassie staining of SDS gels

The gels were then stained with Coomassie blue solution for one hour/overnight and then de-stained in the Coomassie destaining solution for protein visualization.

2.2.3.4 Protein transfer to membrane

Proteins were transferred from the acrylamide gel to a Polyvinylidene Fluoride (PVDF) membrane (Thermo Fisher scientific, Leicestershire, UK) using a Trans-Blot SD Semi-dry Transfer cell (Biorad, Hertfordshire, UK). Firstly, the gel and two sheet of extra thick filter paper were soaked in transfer buffer and the PVDF membrane in methanol prior to transfer. A sandwich was formed onto the platinum anode bed in the followed order; Filter paper, PVDF membrane, gel and another filter paper. Small air bubbles trapped in the sandwich were removed by gently rolling a glass tube on the filter paper. Finally, the cathode was placed on top of the stack and the transfer was at 25 V for 30 min.

2.2.3.5 Membrane immunolabelling

Membrane were placed in a square petri dish and blocked with 5% powdered milk in 1x Tris-Buffered Saline Tween-20 (TBST) for 1 h with shaking. The primary antibody was then diluted to the recommended dilution in 5% powdered milk and added to the membrane before leaving overnight at 4°C. The membrane was washed 4 times for 15 min with 1 x TBST and incubated with the secondary antibody conjugated to

horseradish peroxidise (HRP) at the appropriate dilution in 5% powdered milk for 2 h in the dark. After incubation, the membrane was washed 4 times in 1 x TBST for 10 min and finally in 1 x PBS.

2.2.3.6 Detection of HRP signal

Detection was according to the manufacturers' instructions; the membrane were activated with Supersignal® West Pico Chemiluminescent Substrate (Pierce, UK) for 3 min. The membrane was placed between two sheets of clear polythene and placed in a radiography cassette (IEC 60406, GRI, UK). In a dark room, x-ray film was placed in the cassette and the film exposed for a variable amount of time which were sample and antibody dependent. The film was then developed by using SRX-101A X-ray processor (Konica Minolta, Banbury, UK). Protein sizes were determined by placing the film over the membrane and marking the position of the marker.

2.2.3.7 Reprobing of the membrane

The membranes were washed with dH_2O for 5 min then incubated with 0.2 M NaOH for 5 min and rinsed again with dH_2O for 5 min. The membranes were then blocked in 5% milk in TBST for 1 h before labelling again with a different antibody.

2.2.4 Bioinformatics

2.2.4.1 Databases/internet resources used to identified MORN encoded proteins

TritrypDB (http://tritrypdb.org/tritrypdb/), Pfam (http://pfam.xfam.org/) and SMART (http://smart.embl-heidelberg.de/):- data bases with genomic resources for *Leishmania*.

2.2.4.2 Tools and Software

NCBI was utilized to confirm partial sequenced inserts by blast analysis. http://blast.ncbi.nlm.nih.gov/Blast.cgi

Mega5 was used to generate phylogenic trees from homologs of LmaMORN5 and LmaMORN6.

Clustalw2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/) is a DNA or protein multi sequence alignment program, used to aligned *L. major* against L. *braziliensis* homologous sequences.

PAL2NAL (http://www.bork.embl.de/pal2nal/) was used to converts alignment of *L. major* versus *L. braziliensis* and corresponding DNA sequences into a codon alignment, which were further subjected to the calculation of synonymous (d_S) and non-synonymous (d_N) substitution rates. This measurement is known as dN/dS and it quantifies selection pressures by comparing the rate of substitutions at silent sites (dS), which are presumed neutral, to the rate of substitutions at non-silent sites (dN), which possibly experience selection. The ratio dN/dS is expected to exceed unity only if natural selection promotes changes in the protein sequence; whereas a ratio less than unity is expected only if natural selection suppresses protein changes.

3 Results

3.1 *In silico* screening identified 22 proteins containing MORN repeats in *L. major*

As a strategy to identify proteins containing MORN-repeat in *L. major*, the SMART (http://smart.embl-heidelberg.de/), TritrypDB (http://tritrypdb.org/tritrypdb/), and Pfam (http://pfam.xfam.org/) databases were searched for genes encoding MORN motifs. 20 MORN proteins were identified from the SMART database, 21 in the TritrypDB and 22 in Pfam. A brief description of the 22 *L. major* MORNs (LmaMORNs) are listed in Table 3.1 below. These conserved hypothetical LmaMORNs which are all uncharacterized so far, are numbered here as 1 and from 5 to 25 (Table 3.1). LmaMORN1 corresponds to its ortholog in *T. gondii* (TgMORN1) but TgMORN2 has no homolog in *L. major* (Gubbels et al., 2006). MORN3 (Zhang et al., 2015) and MORN4 (Mecklenburg et al., 2015) have also been identified and named but lack identifiable homologs in *L. major* and are therefore omitted in the numbering scheme I have adopted. Amongst the 22 proteins containing MORN-repeats, LmaMORN 1, 11, 12, 13, 14, 15, 16, 17, 18, 20, 22, 23, 24 have MORN repeats ranging from 3 to 15 in number and no other major functional domain annotation. These MORN-repeats were found to be present anywhere across the protein's length, indeed in some cases the proteins appear almost exclusively composed from MORN repeats. LmaMORN 5, 6, 7, 8, 9, 10, 19, 21 and 25 are annotated to contain other functional domains including a kinase (LmaMORN5), two kinesins (LmaMORN 6 and 7), FYVE motif (LmaMORN 8), transmembrane domains (LmaMORN 10) and two with VPS9 motifs (LmaMORN 9 and 25) respectively. Finally LmaMORN 5, 9, 19 and 21 are annotated by signal P (HMM and NN) to be extracellular/secreted.

MORN	Gene Name	Description	Size (kDa)	Diagram (SMART database)
1	LmjF30.3310	Hypothetical Protein, Conserved	40.8	
5	LmjF05.0390	Protein kinase, Putative	77.2	
6	LmjF32.0420	Hypothetical Protein, Conserved	80.7	
7	LmjF31.2710	Phosphatidyl inositol -4-phosphate 5 kinase-like protein	119.5	
8	LmjF26.1420	Hypothetical Protein, Conserved	257.0	
9	LmjF25.2330	Hypothetical Protein, Conserved	92.4	
10	LmjF16.0100	Hypothetical Protein, Conserved	77.4	
11	LmjF36.1810	Hypothetical Protein, Conserved	100.6	
12	LmjF22.1400	Hypothetical Protein, Conserved	46.7	
13	LmjF36.3780	Hypothetical Protein, Conserved	36.0	
14	LmjF20.0580	Hypothetical Protein	45.3	
15	LmjF05.1080	Hypothetical Protein, Conserved	80.1	
16	LmjF20.0520	Hypothetical Protein, Conserved	102.2	
17	LmjF11.0090	Hypothetical Protein, Conserved	57.2	
18	LmjF20.1100	Hypothetical Protein, Conserved	203.5	
19	LmjF20.0130	Hypothetical Protein, Conserved	48.7	
20	LmjF10.0195	Hypothetical Protein, Conserved	23.3	
21	LmjF03.0970	Uncharacterised protein	39.8	
22	LmjF27.2305	Uncharacterized protein	19.3	
23	LmjF29.1700	Uncharacterized protein	129.7	
24	LmjF22.1270	Uncharacterized protein	47.0	
25	L1994.08	Uncharacterized protein, Putative	82.7	

Table 3.1. The family of MORN-repeat proteins in *L. major*. Diagrams in this table are adapted from the SMART database except for the last two, which were absent from that database. The MORN repeats are represented with a purple rectangle with MORN written across it while the grey rectangles with MORN across are extra domains identified according to Pfam. The green sections are coiled-coil regions, pink sections represent low complexity regions and signal peptides are represented in red. Other functional domains are represented as follows: FYVE (pink oval shape), Kinesin (blue oval), kinase (pentagon shape), VPS9 (black square) and transmembrane (blue rectangle).

3.2 Selection of LmaMORN5 and LmaMORN6

TbMORN1 was identified as a component and marker of a novel cytoskeleton structure called the bilobe. However, the interesting combination of MORN-repeats with other functional domains (Table 1) suggests a wide variety of functions. I sought to investigate whether these modular MORN proteins with apparently diverse functionality also target to the bilobe. Two LmaMORN proteins with discrete functional characteristics were chosen for comparison in this study, LmaMORN5 (a putatively secreted kinase) and LmaMORN6 (a kinesin). LmaMORN5 gene (LMJf05.0390) is 719 amino acids long with an estimated molecular mass of 77.2 kDa. It is predicted to contain a signalling peptide with a predicted cleavage site between residues 27 and 28 (signalP), followed by 7 MORN-repeats on its N-terminus and carries a kinase domain at its C-terminus. This is interesting because protein kinases are well known for their role in signal transduction. Therefore this combination of MORNs, Kinase and signalling peptide may indicate a multi-functional role perhaps serving to phosphorylate signalling proteins in both parasite and host. On the other hand, LmaMORN6 (LmjF32.0420) contains 5 MORN-repeats at its C-terminus and a domain that shows homology to a kinesin motor at its N-terminus. Kinesins are important components of the microtubule cytoskeleton, implying an intracellular housekeeping role. LmaMORN6 gene is encoded by 718 amino acids with an estimated molecular mass of 80.7 kDa.

3.3 Two sections of LmaMORN5 were found to be under selective pressure

Pathogen secreted proteins which interact directly with the host are often under a direct selection pressure which is not experienced by proteins with predominantly housekeeping role. To investigate whether this premise as true of LmaMORN5 and LmaMORN6, their gene sequences and those of their homologs available in TritrypDB database were used to generate a phylogenetic tree using MEGA software. The results showed divergence according to genus, subgenus and species (Figure 3.1). It is interesting to note that among the homologs of LmaMORN5 represented in the phylogenetic tree, L. major and *L. mexicana* were annotated to carry signal peptides (blue star). Protein sequences of LmaMORN5 and LmaMORN6 were then aligned against homologs in *L. braziliensis,* a different subgenus, using ClustalW (Figure 3.2). The alignment results showed localized variation in amino acid sequence. LmaMORN5 shows 81.4% identity, 86.4% similarity and 1.5% gaps with the majority of the alterations founded in two regions, region 1 were amino acid 1-95 and region 2 were amino acids 387-432 (Figure 3.2B). The ratio of amino acid substitution (dN/dS) of the altered regions were measured using the PAL2NAL software. The results shows significant dN/dS 1.1663 and 1.5243 for region 1 and region 2 respectively which implies these sections maybe under selective pressure (Figure 3.2). Region 1 includes the signal peptide, supporting gain of an important extracellular function for LmaMORN5. In contrast. LmaMORN6 shows 85.4% identity, 91.9% similarity and no gaps so that evolution under selective pressure appears to be insignificant for this protein when measured by dN/dS consistant with a housekeeping role.

A. LmaMORN5

LdBPK_050390.1 L. donovani
LinJ.05.0390 L. infantum
LmjF.05.0390 L. major ✦
LmxM.05.0390 L. mexicana ✦
LtaP05.0400 L. tarentolae
LbrM.05.0400 L. braziliensis
LbrM2903_05_0370 L. braziliensis
CfaC1_05_0450 C. fasciculata
Tb927.10.10350 T. brucei
TcCLB.511633.70 T. cruzi
TCDM_11204 T. cruzi

0.05

B. LmaMORN6

LdBPK_320430.1 L. donovani
LinJ.32.0430 L. infantum
LmjF.32.0420 L. major
LmxM.31.0420 L. mexicana
LtaP32.0450 L. tarentolae
LbrM.32.0490 L. braziliensis
LbrM2903_32_0530 L. braziliensis
TCDM_00488 T. cruzi
TcCLB.506213.90 T. cruzi
TcCLB.511287.20 T. cruzi

0.1

Figure 3.1. Phylogenetic Tree based on the gene sequence of few homologs of LmaMORN5 and LmaMORN6. *L. mexicana* is the only homolog annotated for secretion (blue star).

42

A

```
# Aligned_sequences: 2
# 1: Major
# 2: Braziliensis
# Matrix: EBLOSUM62
# Gap_penalty: 10.0
# Extend_penalty: 0.5
#
# Length: 730
# Identity:     594/730 (81.4%)
# Similarity:   631/730 (86.4%)
# Gaps:          11/730 ( 1.5%)
# Score: 3189.0
#
#
#=======================================
CLUSTAL 2.1 multiple sequence alignment
```

Major   --MNSTINSKGASAAAAATAAVPLHGGDSHRMGAAATAGGSERANWNNPSFVAAPRNSSG 58
Braziliensis MSLNSTTNNKCTSTAIASAPAPPSHGGASHGIGAATTASGQVRASWNTSSPEAAQDNSST 60
             .:** *.* :*:* *::.* * *** ** ;***;**:*. **.**;* ** *** ***

Major   TINVDGYPPAQDHQQPSHPLLLSHSTDVDDAGNGAQHAAVN|PLEYCGVRLLPGVEQIDF 118
Braziliensis AVKRDGCPPSQTHLQPPSPSLLSHSTDAGEEGGDAQNAFST|PLEYCGVHLLPGVEQIDF 120
             ::: ** **;* * **. * ******..: *.*:*. .|****** *******

Major   DEGYYVGTIDENGEMAGYGKATWHSGDTYEGEWLNGMMHGKGTYTWADGDYYQGDYVRGR 178
Braziliensis DEGYYVGTIDENGEMAGYGKATWHSGDTYEGEWLDGMMHGKGTYTWADGDYYQGDYVRGR 180
             **************************************;*************;*******

Major   MEGRGEMKDATGLYTGEWADDMRQGYGRMLYAGGNVYEGEWLAGMRHGSGKLVEPAAHVM 238
Braziliensis MEGSGEMKDATGLYTGEWADDMRQGHGRMVYAGGNVYEGEWLAGMRHGAGKLVEPAAHVT 240
             *** .********************;***;*******************.**********;

Major   YEGEFNRNEKEGRGVQTNSDGDVYEGEFARGKPNGRGTYLWADGARYIGMFKDGVKHGDG 298
Braziliensis YEGEFNRNEKEGRGIQTNVDGDVYEGEFAHGKPNGRGTYLWADGARYIGMFKDGVKHGDG 300
             **************;***.*********:*******************************

Major   CEWLANGDWVAGLFVDGEHVDSQSTLHKATVLTPDSSDADGGSEDGAGDGGGGGSRGSIG 358
Braziliensis CEWLANGDWIAGLFVNGEHVDSHSTHHVATVFTTDSSDADDGSEVGAGDRRALNSRKNAS 360
             *********:*****:******:*** * ***;*.******.*** **** ..;** ..

Major   LSESTAAAVLAPLDAAKLRRIADQVHAPSFSI|GSATAMRSLRSSLRKNSSSNYQDFSGL 418
Braziliensis LSDSTAAAVLAPLNAEKLRRIADQVRAPSFSI|TSSMILRNLRDPSCTTGSSHYRDFNDT 420
             .********:*:*********:******|.*;. ;*.**:.. ...**;*;.**..

Major   GGVTIEEASSSFLRSLA|PQMLLDDSDLEGWTPLKTIGKGSFGAVYTALLRNGRTVCCKV 478
Braziliensis DSVIADEVTSPFSRALV|PQMLLDDSDLEGWTPLKTIGKGSFGAVYTALLRNGRTVCCKV 480
             ..* .;*:.:*.* *.*|************************************

Major   IELGTVESEEEMDKLRNEIALMRRLRHPNCVQYYGSLEDKVKNTLNIFMEYVSGGTLTSF 538
Braziliensis IELGSVESEEEMEKLRNEIALMKRLRHPNCVQYYGSLEDRARNTLNIFMEYVSGGTLTSF 540
             ****;*******:*********:****************;.;*****************

Major   VTKFKSIPLETLRQWVYQMVCGVKYLHECGIVHRDIKGDNVLVSVDGIVKLADFGCSKAI 598
Braziliensis VAKFKSIPLETLRQWVYQMVCGVKYLHECGIVHRDIKGDNVLVSVDGIVKLADFGCSKAI 600
             *;***

Major   DDVCSATHGCSTMVGTPYWMAPEVIKCEAGGYGVKSDIWSIGCTIVEMLTGKPPWPECNS 658
Braziliensis DDVCSATHGCSTMVGTPYWMAPEVIKCEAGGYGVKSDIWSIGCTMVEMLTGKPPWPECNS 660
             ;************

Major   MWAAVYKIANSTGLPTEIPADIDPKLMDLLQKCFERDPKLRPTAAGMLSHPFLAKVTEGV 718
Braziliensis MWAAVYKIANSTGLPTEIPADVDPELMDLLQKCFERNPKLRPTAAEMLKHPFLVKVTEGV 720
             *********************;**:***********.********* **.****;******

Major   A--------- 719
Braziliensis TSPMEGNRSE 730
             :

Synonymous (d_S) and non-synonymous (d_N) substitution rates
$S = 91.6$
$N = 205.4$
$d_S = 0.3849$
$d_N = 0.4490$
$d_N/d_S = 1.1663$

Synonymous (d_S) and non-synonymous (d_N) substitution rates
$S = 27.2$
$N = 104.8$
$d_S = 0.3845$
$d_N = 0.5861$
$d_N/d_S = 1.5243$

B

Region 1   Region 2

MORN MORN MORN MORN MORN MORN MORN   S_TKc

0   100   200   300   400   500   600   700
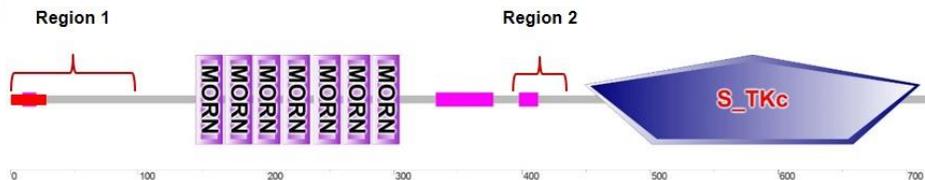
Figure 3.2: Two sections of the LmaMORN5 sequence are under selective pressure. The protein sequences for LmaMORN5 were aligned with the *L. braziliensis* homolog using ClustalW2. There were significant divergences in two sections (Boxed in red). These two sections were tested for dN/dN and were found to be significant as show in the diagram above. (B). Schematic diagram of LmaMORN5 showing the two sections under selective pressure.

3.4 Cloning and vector constructs

The ORFs from LmaMORN5 and LmaMORN6 were excised from the cloning vector with BglII and BamHI and cloned into the BamHI cloning site of the pXG-/GFP+ expression vector placing GFP tag at the 3' end of the cloned gene, generating C-terminal GFP fusion constructs for episomal expression in *L.major* (pXG-/GFP+LmaMORN5 and pXG-/GFP+LmaMORN6) (Figure 3.3A).
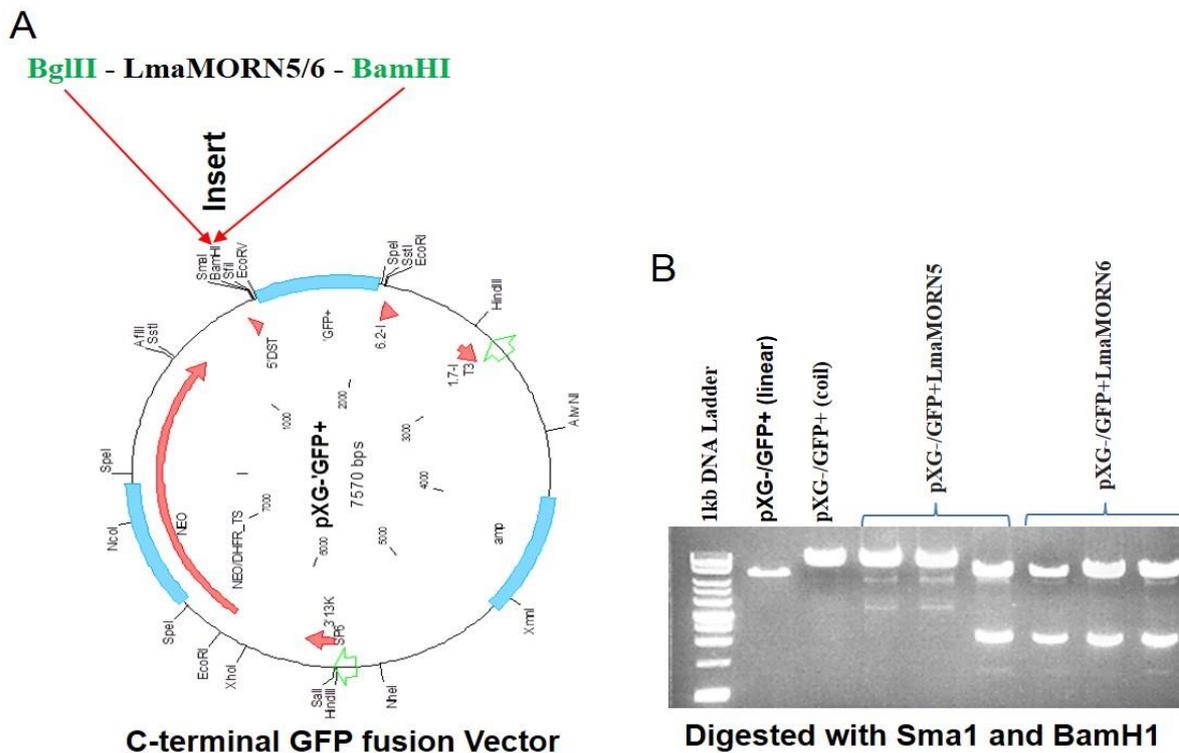

Figure 3.3. Construction of pXG-/GFP+LmaMORN5 and pXG-/GFP+LmaMORN6. (A) *Leishmania* expression vectors for C-terminal GFP fusion were generated by inserting LmaMORN5 or LmaMORN6 gene into the BamH1 of the polylinker site of pXG-/GPF+. (B) Inserts and correct orientation were identified by cleavage of DNA construction by two restriction endonucleases, Sma1 and BamH1. From the three colonies of pXG-/GFP+-LmaMORN5, only one is in the correct orientation (which was digested). The three colonies of pXG-/GFP+-LmaMORN6 were all digested.

The overhangs of the BglII and BamHI ends can ligate each other and therefore inserts can ligate in either orientation within the vector. The correct orientation has BamHI and BglII sticky ends ligating with each other at the 5', destroying the restriction site while the 3' is BamHI/BamHI conserved. To confirm the insert and correct orientation, colonies were amplified and cleaved with both SmaI (which is 5' of BglII-BamHI ligation) and BamHI. The amplified colonies with the correct orientation were digested (Figure 3.3B). These constructs were also sequenced just across the fusion between insert and the GFP gene, and their orientation and reading frame were checked manually. The GFP gene in the pXG-/GFP+ vector does not have a start codon and can therefore only be expressed with another gene fused to its 5' end in the correct orientation.

3.5 Expression of LmaMORN5-GFP and LmaMORN6-GFP in *L. major*

To confirm expression of the fusion proteins, mid log promastigotes of *L. major* Friedlin were transfected with pXG-/GFP+LmaMORN5, pXG-/GFP+LmaMORN6 and pXG-/GFP2+ a control plasmid which expresses the whole GFP gene. Chimeric and control GFP transfectants began fluorescing green under UV light from approximately three weeks post transfection (Figure 3.4A). This result confirmed the correct orientation and GFP fusions. Western blot was employed for further confirmation using poly clonal α-GFP anti-bodies to detect the GFP chimera protein (Figure 3.4B). The lysate of the parental *L. major* (lane 1) showed no signal while a 27 kDa band was detected for the GFP only lysate (lane 2). Lane 3 was LmaMORN6-GFP with an expected band size of 107.7 kDa. Lane 4 was LmaMORN5-GFP showing a band size somewhat bigger than the predicted 104.2 kDa of the polypeptide plus GFP tag (Figure 3.4A).
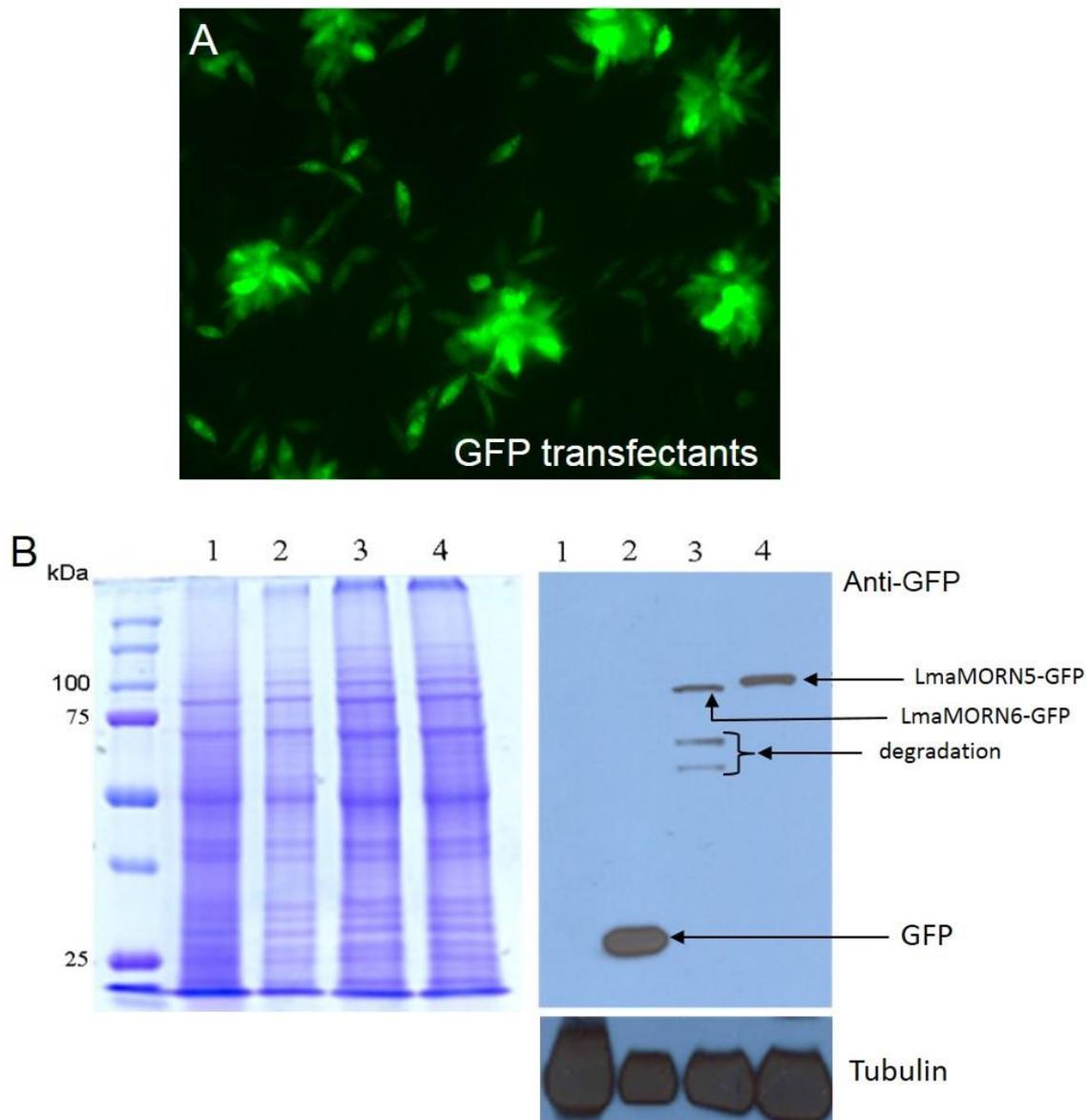
Figure 3.4. Expression of LmaMORN5-GFP and LmaMORN6-GFP chimera proteins. (A) GFP transfectants three weeks post transfection. (B) Lysates of 4×10^6 cells of *L. major* parental cell line and all mutants were analysed by immunoblotting. A corresponding coomassie stained gel was produced to shows loading. Lane 1 was *L. major* parental cell line lysate with no GFP signal. Lane 2 was lysate of GFP only transfectants with band size of 27 kDa. Lane 3 were LmaMORN6-GFP with predicted band size of 107.7 kDa and lane 4 were LmaMORN5-GFP transfectants showing band sizes bigger than the predicted 104.2 kDa.

This may be the result of LmaMORN5 having been post-translationally modified by glycosylation, consistent with it being a secreted protein. Glycosylation prediction showed 12 N-linked and 48 O-linked glycosylation on asparagine and serine/threonine residues respectively (Hamby and Hirst, 2008). These results taken together confirmed LmaMORN5 and LmaMORN6-GFP expression and GFP tag acted as a reporter, important for further investigations. Transfectants were stable and grew well with no obvious morphological or motility defects. It was however observed that the parental cell line and GFP control cultures were more confluent on passage day than LmaMORN5/LmaMORN6 cultures.

3.6 LmaMORN5 and LmaMORN6 transfected promastigotes showed slow growth

Growth rate of transfectants against controls were measured by daily calculation of culture density and the mean value from triplicate cultures were presented as growth curve (Figure 3.5). The results showed parental *L. major* cell line and the GFP transfectants grew exponentially 48 h after inoculation and reached peak cell density of 3.9×10^7 cells/ml and 3.1×10^7 cells/ml respectively by day 5. In contrast, LmaMORN5-GFP and LmaMORN6-GFP transfectants showed somewhat slower growth, beginning exponential growth 72 h after inoculation and peak cell density of 3.1×10^7 cells/ml and 2.5×10^7 cells/ml respectively by day 7 (Figure 3.5A). The growth delay was most clearly shown in a log scaled graph (Figure 3.5B). Parental and GFP control cells exponential growth started on day 2 while for LmaMORN5 and LmaMORN6 transfectants exponential growth began on day 3. It is worth noting that while LmaMORN6 reached similar peak cell density to the control lines, LmaMORN5 showed a reduction in peck density. Thus, the over-expression of LmaMORN5 and LmaMORN6 in *L. major* promastigotes showed a delay growth phenotype.
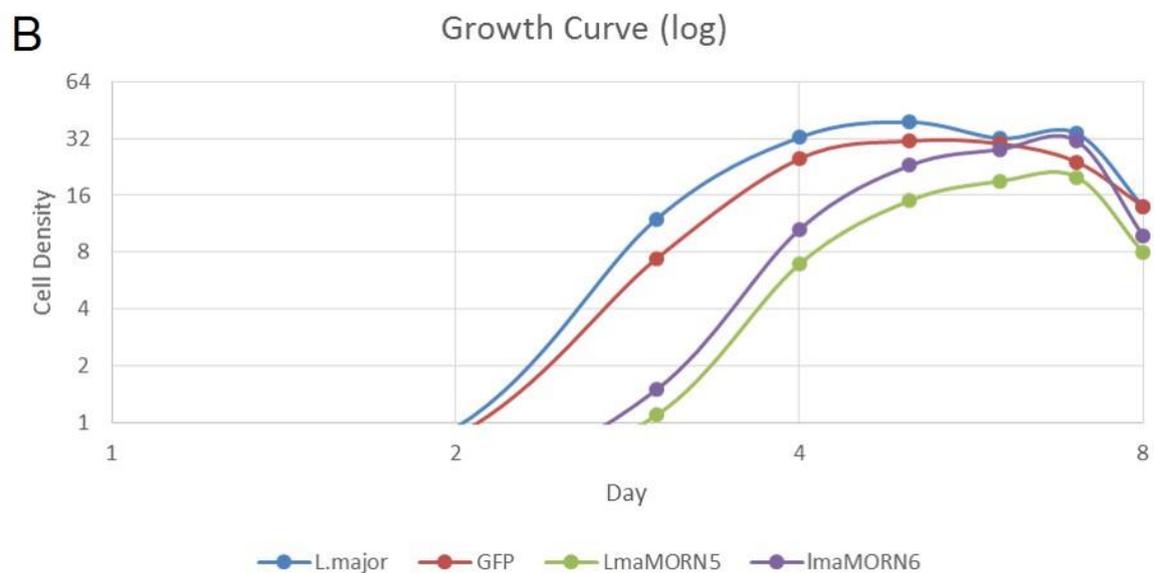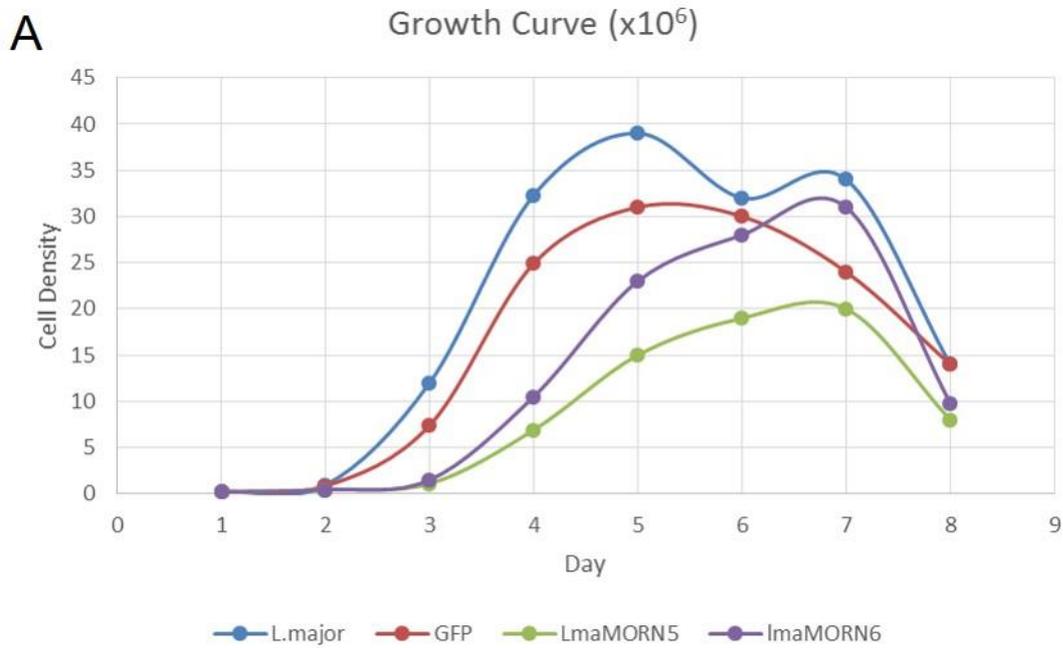
Figure 3.5. LmaMORN5 and LmaMORN6 mutants showed slow growth rate. 10^6 log phase promastogotes were inoculated into 5ml fresh media and counted every day for 8 days. LmaMORN5-GFP and LmaMORN6-GFP mutants' shows a 24 h delayed growth and 48 h longer to reach peak density when compared with controls.

3.7 LmaMORN5-GFP and LmaMORN6-GFP localize to the base of the flagellum and proximal to the kinetoplast.

To investigate the intracellular distribution and localisation, transfectants were fixed and mounted for microscopy. The GFP-only transfectants showed a diffuse GFP expression (Figure 3.6A). LmaMORN5-GFP and LmaMORN6-GFP chimera proteins are seen to predominantly accumulate at two foci; firstly, at the base of the flagellum (arrow head) and secondly in close proximity to the anterior face of the kinetoplast (arrow) (Figure 3.6B-C). Additionally, LmaMORN6-GFP also localized along the length of the flagellum (red arrow) (Figure 3.6C) and sometimes appeared to accumulate at the distal tip (broken arrow) (Figure 3.6D).

The localization at the base of the flagellum resembles the bilobe due to its structure and position, while that proximal to the kinetoplast suggested basal bodies. The fact that 2N2K cells showed 2 distinct localizations proximal to each kinetoplast (arrows) further supported a basal body localization (Figure 3.6E).

3.8 LmaMORN5 and LmaMORN6-GFP chimera proteins were intimately associated with the detergent-extracted cytoskeleton

The localisation results in figure 3.6 above suggested that LmaMORN5 and LmaMORN6-GFP chimera proteins were associated with cytoskeletal components like the basal bodies and the flagella. In order to investigate this further, detergent-extracted cytoskeletons at 4°C and 37°C were analysed by fluorescence microscopy and immunoblot. Microscopic images showed that LmaMORN5-GFP and LmaMORN6-GFP remained associated with the cytoskeleton post 4°C and 37°C triton-X100 extraction. No GFP signal was detected from the cytoskeleton of GFP only cells (Figure 3.7A).
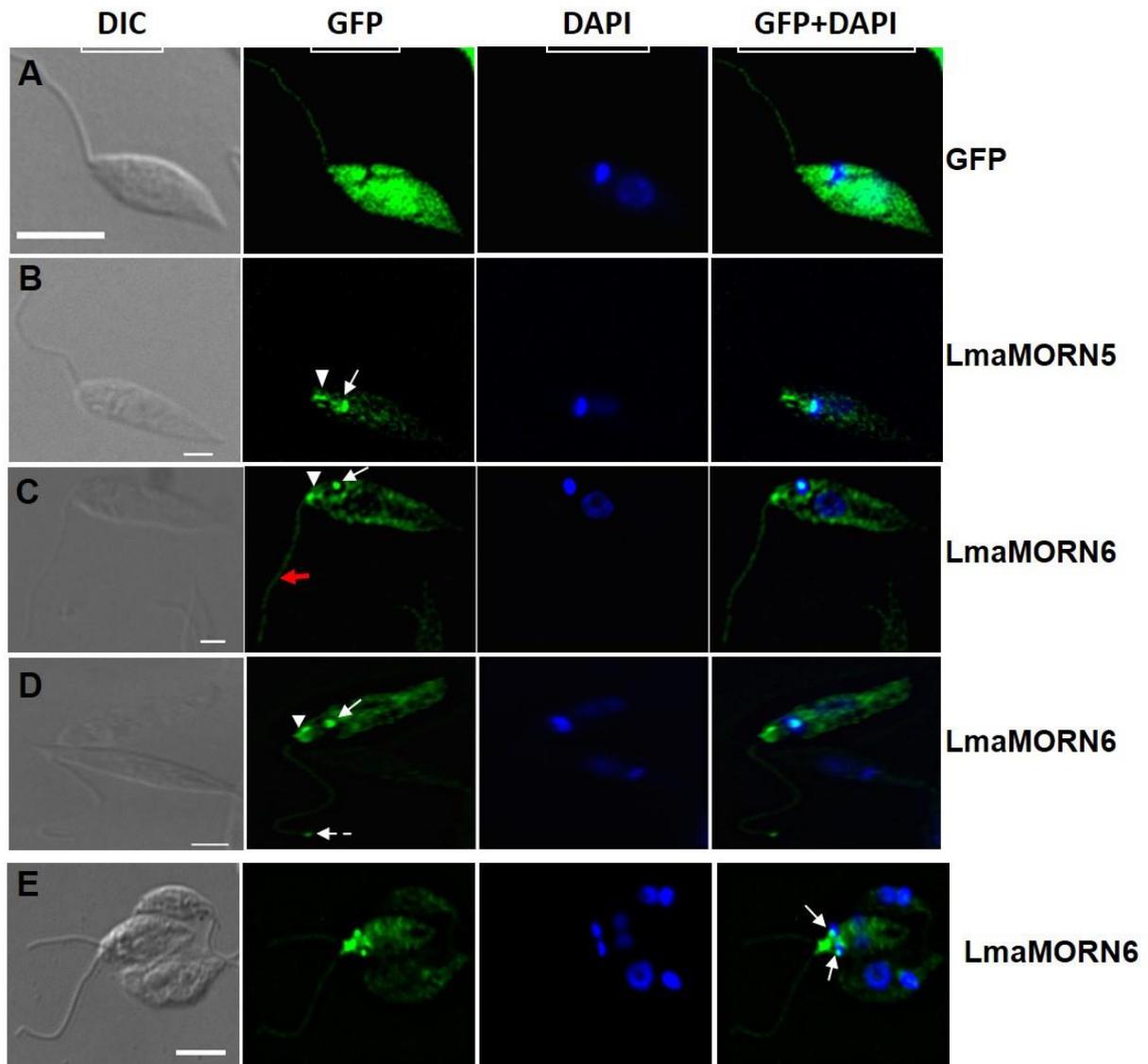
Figure 3.6. LmaMORN5-GFP and LmaMORN6-GFP localizes at the base of the flagellum, proximal to the kinetoplast and also along the flagellum in the case of LmaMORN6-GFP. Mutants were PFA fixed, stained with DAPI and mounted for microscopy. (A) GFP control mutant, showing diffused GFP fluorescence. (B) LmaMORN5-GFP mutant showing bright foci of fluorescence at the base of the flagellum (arrow head) and adjacent to the kinetoplast (arrow). (C) LmaMORN6-GFP mutant also showing bright foci of green fluorescence at the base of the flagellum (arrow head), adjacent to the kinetoplast (arrow) and also along the length of the flagellum (red arrow) and sometimes accumulates at the tip of the flagellum (broken arrow) (D). (E) 2N2K LmaMORN6 transfected cell showing two distinct localization next to each kinetoplast.
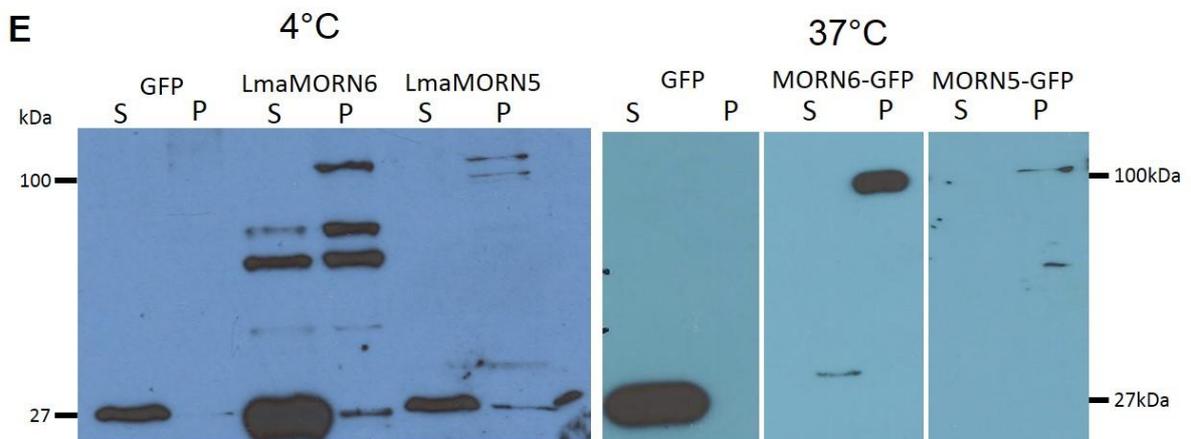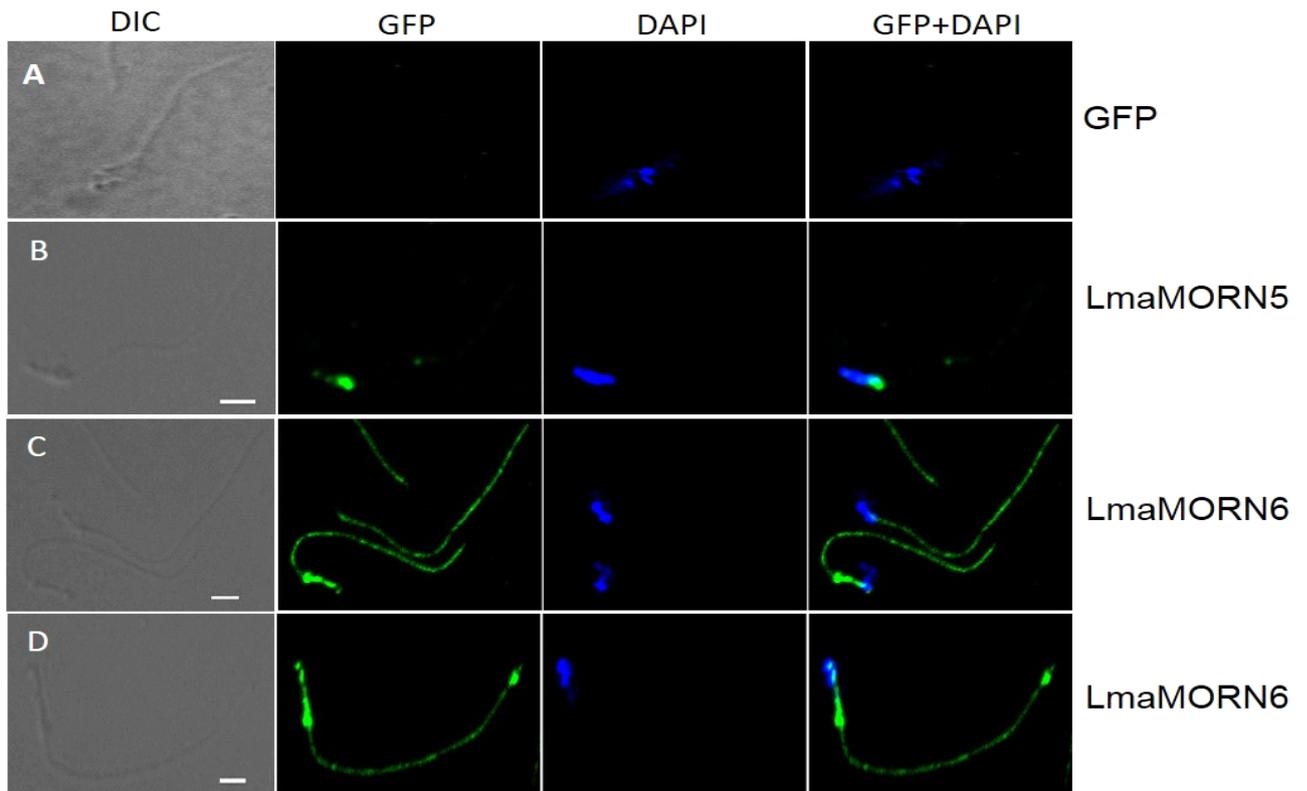
Figure 3.7. LmaMORN5-GFP and LmaMORN6-GFP chimera proteins remain associated with the detergent-extracted cytoskeleton. (A) GFP mutant with no GFP fluorescence (B) LmaMORN5-GFP protein localizes at the base of the flagellum. (C) LmaMORN6-GFP decorating the entire remaining flagellar cytoskeleton and **(D)** localization at the tip of the flagellum in about 1% of its population. (E) Detergent extracted cell lines at 4°C and 37°C analysed by immunoblotting (S = supernatant and P = pellet). Correct band size were detected in the pellet of LmaMORN5 and LmaMORN6-GFP and not in the supernatants. No or little band was detected in the pellet of the GFP only control.

LmaMORN5-GFP signal was detected from the base of the flagellum to the basal body (Figure 3.7B). LmaMORN6-GFP was associated with the entire flagellar cytoskeleton post detergent extraction (Figure 3.7C) and was sometimes observed to accumulate at the distal tip of the flagellum (Figure 3.7D).

Analysis by immunoblotting post 4°C and 37°C Triton X-100 extraction shows little or no protein in the pellet of the GFP-only control. LmaMORN6-GFP lane shows the expected 107 kDa band in the detergent insoluble pellets. The lower bands present in both pellet and supernatant lanes may be due to degradation. LmaMORN5-GFP also shows the expected band size in the pellets lane only (Figure 3.7E). Together these results showed that both LmaMORN5-GFP and LmaMORN6-GFP chimera proteins are associated with the parasite's cytoskeleton, especially the bilobe, basal body and flagellum.

3.9 LmaMORN5 and LmaMORN6-GFP chimera protein significantly Co-localized with YL1/2

In order to identify the localization proximal to the kinetoplast, whole and detergent-extracted transfectants were stained with YL1/2 antibody. In the whole cells, GFP transfectants showed YL1/2 staining of the anterior part of the cell body and the basal bodies (Figure 3.8A). LmaMORN5-GFP and LmaMORN6-GFP chimera protein showed significant co-localisation with YL1/2 staining (Figure 3.8B and C). In the detergent-extracted cytoskeleton, GFP-only transfected cells showed YL1/2 staining of the basal body and part of the flagellum (Figure 3.8D). LmaMORN5-GFP and LmaMORN6-GFP chimera protein again co-stained with YL1/2 (Figure 3.8E-F).

I therefore conclude that the localization of both LmaMORN5 and LmaMORN6 proximal to the kinetoplast reflects basal body association.
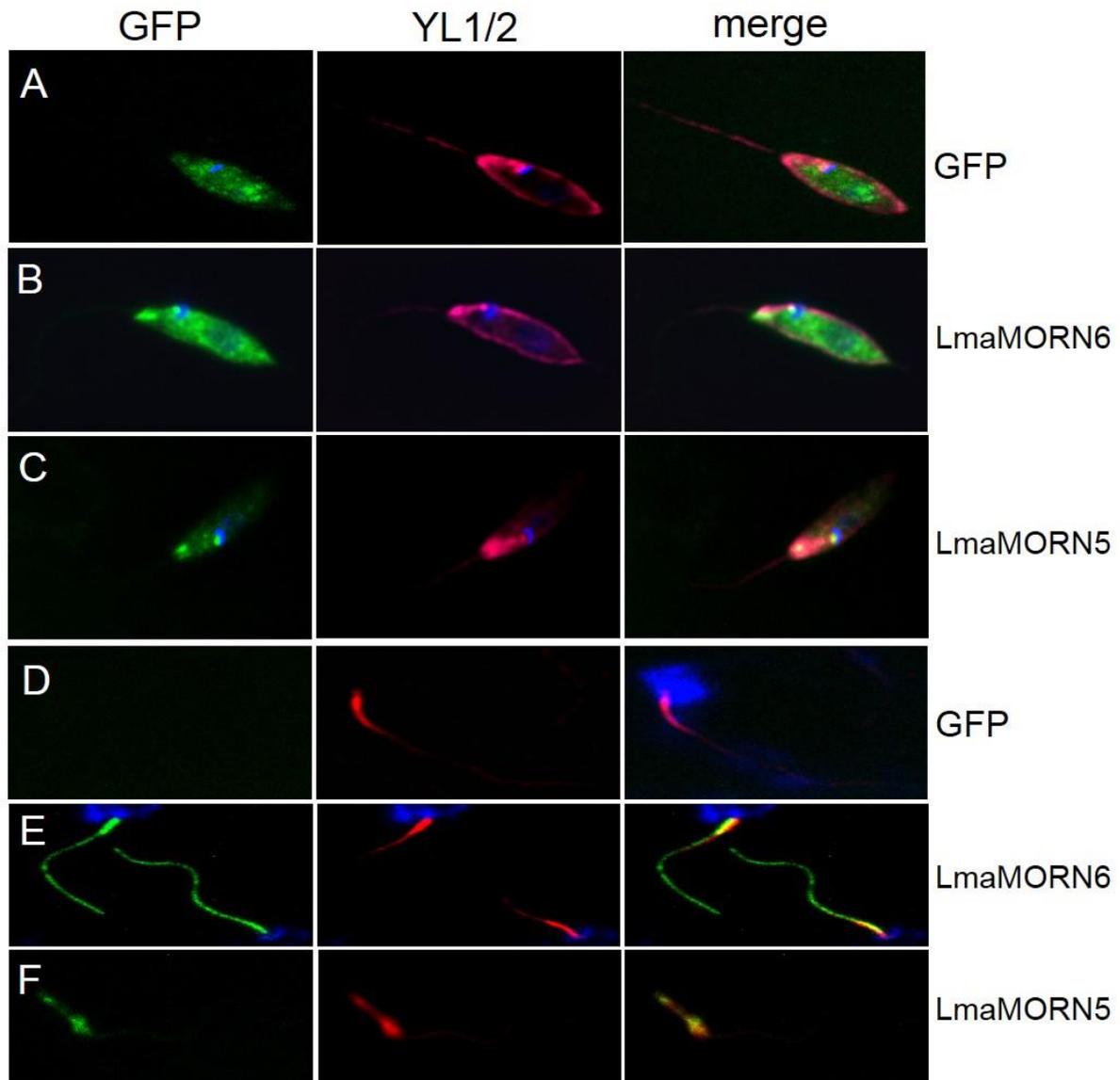
Figure 3.8. The localization proximal to the kinetoplast significantly overlaps with the YL1/2 basal body staining. Transfectants were stained with YL1/2 and DAPI for microscopy. (A) GFP Mutant (control) showing YL1/2 staining the anterior of the cell body and the basal body which lies next to the kinetoplast. (B-C) LaMORN6-GFP and LaMORN5-GFP localization proximal to the kinetoplast significantly overlaps with YL1/2 staining. (D-F) were corresponding triton X-100 treated mutants with same results.

3.10 LmaMORN5 and LmaMORN6-GFP chimera protein localization overlaps with that of Bilbo1

TbMORN1 localizes at the bilobe structure and partially overlaps with Bilbo1 in *T. brucei* (Morriswood et al., 2009). I considered whether LmaMORN5 and LmaMORN6 should also overlap with Bilbo1. This was investigated by immunofluorescence of detergent-extracted transfectants with poly clonal anti-Bilbo1. GPF-only cells were co-stained with tubulin to highlight cytoskeleton and Bilbo1 was shown to stain the flagellum but more strongly at the neck/ junction were the flagellum leaves the cell body and also on the basal body (Figure 3.9A). LmaMORN5-GFP and LmaMORN6-GFP detergent-extracted cells displayed visible localization at the base of flagellum partially overlapping Bilbo1 staining (Figure 3.9B-D). This further supports the notion that LmaMORN5-GFP and LmaMORN6-GFP localization at the base of the flagellum targets the bilobe.

3.11 LmaMORN5-GFP secreted into cytoplasm of infected macrophages

To confirm the possibility of LmaMORN5-GFP secretion as annotated in the bioinformatics, concentrated supernatant of stationary phase LmaMORN5-GFP and GFP-only transfectants were screened by immunoblotting. However no band was detected in the supernatants of LmaMORN5-transfected cells (Figure 3.10A, lane 4). This showed that LmaMORN5-GFP was not readily detectable in the supernatant of promastigote cells. Macrophages infected with GFP only, LmaMORN5 and LmaMORN6 transfected promastigotes showed some interesting results. All cell lines were able to invade and establish infection in macrophages. The GFP fluorescence of GFP control parasites and LmaMORN6-GFP chimera were mainly confined within the parasitophorous vacuoles of infected macrophages (Figure 3.10B-C). Whereas LmaMORN5-GFP chimera not

only stained the parasitiphorous vacuole but also seems to have diffused and spread into the cytoplasm (Figure 3.10D). This corroborates the secretory annotation of LmaMORN5 protein and also indicates that its secretion seems to the induced by the invasion process or by amastigotes.
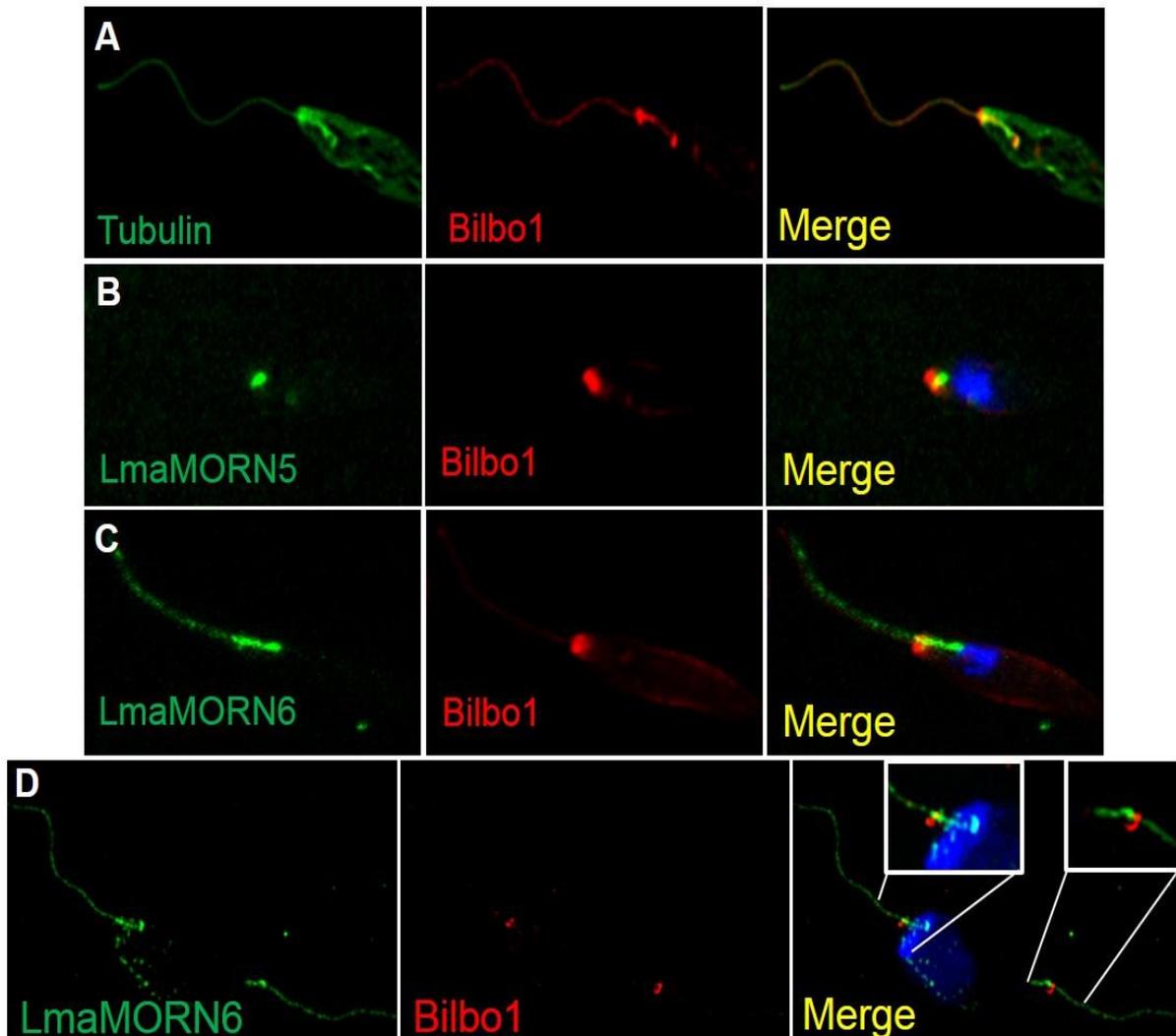
Figure 3.9. LmaMORN5-GFP and LmaMORN6-GFP bilobe localisation slightly overlaps with Bilbo1. Detergent-extracted cells costained with poly Bilbo1. (A) GFP only cells costained with Tubulin shows Bilbo1 staining of flagellar collar and basal body. (B) LmaMORN5 Bilobe localization partially overlap with Bilbo1. (C-D) are LmaMORN6 also showing overlap with Bilbo1.
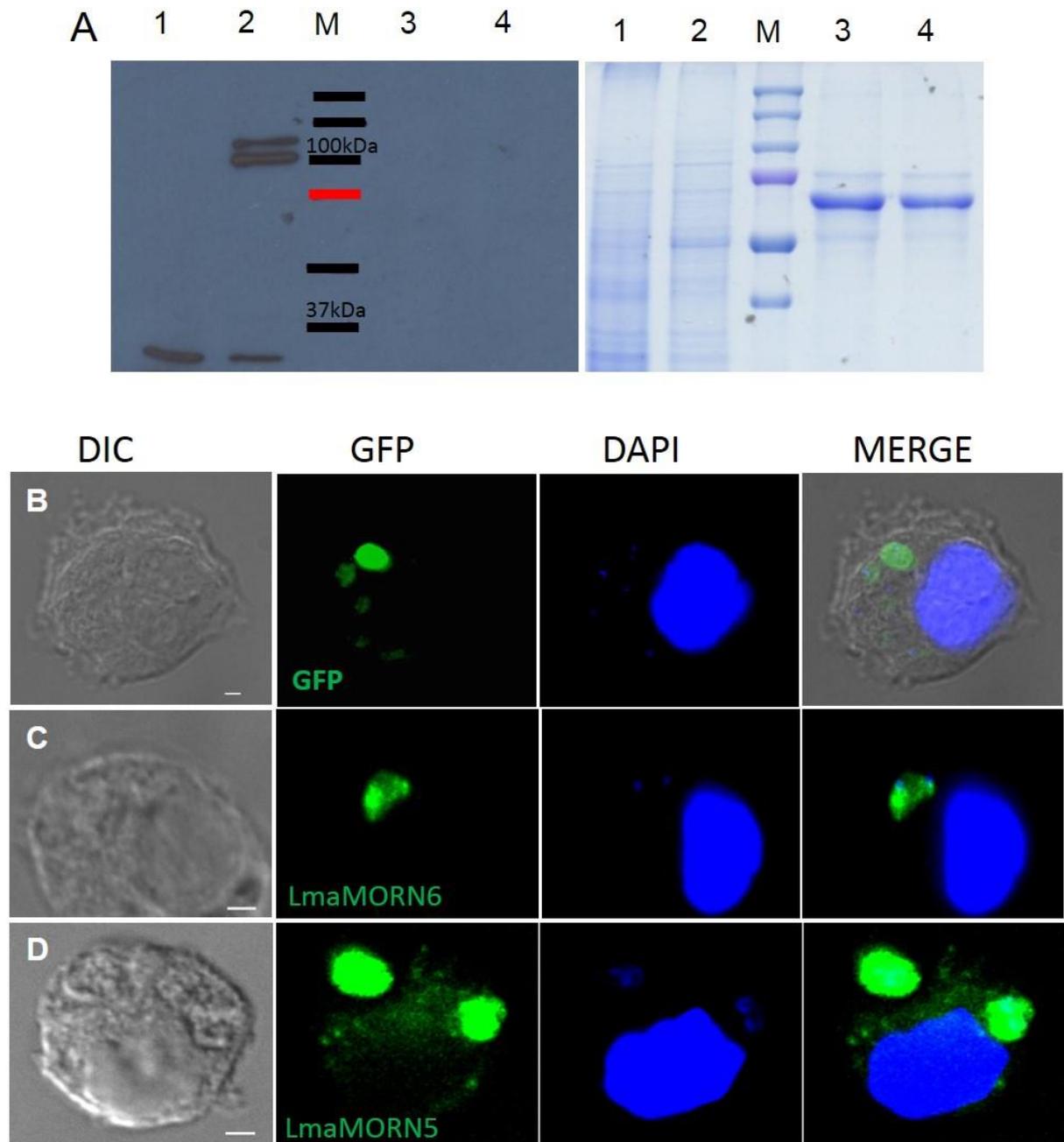
Figure 3.10. LmaMORN5-GFP detected in the cytoplasm of infected macrophage. (A) Lane 1 and 2 were whole cell lysate of GFP and LmaMORN5-GFP mutants respectively while lanes 3 and 4 are their corresponding supernatants after incubation in supplement free RPMI for 5 h. Lane1 showed GFP band at 27kDa and lane 2 a band of 107kDa for MORN5-GFP but no band was detected in lane3 and 4. The corresponding coomassie stain was to show loading. (B) GFP promastigote mutants infected macrophages and the GFP protein stained the parasitophorous vacuole. (C) LmaMORN6-GFP protein also localizes within the vacuole. (D) LmaMORN5-GFP chimera protein predominantly localizes within the vacuole, but also diffused into the cytosol.

3.12 LmaMORN5 mutants showed the highest rate of infection

The secretion of LmaMORN5 into the cytosol of infected macrophages indicates a role in the parasites infection process (Figure 3.10D). In order to investigate further, macrophages were infected with GFP, LmaMORN5, LmaMORN6 transfected promastigotes and *L. major* parental cell line. Internalised parasites were counted in 100 macrophages for each parasite line. The results show that GFP and parental parasites were similar in terms of a parasite:macrophage ratio of 1:10, LmaMORN6 at 2:10 while LmaMORN5 show a staggering 6:10. This indicates that the overexpression of LmaMORN5 might have increased the parasite's infectivity by 6-fold (Figure 3.11). Therefore LmaMORN5 may play a role in the parasite's infection process.
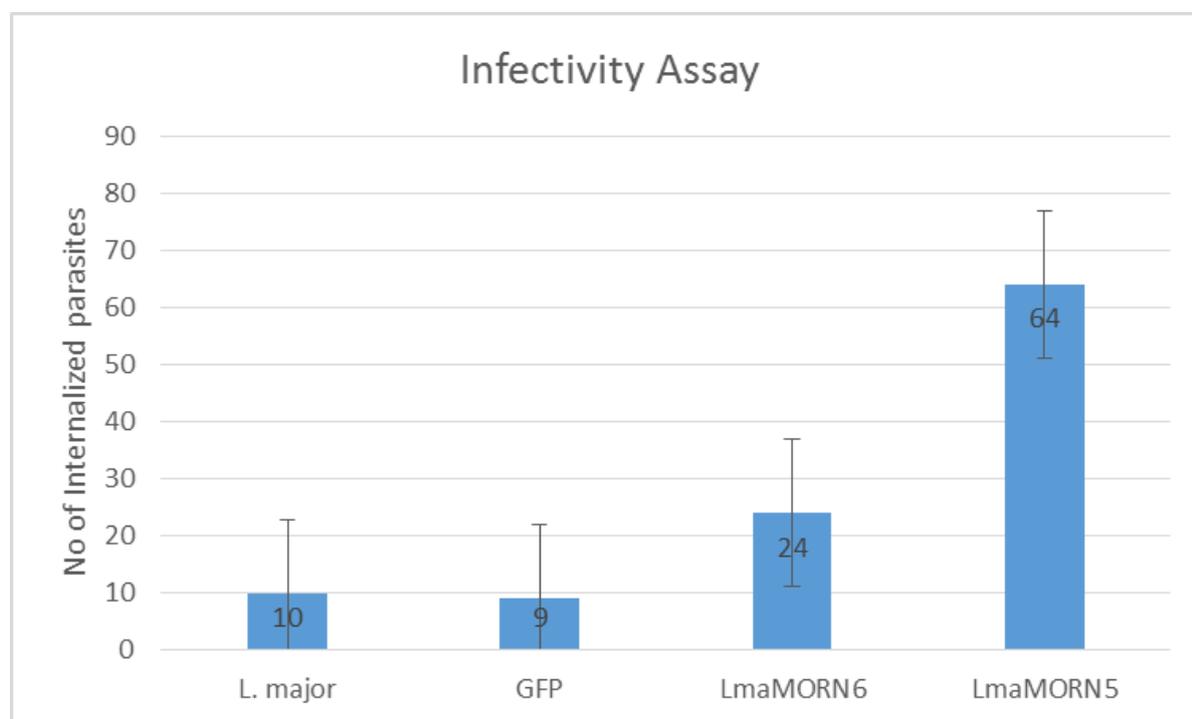
Figure 3.11. LmaMORN5 increases the parasites infectivity by 6 folds. The Infection rate was calculated by counting internalised parasites in 100 Raw 264.7 cells. LmaMORN5 mutants shows the highest amount of internalized parasites, 3x LmaMORN6 and 6 times that of control cells.

4 Discussion

4.1 Bioinformatics

Proteins containing MORN-repeats are present and conserved across the tree of life, implying that MORN proteins play an important functional role from a biological perspective in a wide range of organisms. The research studies to date have reported a high degree of variability in the role MORNs play in various organisms. Most studies of the MORN-repeats within the trypanosomatids have been in *T. brucei* and have mainly concerned themselves with MORN1 protein. Here I report for the first time proteins containing MORN-repeats in *Leishmania major*, a protozoan parasite of significant public health importance. A total of 22 proteins were annotated as containing MORN-repeats in *L. major,* comprising 1.4% of its genome (Table1), yet none have been characterised so far. These uncharacterized LmaMORN proteins thus present an opportunity to dissect the cell biology of the *Leishmania* parasite and also to further investigate the function and evolution of MORN proteins in this genus.

Seven of the LmaMORN proteins identified contain other interesting functional protein domains, two of which (LmaMORN5 and LmaMORN6) were chosen for further investigation. The two proteins are similar in carrying MORN-repeats but their varied extra functional domains indicate a different role for each. LmaMORN5 may be involved in cell signalling due to its predicted secretion and the presence of a kinase domain. This kinase domain was annotated by the SMART database as a serine/threonine kinase and more specifically putatively classified as STE11 (Parsons et al., 2005). STE group proteins are serine threonine

eukaryotic protein kinases that are especially numerous in the trypanosomatids, involved in the Mitogen-activated protein kinase (MAPK) signaling cascade. MAPK transduce external stimuli and other signals to the nucleus thereby regulating cell functions including proliferation, gene expression, differentiation, mitosis, cell survival, and apoptosis (Pearson et al., 2001). The STE are most studied in yeast, where they regulate the cell cycle and mating behaviour in response to pheromone stimulation. The mating MAPK pathway consists of three steps; MAPKKK phophorylates its substrate MAPKK, which in turn will phosphorylate the MAPK substrates. The STE11 family proteins often function as MAPKKK in the MAPK cascade. It was not possible to predict a complete MAP kinase signalling pathway in trypanosomatids (Naula et al., 2005). However, 21 STE11 proteins have been predicted to be encoded in the *L. major* genome (Parsons et al., 2005). 10 MAPK (LmxMPK–LmxMPK9) and 1 MAPKK (LmxMKK) proteins have been identified and partially characterised in *Leishmania mexicana* (Bengs et al., 2005, Wiese et al., 2003a, Wiese et al., 2003b, Wiese and Gorcke, 2001, Wiese, 1998). LmaMORN5 is therefore the first leishmanial STE11/MAPKKK characterized and also the only protein kinase annotated to contain MORN-repeats in *Leishmania*. A kinase specificity experiment will be required to confirm the predicted classification of LmaMORN5 as STE11.

In contrast, LmaMORN6 contains a putative N-terminus kinesin motor domain, implying anterograde transport along microtubules. The structure of LmaMORN6 is similar to proteins belonging to the kinesin-3 family, which are N-terminal kinesin motor proteins with domains like FHA or PH at the tail region. One study has shown that a kinesin-3 (UNC 104) binds to its cargo through its pleckstrin homology (PH)-domain in the tail region, which interacts specifically

with PI(4,5)P$_2$ (Klopfenstein et al., 2002). Similarly MORN domains have been shown to predominantly bind to Diacylglycerol (DAG) and PA but also relatively slightly to PI, PI4P and PI(4,5)P2 (Habicht et al., 2015, Ma et al., 2006). This varied affinity for different lipids may imply deferential multiple binding targets. Therefore, the MORN domain in such protein combinations may act as a means of directing localization/interaction.

Further investigation showed that although MORN5 genes were conserved across kinetoplastids, LmaMORN5 contains two local regions (1 and 2), which appear to be under selective pressure. Region 1 spans the signal peptide, thus indicating a potentially important extracellular role with a possible function in host-parasite interactions. This reinforces the impression that gain of function by a dramatic change in compartmental localization may have occurred in response to selective pressure like the adoption of an intracellular lifestyle. Region 2 spans across a low-complexity region (LCR), which have been reported to show significant divergence across protein families. LCR-containing proteins tend to have more binding partners across different protein-protein interaction networks than proteins that have no LCRs (Coletta et al., 2010). In contrast, LmaMORN6 showed more conservation, with no specific regions under selective pressure identified. This detailed analysis of selective pressure, with LmaMORN5 appearing to be under positive selective pressure and LmaMORN6 appearing to be under stabilizing selective pressure, could be an indication of a role in virulence for the former, and a more standard housekeeping role for the latter.

4.2 Expression and Growth of transfectants

GFP-tagged fusion proteins were stably expressed in *L. major*. A western blot analysis of total cell lysates confirmed the expression of LmaMORN5 and LmaMORN6 chimera proteins. The molecular size detected for LmaMORN5 was bigger than predicted, which indicates a possible posttranslational modification consistent with its secretory annotation. A combination of sixty N-linked and O-linked glycosylation were predicted on LmaMORN5 sequence (Hamby and Hirst, 2008). This is similar to the heavily secreted phosphatase of *L. Mexicana* which was shown to contain both N- and O-linked glycosylation (Ilg et al., 1994). Protein glycosylation has crucial roles in most physiological processes and diseases, including cell signaling, cellular differentiation and adhesion. Further experiments are required to confirm the presence and type of glycosylation for LmaMORN5.

Growth rate assays showed that promastigote cells transexpressing LmaMORN5 and LmaMORN6 chimeras were slower in growth than control cells. This is consistent with previous findings where disrupting the endogenous levels by silencing of the TbMORN1 protein caused a slow growth phenotype in procylic *T. brucei* and was lethal in bloodstream forms (Morriswood et al., 2009). The authors also observed a slight increase in 2K2N cells, which indicates delayed cytokinesis. Another experiment in *T. gondii* showed that MORN1 null mutants failed to assemble a basal complex, which resulted in inefficient constriction and failed division of the apicoplast, thereby supporting a critical role for MORN1 in mitosis and cytokinesis of *T. gondii* (Lorestani et al., 2010). Thus, the MORN domain may have a role in mitosis and cytokinesis, possibly in association with the microtubules or means of binding to the organelles involved in division.

4.3 Subcellular localization of LmaMORN5 and LmaMORN6

Immunofluorescence data of whole-cell transfectants showed chimeric protein accumulation/localization at the base of the flagellum, proximal to the kinetoplast and also along the flagellum in the case of LmaMORN6.

The localization at the base of the flagellum correlates with the position and shape of the bilobe structure. The bilobe, redefined as hairpin-shaped, is present at the top of the flagellar pocket and extends toward the anterior of the cell (Esson et al., 2012, Gheiratmand et al., 2013). It co-purifies with the trypanosome flagellum, implying intimate association (Morriswood et al., 2009). Four proteins, Tbcentrin2, Tbcentrin4, TbLRRP1 and TbMORN1 were reported to stably localize to the bilobe, however, only TbMORN1 and TbLRRP1 were exclusive to it. Here too the bilobe along with LmaMORN5 and LmaMORN6 chimera localization remained intact with the flagellum post detergent extraction (Figure 3.7B-D), indicating close association. Furthermore, the posterior part of TbMORN1 was also found to partially overlap with Bilbo1 in *T. brucei* (Morriswood et al., 2009). Bilbo1 is a protein component of the flagellar pocket collar. Here for the first time, the presence of Bilbo1 in *L. major* was reported and was also found to partially overlap with the localization of LmaMORN5 and LmaMORN6 chimeras at the base of the flagellum (Figure 3.9). This further supports the idea that LmaMORN5 and LmaMORN6 localization at the base of the flagellum occurred at the bilobe. Therefore we report here for the first time two other MORN proteins (LmaMORN5 and LmaMORN6) that also target and localize to the bilobe but unlike TbMORN1 are not exclusive to it.

Specific silencing of bilobe components in procyclic trypanosomes have diverse effects including impaired Golgi duplication, nuclear replication, and cytokinesis and FAZ biogenesis (Zhou et al., 2010, Shi et al., 2008, He et al., 2005). This suggests a critical role in cytoskeletal organization and cell division for the bilobe. A polo-like kinase that also transiently localized to the bilobe was shown to regulate the biogenesis of the bilobe itself, probably by phosphorylating the centrin2 (de Graffenried et al., 2008). The bilobe may therefore interact with a range of effector molecules important for its various roles, and MORN domains may be responsible for targeting such molecules to it. For example, LmaMORN5 which also carries a kinase domain, may similarly be involved in phosphorylating components of the bilobe, whereas LmaMORN6, with its kinesin motor, could be involved in intraflagellar transport and/or cytokinesis.

The localization proximal to the kinetoplast correlates with the structural position of the basal body in *L. major,* which were confirmed by its co-localization through YL1/2 staining (Figure 3.8B-C). The basal body is the main MTOC in kinetoplasts and with a co-ordinating role for duplication and segregation of organelles. A study in *T. gondii* showed that overexpression of MORN1 resulted in severe but specific defects in nuclear segregation and daughter cell formation (Gubbels et al., 2006). TgMORN1 was shown to be localized at the basal complex and was required for basal complex assembly in *Toxoplasma gondii* (Heaslip et al., 2010, Lorestani et al., 2010). These observations are consistent with LmaMORN5 and LmaMORN6 overexpression at the basal body partially disrupting function, resulting in the slow growth phenotype observed in figure 3.5.

The basal body is the nucleation site for the new flagellum and a base for flagellum growth and therefore may suggest that the MORN chimera proteins are trafficked initially to the bilobe and basal body before being sorted for additional destinations via secondary targeting motifs (N'terminal signal peptide) or functional domains (kinesin motor). Similarly, IFT particles were shown to accumulate around the basal bodies in *Chlamydomonas,* which may further suggest that the basal bodies act as a gate that facilitates these materials into the flagellum (Deane et al., 2001).

LmaMORN6 alone significantly localizes along the flagellum and occasionally accumulates at the tip, which may be attributed to the presence of the kinesin domain. This further strengthens the putative involvement of LmaMORN6 in flagella-related functions. The flagellum, being a highly complex organelle, is essential for motility, has a role in cell morphogenesis, and is crucial for cell polarity and division. Furthermore, the flagellum is supported by a variety of molecular motor proteins through highly active intraflagellar transport (IFT). IFT is a motility in which particles move underneath the flagellar membrane (Kozminski et al., 1993). Anterograde (outward) and retrograde (inward) movements of these IFT particles are mediated by kinesin and dynein respectively (Kozminski et al., 1995, Pazour et al., 1998). LmaMORN6, though not characterized into any kinesin family yet, is more likely to be a flagella-associated kinesin due to its localization. The occasional accumulation of LmaMORN6 chimera protein at the tip of the flagella is reminiscent of kinesin-13, which was proposed to localise at the distal tip of the flagella axoneme, where it may depolymerise axonemal MTs and co-operate with the IFT machinery to control the length of the flagellum (Dawson et al., 2007, Blaineau

et al., 2007). It may be that this α-GFP accumulation phenomena may be due to the localization of LmaMORN6 chimera at an expanded flagellar tip, a common characteristic of the heptomonad promastigotes. There is evidence that promastigotes undergo several major developmental forms including heptomonad promastigotes (Gossage et al., 2003). I therefore suggest that LmaMORN6 may be a flagellar protein and may have a role in flagellar transport.

4.4 LmaMORN5 and LmaMORN6 chimera association with detergent resistant structures

LmaMORN5-GFP and LmaMORN6-GFP chimera proteins were found to remain in the detergent insoluble pellet/cytoskeleton post 4°C and 37°C triton X-100 extraction (Figure 3.7). These results were evidence that both LmaMORN5 and LmaMORN6 were strongly associated with the parasite's cytoskeleton, implying some structural role. Detergent extractions of MORN proteins from previous studies have shown that MORN proteins are associated with the cytoskeleton and also membrane organelles (Gubbels et al., 2006, Ferguson et al., 2008, Morriswood et al., 2009). *Leishmania* detergent resistant membranes (DRMs) are defined as being enriched with sphingolipids, sterols and GPI-anchored molecules. GPI proteins such as glycoinositol phospholipids (GIPLs) have been implicated in the viability of the amastigote stage of the *Leishmania* life cycle (Ilgoutz et al., 1999). It is interesting to note that like GIPL, LmaMORN5 was predicted to be glycosylated and shown to be secreted by amastigotes/during infection and that the MORN domain it carries has been shown to interact directly with phospholipids to facilitate membrane association. Taken together these properties may imply raft association. Unfortunately, it was not possible

to show whether these proteins reside in the DRMs of LmaMORN5 and lmaMORN6 transfected cells, in part due to their high rate of degradation at 37°C detergent extraction. The role of MORN domains in combination with functional domains like kinase and kinesin might be to facilitate binding to the cytoskeleton and/or membrane organelles to effect responses.

4.5 LmaMORN5 is secreted into the cytoplasm of host cells and significantly increases parasite infectivity in macrophages

LmaMORN5 was not detected in the supernatant of late phase promastigotes (Figure 3.10A). This may suggest that it is only secreted by the amastigote stage or during the infection process. To test for this, transfectants were used to infect macrophages and then analysed by microscopy. Firstly, all *Leishmania* lines successfully invaded and established infections in macrophages, with the GFP/α-GFP staining the parasitophorus vacuole (Figure 3.10). Unlike the other proteins studied, the LmaMORN5-GFP chimera protein was not confined to the parasitophorus vacuole, but was found to diffuse into the cytoplasm as well (Figure 3.10D). This result confirmed the secretory annotation of LmaMORN5 and also suggested that its secretion may be triggered by the invasion process, or that it is released by amastigotes. It has been reported that *L. major* promastigotes release both constitutive and inducible protein kinases. The inducible protein kinase (CK2) were only secreted by incubating parasites with their substrates (Casein) (Sacerdoti-Sierra and Jaffe, 1997). LmaMORN5 secretion was only observed inside the macrophage, implying that it may be an induced protein kinase and may have a role in host-parasite interactions.

Further experiments are necessary to identify substrates and a possible role in the complex kinase signalling pathway.

The secretory nature, kinase domain and selective pressure of LmaMORN5 collectively indicate a very important role for this protein and its potential function as a virulence factor in the parasite infection process. Comparative study of the infectivity of the transfectants showed LmaMORN5-expressing cells to have the highest number of internalized or amastigote cells, equalling six times that of the controls (Figure 3.11). A similar secreted protein kinase (CK2) that phosphorylates casein was reported to also increase the parasite's infectivity in *L. braziliensis* (Zylbersztejn et al., 2015). *L. donovani* parasites overexpressing LdCK1.4 (a protein kinase) caused early differentiation into the metacyclic phase and gave significantly higher infections of mouse peritoneal macrophages compared to parental cell lines (Dan-Goor et al., 2013). Therefore, overexpression of LmaMORN5 may play a vital role in speeding up metacylogenesis and/or parasite survival inside the vertebrate host cells. Phosphorylation of host serum or host cell proteins may be involved in the regulation of leishmanicidal processes. It will be important to compare the number of metacylics between the transfectants in order to establish if LmaMORN5 has any role in metacyclogenesis. These results also further support the suggestion that LmaMORN5 may be a virulence factor.

Knockout experimentation may be crucial towards further characterizing and confirming the role of LmaMORN5. If cells are not viable then analysing the RNAi of its homolog in *T. brucei* will be an important experimental step towards understanding and identifying the true nature and function of this protein in the host-parasite interactions of leishmaniasis.

In general, I suggest that the remaining 20 LmaMORNs should be expressed in *L. major* and their localization determined. Further functional experiments involving truncations, domain deletions and mutagenesis will be crucial in establishing the role of MORN-repeats in *Leishmania*.

5 Abbreviations

BSA	bovine serum albumin
CIP	calf intestinal phosphate
DAPI	4', 6- diamidino-2-phenylindole
DMEM	dulbecco's minimum essential medium
DRM	detergent rich microdomain
E.	escherichia
FCS	foetal calf serum
FC	flagellar pocket
GFP	green fluorescent protein
GPI	glycophosphoinositol
H	hour
HRP	horse radish peroxide
IFT	intra-flagellar transport
IP3	inositol triphosphate
kDa	kilodalton
L	lysate
LmaMORN	*L. major* MORN
min	minute(s)
MTOC	microtubule organising centre
MT	microtubule
MORN	membrane orientation and recognition nexus
ORF	open reading frame
P	pellet
PBS	phophate buffered saline
PCR	polymerase chain reaction
PFA	paraformaldehyde
PFR	paraflagellar rod
PLK	polo-like kinase
PIPK	phosphatidylinositolphosphate kinase
RT	room temperature
RNAi	RNA interference
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
S	supernatant
T.	trypanosoma
TBST	tris buffered saline tween-20
W	wash
WHO	the world health organisation

6 List of Figures

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8 References

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Appendix

Publication and Communication

Paper

Jobe, Momodou, Anwuzia-Iwegbu, Charles, Banful, Ama, Bosier, Emma, Iqbal, Mubeen, Jones, Kelly, Lecutier, Suzanne J, Lepper, Kasimir, Redmond, Matt, Ross-Parker, Andrew, Ward, Emily, Wernham, Paul, Whidden, Eleanor M, Tyler, Kevin M, & Steverding, Dietmar. (2012). Differential in vitro activity of the DNA topoisomerase inhibitor idarubicin against Trypanosoma rangeli and Trypanosoma cruzi. *Memórias do Instituto Oswaldo Cruz, 107*(7), 946-950.

Abstract

British Society for Parasitology meeting. March 2010. Cardiff UK. Membrane orientation and recognition nexus proteins of *Leishmania*. Momodou Jobe and Kevin Tyler. Poster *presentation*