The role of the Sec1/Munc18 protein, Vps45, in *Leishmania mexicana*

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Abstract

Autophagy is an important process that allows the degradation and recycling of the cytoplasm, proteins and damaged organelles to occur as a survival and remodelling mechanism. This process occurs through membrane fusion driven by SNARE protein complex formation tightly regulated by SM proteins. The SM protein Vps45 has been shown to have a role in regulating SNARE interactions and vesicle trafficking from the trans-Golgi network (TGN) into the endosomal pathway in the yeast *S. cerevisiae.* The absence of Vps45 has shown to cause yeast cells have a longer doubling time, temperature sensitivity and accumulation of vesicle clusters near the vacuole not found in wild type cells. The absence of Vps45 also causes a significant reduction in Tlg2, the SNARE proteins Vps45 interacts with known to be essential for the regulation of autophagy.

The life cycle of *Leishmania* involves both the sand fly and the mammalian host. Due to this extreme change in environments, *Leishmania* parasites need to be capable of adapting to the changing conditions in order to survive. Current research carried out in *Leishmania* on autophagy has been shown to be initiated in two conditions; during cellular differentiation and starvation conditions. The main aim of this project is to understand the role of Vps45 in *Leishmania* and examine whether its functions and interactions are conserved as they are in yeast.

The results of this study have shown that there are two Vps45 copies in *Leishmania*. The deletion of Vps45 has shown to cause a small change in the growth of *Leishmania*, however, interestingly, the deletion of one Vps45 copy and the presence of a heterozygote copy of the second copy of Vps45 showed to have a significant reduction in the growth of *Leishmania*. Due to the difficulty of working with *Leishmania*, endogenous tagging and over-expression of Vps45 were not achieved.

The potential application of this project involves understanding the interaction of *Leishmania* with its host as well as understanding its survival mechanisms through autophagy. As these parasites are capable of withstanding and surviving within the harsh environment of the host, investigating these important pathways may be highly beneficial in the production of improved diagnostics methods as well as treatments.

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Authors declaration

Other than the LmxM.36.2230 knockout cell line, primers for endogenous tagging of LmxM.36.2230, the construction of the pSSU plasmid all carried out by Kirstin Spence, the use of JM6571 Leishmania cells from Jeremy Mottram group, I declare that this thesis is a presentation of original work and I am the sole author, unless otherwise stated. This work has not previously been presented for an award at this, or any other, University. All sources are acknowledged as References.

1. Introduction

1.1 Introduction to leishmaniasis

1.1.1 Leishmaniasis

Leishmaniasis is a vector-borne neglected infectious disease predominantly affecting communities in deprived, tropical and sub-tropical regions worldwide (Steverding 2017). According to the World Health Organisation (WHO), infection with leishmaniasis currently threatens 350 million people in 98 countries and causes approximately 70,000 deaths per year (Stockdale and Newton 2013) (Torres-Guerrero et al. 2017).

There are three distinct forms of leishmaniasis, caused by different species of *Leishmania*, with the most common form known as cutaneous leishmaniasis (CL) (differentiated by localised, diffuse and disseminated) as well as mucocutaneous leishmaniasis (MCL) and visceral leishmaniasis (VL) also known as kala-azar (Reithinger et al. 2007) (Aronson et al. 2017a). The form of leishmaniasis acquired is also dependent on the type of phagocytic cells that have been invaded as well as the species of *Leishmania* (Steverding 2017). *Leishmania* parasites in CL and MCL mainly infect macrophages within the skin, causing symptoms such as local swelling which can turn into pustules and ulcers (Torres-Guerrero et al. 2017). VL on the other hand, mainly infects macrophagic cells within the blood of the liver and spleen causing symptoms such as hepatomegaly, anaemia and thrombocytopenia (Alves et al. 2018) (Steverding, 2017). VL is known to be the most life-threatening form and can be fatal in 95% of cases unless treated (Steverding, 2017).

The diagnosis of *Leishmania* involves the detection of parasites from tissue samples in CL (skin lesions) using light microscopy (Goto and Lindoso 2010). The most common method of detecting VL is through the detection of parasites from the bone marrow. DNA and antibody assays can also be used to diagnose leishmaniases (Sundar and Rai 2002). Diagnosing leishmaniasis can be difficult and it is important to identify the specific *Leishmania* species in order to provide the right treatment.

There are several drugs available to treat leishmaniasis, many of which are toxic and have dangerous side effects. The most common drug of choice are the pentavalent antimony compounds (methylglucamine antimoniate and sodium stibogluconate) taken by intramuscular or intravenous injections (Chakravarty and Sundar 2019) (Aronson et al. 2017b). The mechanism of action in not well understood but the drugs are known to target amastigotes by inhibiting the glycolytic and oxidative pathways (Chakravarty and Sundar 2019). Although the drugs can be effective, they can cause high toxicity to the heart, kidneys and liver (Sundar and Rai 2002) (Haldar, Sen, and Roy 2011). Drugs such as pentamidine and amphotericin are also used, although resistance to those by *Leishmania* is largely emerging (Basselin et al. 2002). Unlike these drugs, miltefosine, the only oral drug available known to treat different types of *Leishmania* is also available (Dorlo, Balasegaram, Beijnen, et al. 2012). It was first developed as an anticancer drug but studies have shown its efficiency in treating leishmaniasis (Sunyoto, Potet, and Boelaert 2018). Although treatment using miltefosine has been successful, its known for its high reproductive toxicity in women (Dorlo, Balasegaram,

Lima, et al. 2012). Therefore, investigating *Leishmania* is highly important in producing new and improved treatment options.

1.1.2 Classification and Taxonomy

Leishmania parasites are flagellated, hereoxenous, intracellular protozoans of the genus *Leishmania* in the family Trypanosomatidae, characterised by the presence of circular DNA organelles known as kinetoplasts, unique to Kinetoplastida parasites (Table 1) (Akhoundi et al. 2016) (Torres-Guerrero et al. 2017). Female sand flies within the Phlebotomus (old-world localised in Europe, Africa and Asia) and Lutzomyia (new world localised in Amercias and Oceania) genera are the main vectors responsible for the transmission through a blood bite (Georgiadou et al., 2015) (Steverding 2017) (Stockdale and Newton 2013). According to Steverding (2017), there are more than 98 species of sand flies known to transmit *Leishmania*, 30 of which are known to transmit leishmaniasis among humans (Georgiadou, Makaritsis and Dalekos, 2015).

Table 1: Taxonomy of *Leishmania* (Akhoundi et al., 2016).

Taxonomy of *Leishmania*	
Kingdom	Protista
Phylum	Euglenozoa
Class	Kinetoplastea
Sub-class	Metakinetoplastina
Order	Trypanosomatida
Family	Trypanosomatidae
Sub-family	*Leishmaniinae*
Genus	*Leishmania*

There are currently 53 species of *Leishmania* known, 20 of which are pathogenic to humans (Quiroga et al. 2017). The genus *Leishmania* is divided into three sub-genera depending on the parasite development in the sand fly, they are known as *L. Leishmania*, *L. Viannia* and *L. SauroLeishmania* (Ramírez et al. 2016). The two most pathogenic species to humans are known as *L. infantum* (predominant in The Middle East, Mediterranean, Central Asia and America) and *L. donovani* (predominant in India and East Africa) (Table 2) (Stockdale and Newton 2013) (Ambit et al. 2011).

Table 2: A list of *Leishmania* species, their known sand fly vectors and their geographical (Pace 2014).

Table 1 Disease manifestations and transmission of predominant *Leishmania* species found in the New and Old Worlds.[4–6]

Leishmania (*L*) species	Sandfly vector (Phlebotomus [*P*] or Lutzomyia [*L*]) species	Main affected areas	Reservoir	Disease manifestations
L. aethiopica	*P. longipes* *P. pedifer*	Ethiopia, Kenya	Hyraxes	Cutaneous, diffuse, mucosal
L. amazonensis	*L. flaviscutellata*	East Andes	Rodents	Cutaneous, disseminated
L. braziliensis	*L. ovallesi* *L. wellcomei* *L. neivai* *L. whitmani*	East and West Andes	Rodents, marsupials, dog	Cutaneous, mucosal
L. donovani	*P. argentipes*	India, Bangladesh, Nepal Bhutan	Human	Visceral
	P. martini *P. orientalis*	Sudan, Ethiopia		
L. guyanensis	*L. umbratilis*	East Andes	Arboreal edentate mammals	Cutaneous, mucosal
L. infantum (same as *L. chagasii* in the New World)	*P. ariasi* *P. perniciosus*	Mediterranean region	Dog	Visceral, cutaneous
	L. longipalpis	Latin America		
L. major	*P. duboscqi*	Sub-Saharan Africa	Rodents	Cutaneous
	P. papatasi	North Africa, Middle East Iran, Pakistan, India	Gerbils, Rodents	
L. mexicana	*L. olmeca olmeca*	West Andes	Rodents, marsupials	Cutaneous, diffuse, mucosal
L. panamensis	None proven	West Andes	Arboreal endentate mammals	Cutaneous, mucosal
L. peruviana	None proven	Peru	Rodents, marsupials, dog	Cutaneous, mucosal
L. tropica	*P. sergenti*	North Africa, Middle East, Iran, Afghanistan	Human	Cutaneous
	P. arabicus *P. guggisbergi*	North and sub-Saharan Africa	Hyraxes	

1.1.3 Life cycle of *Leishmania*

The life cycle of *Leishmania* parasites involves two hosts; the sand fly vector and the mammalian reservoir (Dostálová and Volf 2012). Transmission begins when an infected sand fly takes a blood meal and thus injects infective metacyclic promastigotes into the mammalian host. This injury causes the recruitment of immune cells such as neutrophils and macrophages which then take up the parasites (Walker et al. 2013). As neutrophils are short-lived immune cells, macrophages are the final host immune cells which *Leishmania* parasites multiply and differentiate in (Walker et al. 2013).

Upon the interaction of macrophages with parasitic cells, internalisation of the metacyclic promastigotes occurs via phagocytosis causing the formation of a phagosome containing *Leishmania* parasites which differentiate into amastigotes (Figure 1) (Liu and Uzonna 2012) (Gossage, Rogers, and Bates 2003) (Bates 2007). The phagosome fuses with the lysosome and forms into a phagolysosome, where the destruction of pathogens usually occurs through essential molecules (Carneiro et al. 2016) (Novais et al. 2014). However, amastigotes are able to withstand the harsh environment of the phagolysosome due to the initiation of the parasitophorous vacuole membrane surrounding the parasite and begin to replicate via binary fission (Novais et al. 2014). This in turn causes the macrophagic cell to rupture and release the amastigotes which can then further infect other macrophages, or circulate in the blood to be taken up by sand flies during a blood-meal (Figure 1) (Walker et al. 2013).

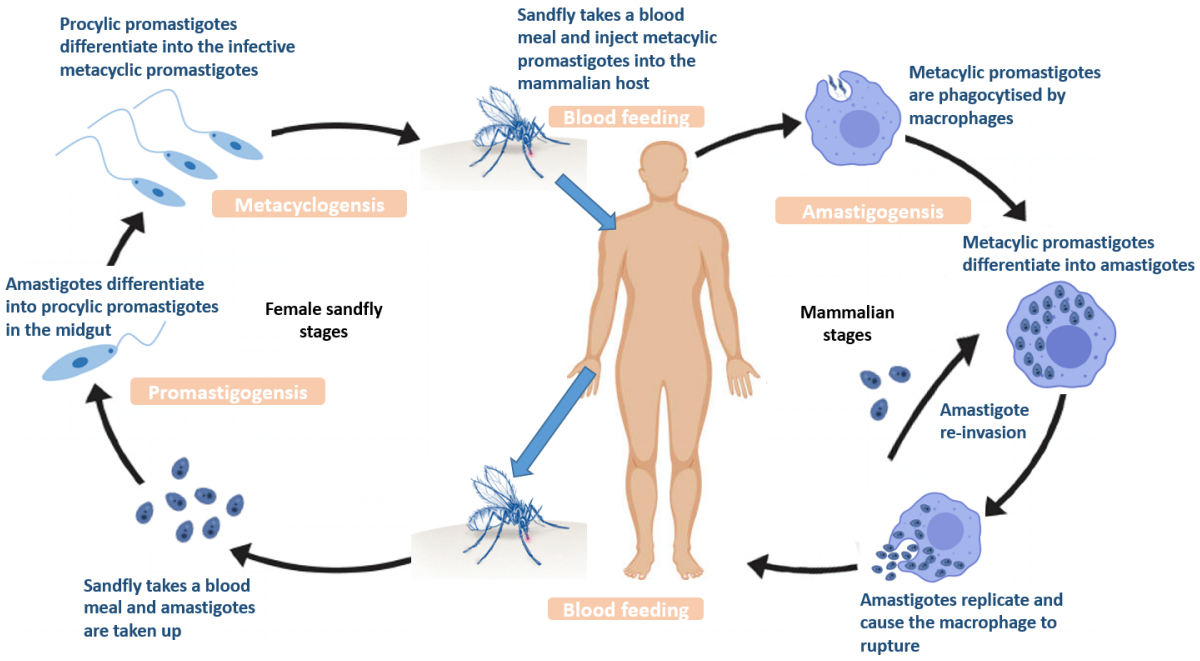

Figure 1: The life cycle of *Leishmania* in the mammalian stage and the female sand fly stage.

Amastigotes taken up by sand flies within the bloodmeal are ingested into the midgut and due to the changing conditions, such as the rise in pH and the reduction in temperature, amastigotes differentiate into procyclic promastigotes which are slightly motile and begin to replicate in the blood within the peritrophic matrix (PM) (Dostálová and Volf 2012)(Bates 2007). Procyclic promastigotes differentiate into a large and slender form known as nectomonad promastigotes that stop replicating and begin to migrate towards the anterior gut, adhering to the gut epithelia (Figure 2) (Sunter and Gull 2017) (Ramalho-Ortigao 2010). These parasites then move to the thoracic midgut and stomodeal valve where they differentiate into a smaller, replicative form known as leptomonad promastigotes (Figure 2) that secrete a substance known as the promastigote secretory gel (PSG) (Inbar et al. 2017). Leptomonad promastigotes that stop division then differentiate into the infective metacyclic promastigotes (Gossage, Rogers, and Bates 2003).

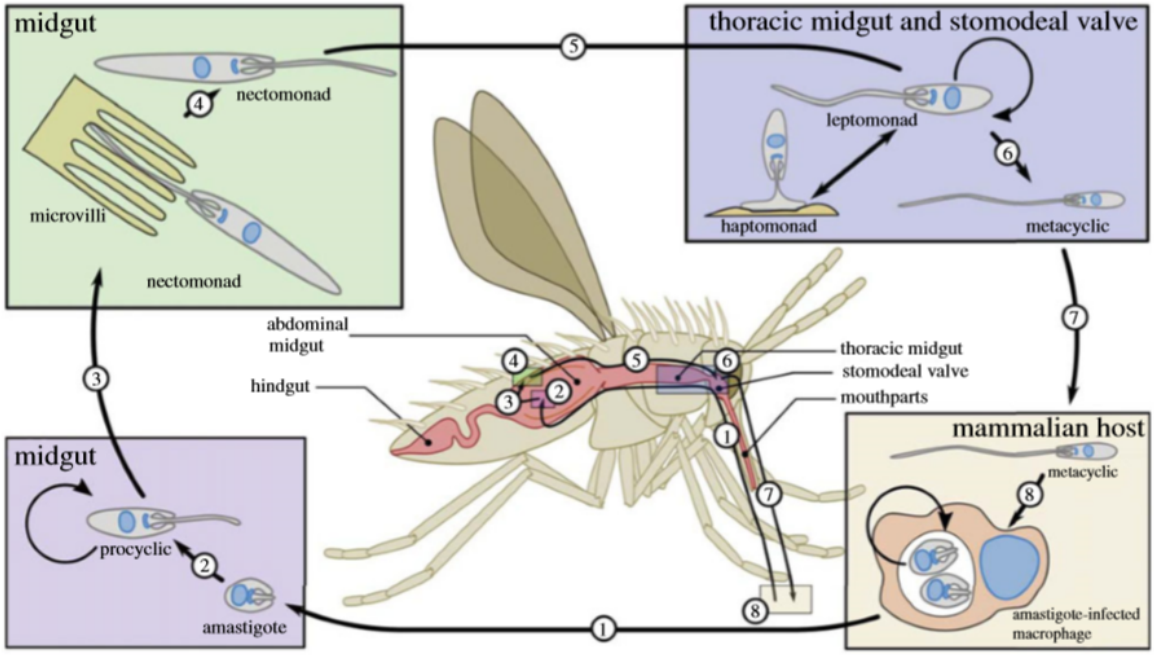

Figure 2: The differentiation of *Leishmania* parasites within the sand fly (Sunter and Gull, 2017).

1.2 Autophagy

1.2.1 Overview

Autophagy is an important process that allows the degradation and recycling of the cytoplasm, proteins and damaged organelles to occur as a survival and remodelling mechanism in eukaryotes (Besteiro et al. 2007). This process occurs through membrane fusion driven by SNARE (soluble N-ethylmaleimide-sensitive fusion protein-attachment protein receptor) protein complex formation, which must be tightly regulated in order to ensure maintenance of the cell (Dulubova et al. 2002) (Archbold et al. 2014). The function of autophagy and its processes and pathways have been clearly understood due to the identification of important autophagy-related genes (ATG) in yeast, allowing the research in other organisms to occur (Lamb, Yoshimori, and Tooze 2013) (Lamb, Dooley, and Tooze 2013).

There are three distinct types of autophagy known as macro-autophagy, micro-autophagy and chaperone-mediated autophagy (Lamb, Dooley, and Tooze 2013). Although the different types of autophagy exist in Leishmania, the most studied type of autophagy is the macro-autophagy form and as a result, macro-autophagy will be referred to as autophagy from hereon.

1.2.2 Macro-autophagy

Macro-autophagy is characterised by the formation of a double-membrane phagophore derived from the endoplasmic reticulum (ER) and the trans-Golgi, although the origin of the phagophore is not well understood (Xie and Klionsky 2007). The process of autophagy begins with ATG proteins, many of which have been identified through a genetic screen carried out in yeast (Tsukada and Ohsumi 1993) (Thumm et al. 1994) (Ghosh and Pattison 2018). More than 30 ATG genes have been identified, that are classified into six important functional groups known to be contributors of autophagosome formation (induction, nucleation, elongation, maturation, fusion and degradation). They are known as Atg1, Atg8, Atg2-Atg18, Atg16-Atg5-Atg12, Atg14 phosphatidylionositol 3kinase (PtdIns3K) and Atg9 (Ghosh and Pattison 2018) (Xie and Klionsky 2007). Interestingly, not only is Atg9 the only transmembrane protein that has been shown to be conserved across the eukaryotes (mammals, yeast and *Leishmania*), but the loss of its function has been shown to stop the formation of autophagosomes (Zhuang et al. 2017) (Xie and Klionsky 2007).

The phagophore begins with the engulfment of parts of the cytoplasm as well as proteins and organelles which can be degraded leading to the formation of a closed double-membrane vesicle known as an autophagosome (Figure 3) (Levine and Klionsky 2004) (Lamb, Dooley, and Tooze 2013). The autophagosome then fuses with a lysosome, causing the degradation of the damaged cargo inhabiting the vesicle (Parzych and Klionsky 2014). This mechanism is an essential pathway for the survival of *Leishmania* parasites within the host as well as the macrophage (Besteiro et al. 2007). As previously mentioned, the life cycle of *Leishmania* involves both the sand fly and the mammalian host. Due to this extreme change in environments, *Leishmania* parasites need to be capable of adapting to the changing conditions in order to survive.

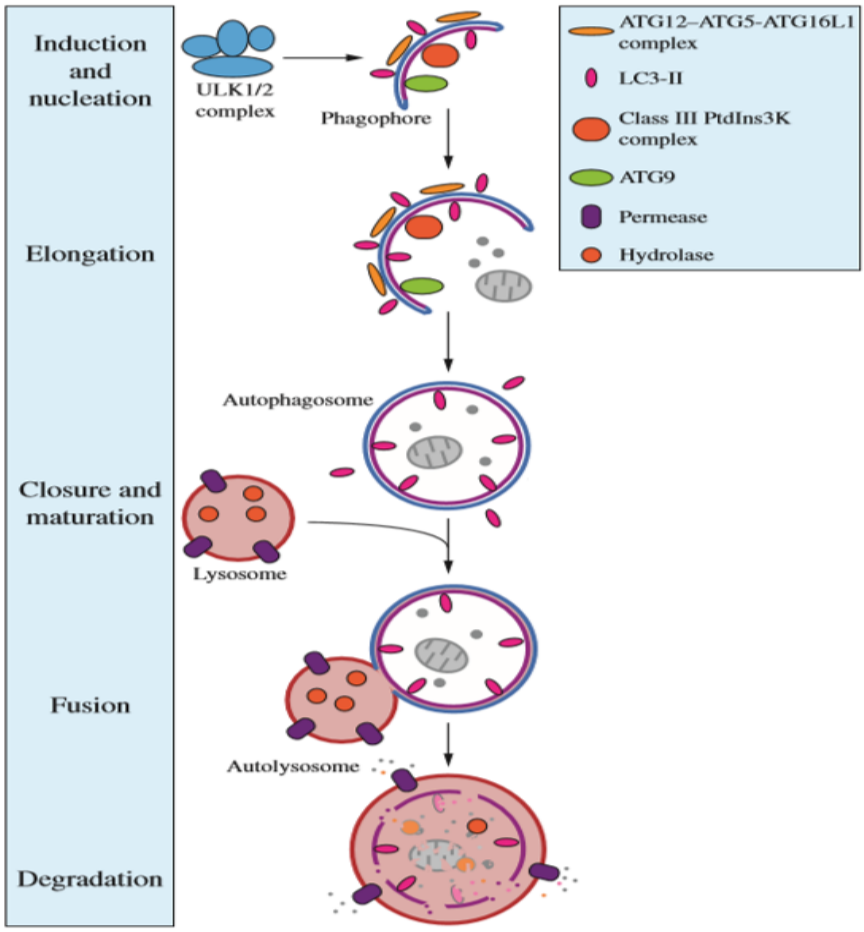

Figure 3: Schematic representation of autophagy. The process begins with the formation of cup-shaped phagophore (Induction and Nucleation) which expands and engulfs parts of the cytoplasm and organelles (Elongation), before forming into a closed double-membrane vesicle (Closure and maturation) and forming into an autophagosome. This vesicle then fuses with a lysosome (Fusion) where the cargo is hydrolysed and broken down (Degradation) (Parzych and Klionsky 2014).

1.2.3 Autophagy in *Leishmania*

Autophagy in *Leishmania* parasites has been shown to be initiated in two conditions; during cellular differentiation and starvation conditions (Dagger et al. 2018) (Besteiro et al. 2007) (Williams et al. 2006) (Gossage, Rogers, and Bates 2003). A study carried out by Inbar et al (2017) in *Leishmania major* (*L. major*) has shown that genes important in the regulation of autophagy are upregulated, particularly during the differentiation of procyclic to metacyclic promastigotes (metacyclogenesis). The ATG protein Atg8, required for the formation of the autophagosome membrane is often used as a marker. Inbar et al (2017) showed that Atg8 is the most upregulated in nectomonad promastigotes before their differentiation to the metacyclic form (Inbar et al. 2017). Metacyclic promastigotes are injected into the host during a blood meal of the fly, upregulation of autophagy may be a way for the parasite to survive within the host due to the changes in conditions (Besteiro et al. 2006). Over-expression of Atg8 in *Leishmania donovani* (infective against humans) has also shown to increase the ability of the parasite to infect macrophages and become resistant to stress, in comparison to the knockout of Atg8 that increased stressed and lowered the ability of the parasite to cause infection, further indicating that the process of autophagy is highly important (Giri and Shaha

2019). Investigating the proteins involved in autophagy further will allow the production of effective drugs that target these pathways.

Further studies carried out on autophagy in *L. mexicana* have shown that the process of differentiation from promastigotes (containing a flagellum) to amastigotes is thought to be through autophagy (Dagger et al. 2018). As shown on Figure 4, the morphology of *Leishmania* cells in the promastigote form and the amastigote form is drastically different. One of the major differences between the vector forms and the mammalian forms is the size of the flagellum (Dostálová and Volf 2012) (Dagger et al. 2018). The flagellum of promastigotes is extremely long and highly motile, in comparison to the short non-motile flagellum hidden within the flagellar pocket in the amastigote form (Wiese, Kuhn, and Grünfelder 2003) (Inbar et al. 2017). This change may be due to the potential roles of the flagellum which includes motility, migration and attachment in the sand fly (Sunter and Gull 2017). As amastigotes reside within the macrophage, reduction of the flagellum is an advantage to the parasite. As well as the flagellum, the cell body of amastigotes is more spherical and smaller in size, in comparison to the larger cell body of the promastigote form (Frank et al. 2015) (Inbar et al. 2017). Dagger et al (2018) has shown that the process of differentiation and size reduction is related to an autophagic-like process, indicating that autophagy is highly important during cellular differentiation (Williams et al. 2006).

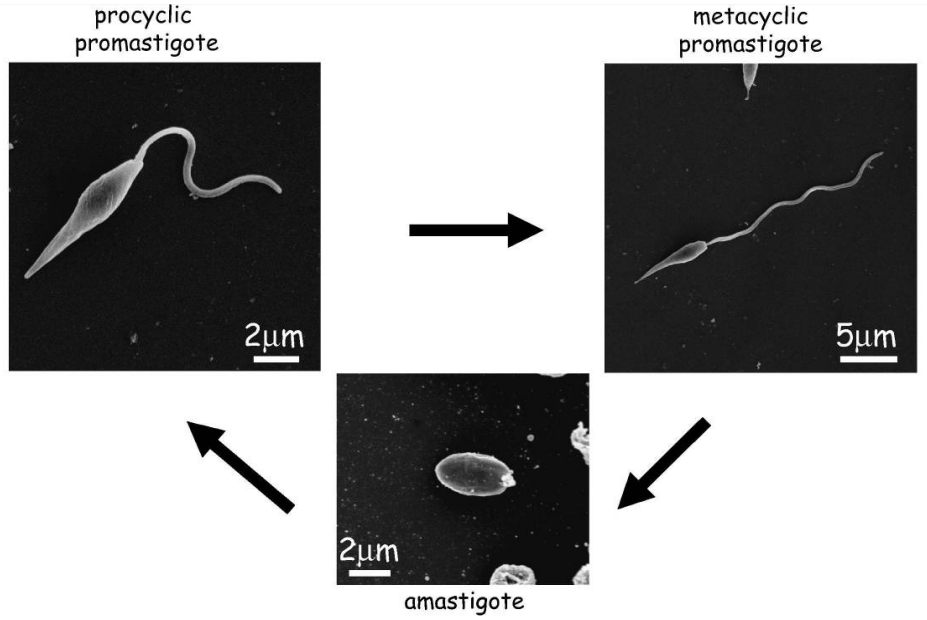

Figure 4: The promastigote (procyclic and metacyclic) form in comparison to the amastigote form (Besteiro et al. 2007).

1.3 Sec1/Munc18 Proteins

1.3.1 SM proteins and SNARE complexes

Vesicle trafficking is an important process that allows important regulations such as autophagy, endocytosis and exocytosis to happen. This process occurs through membrane fusion driven by SNARE protein complex formation, which must be tightly regulated in order to ensure maintenance of the cell (Halachmi and Lev 1996) (Dulubova et al. 2002) (Archbold et al. 2014). The Sec1/Munc18 (SM) protein family regulates intracellular trafficking through high affinity interactions with individual SNARE proteins and SNARE complexes. The proteins were originally identified as essential for membrane trafficking and secretion in yeast, but research has shown that these proteins are highly conserved among different organisms (Bowers and Stevens 2005). There are currently four families of SM protein that have been identified in yeast as Sec1/Munc18, Sly1, Vps33 and Vps45.

1.3.2 Structure of SM proteins

SM proteins are hydrophilic proteins lacking a transmembrane domain indicating their presence in the cytosol (Bryant and James, 2001) (Toonen and Verhage 2003). Due to the conservation of SM proteins present in many organisms, the structural components of SM proteins tend to be very similar being made up of three domains (domain 1, 2 and 3) (Figure 5A), where domain 3 is split into domain 3a and 3b (Archbold et al. 2014). The three domains fold into an arch-shaped structure.

There are two main ways SM proteins are known to bind their specific SNARE partners; a closed syntaxin conformation through the central cleft or through the open syntaxin confirmation by the N-peptide binding site found on domain 1 (Figure 5B) (Eisemann et al. 2020). Studies have shown that SM proteins act as negative regulators of SNARE complexes by holding syntaxins in closed confirmations preventing the formation of complexes and thus the fusion of membranes (N. J. Bryant and James 2001) (Toonen and Verhage 2003). Due to this, it was assumed that increasing the concentration of SM proteins will cause an inhibition of protein fusions (N. J. Bryant and James 2001). However, further studies have shown that in the absence of SM proteins, vesicle transport is blocked (Bryant and James, 2001). This indicates that SM proteins may also have a role in positive regulations by binding to the open conformation of SNARE proteins suggesting a dual role of SM proteins (Toonen and Verhage 2003).

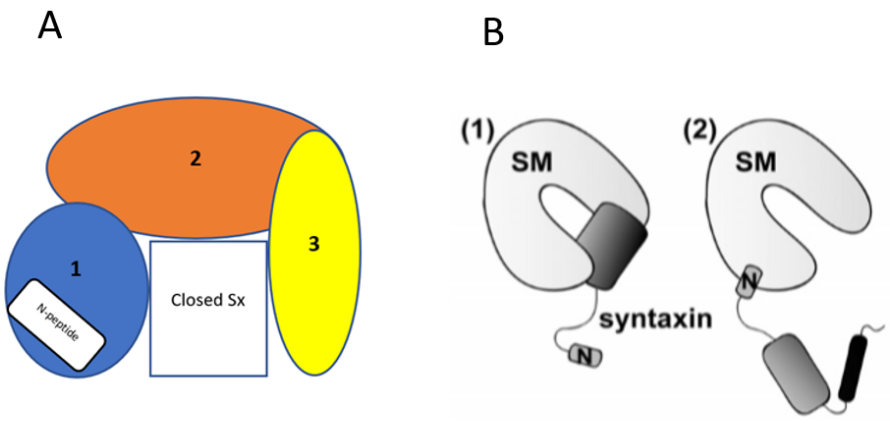

Figure 5: The structure of SM proteins. (A) The three domains of SM proteins as well as the binding site for SNARE proteins. Adapted from Archbold *et al* (2014). (B) Mode 1 and 2 sites at which SNARE proteins bind to SM proteins slightl adapted from Furgason et al (2009).

1.3.3 Vps45

Vps45 has been shown to have a role in regulating SNARE interactions and vesicle trafficking from the trans-Golgi network (TGN) into the endosomal pathway in the yeast *Saccharomyces cerevisiae* (Figure 6) (Carpp et al. 2006). Studies carried out in yeast have shown that Vps45 interacts and regulates the t-SNARE Tlg2 (Nichols, Holthuis, and Pelham 1998)(Abeliovich, Darsow, and Emr 1999) and the v-SNARE Snc2 (Carpp *et al.,* 2006). In the absence of Vps45, yeast cells have a longer doubling time, temperature sensitivity and accumulation of vesicle clusters near the vacuole not found in wild type cells (Shanks et al. 2012) (Cowles, Emr, and Horazdovsky 1994). The absence of Vps45 also causes a significant reduction in Tlg2 and Snc2 (Shanks et al. 2012). Tlg2 has been shown to be important in the regulation of Atg9 in yeast, the only transmembrane protein required for autophagosome formation (Nair et al. 2011a) (Feng and Klionsky 2017).

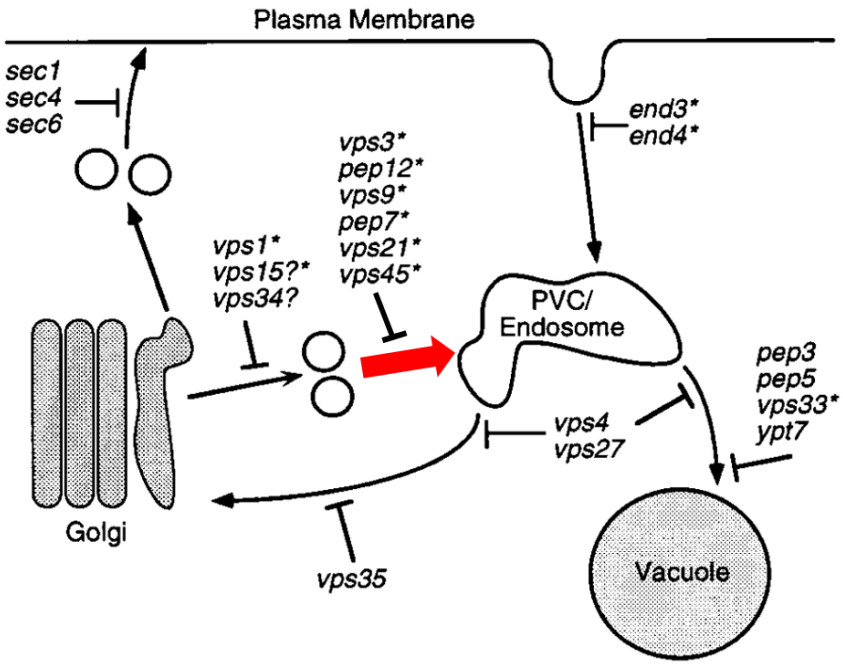

Figure 6: Schematic representation showing different pathways of protein sorting in yeast and proposed protein involved. The red arrow shows the main localisation of Vps45. Slightly adapted from Stepp et al (1997).

1.4 Aims and Objectives

The aim of this project is to determine the function of the SM protein Vps45 in *L. mexicana* and examine whether its role has been conserved in autophagy as it is in yeast. There are three objectives for this project which include:

- Investigating the effect of Vps45 over-expression on *Leishmania* parasites and autophagy.
- Examining autophagy on *Leishmania* parasites in the absence of Vps45.
- Endogenously tagging Vps45 and examining its role in *Leishmania* parasites.

As *Leishmania* parasites are known to cause thousands of deaths worldwide, investigating the different pathways it uses to survive within the harsh environment of the host will be highly beneficial in the production of improved diagnostics methods as well as treatments.

2. Materials and Methods

2.1 Materials

Unless otherwise stated, all chemicals, reagents and antibiotics were bought from Gibco, Invitrogen, Sigma, Fisher Scientific, Macherey Nagel, Promega, PCR Biosystems and New England BioLabs (NEB), Proteintech and InvivoGen. Knockout plasmids were obtained from LeishGEdit. VPS45 Rabbit Polyclonal Antibody was bought from Proteintech.

2.2 Media preparation and Growth Conditions

1 x Media 199 (M199) media with Earle's Salts was used for culturing *Leishmania* parasites at 26 °C in order to mimic the differentiation process within the sand fly's midgut. 500 ml of M199 media was prepared by mixing 100ml of 5 x M199 (dissolving 27.45g of M199 powder and 0.825g of $NaHCO_3$ with 500ml of water), 50ml of fetal bovine serum (FBS), 20ml of HEPES (1M at pH 7.4), 10 ml of Adenine Hemisulfate salt (5mM), 1ml of hemin (2.5mg/ml in 50 mM NaOH) and 319ml of sterile water.

Luria Broth (LB) agar and broth were used for culturing *Escherichia coli.* This was readily prepared within the laboratory. In order to prepare the agar plates, the agar solution was heated, and the appropriate antibiotic was added (Ampicillin 100µg/mL). This was then poured into agar plates and left to dry before being stored in the fridge. In order to grow *E. coli*, bacterial cultures were streaked into agar plates and incubated at 37 °C or transferred into media and incubated in a shaking incubator at 37 °C overnight.

2.3 Molecular Biology

2.3.1 Polymerase Chain Reaction

To amplify PCR product, Q5 (NEB), a high-fidelity DNA polymerase was used. As Table 3 shows, products were placed in a PCR tube and placed in a PCR thermal cycle. Conditions are shown on Table 4.

Table 3: Products and amount needed to carry out a PCR reaction using Q5.

Product	Amount
Q5 Reaction buffer (5x)	10 µl
10 mM dNTPs	1 µl
10 µM Forward primer	2.5 µl
10 µM Reverse primer	2.5 µl
DNA	100 – 1000 ng
Sterile water	Up to 50
Q5 DNA polymerase	0.5 µl

Table 4: Thermocycler conditions for amplifying DNA product.

Steps	Temperature	Time
Denaturation	98 °C	30 seconds
Annealing (two-step cycline)	72 °C	2 minutes
Extension	72 °C	2 minutes
Hold	4 °C	Unlimited

2.3.1.1 Endogenous Tagging

In order to examine the function of Vps45, both copies of Vps45 were endogenously tagged using the pPOTv2 plasmids (pPOTv2_Blast_3xHA for LmxM.36.2230 and pPOTv2_Blast_5xMyc for LmxM.36.0460). Endogenously tagging Vps45 can be used to visualise the protein and examine where within the parasite the protein is localised as well as other proteins it may be interacting with (Dean et al. 2015).

Homologous recombination was used to tag Vps45 at the N-terminus of the target gene (Figure9). Primers were designed to amplify the end 500bp of the 5'UTR, the first 500bp of the ORF and the cassette containing the drug marker and the required tag (Table 4). A fusion PCR was then carried out to produce a DNA product to tag the gene (Figure 9).

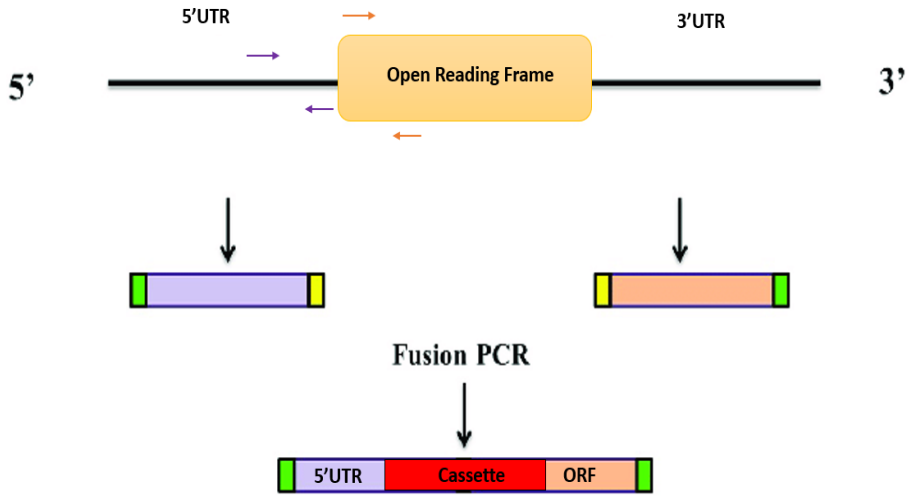

Figure 7: Schematic representation of homologues recombination used to endogenously tag both copies of Vps45 genes.

Table 5: List of primers used to endogenously tag Vps45.

Gene	Description	Forward primer	Reverse primer
LmxM.36.2230	To amplify the last 5'UTR of the gene	AAACACACTTGTGCGTCG	GTAGGCGTCGATAAGGGAA

LmxM.36.2230	To amplify the first 500 bp of the ORF	ATGAACCGCATCTTGCCG	TTCTTCTGGTAGCGAATGCG
LmxM.36.2230	To amplify the cassette containing 3Xha tags and drug resistance	AACAGCTTCTTTTCCCTTATCGACGCCTAC GTATAATGCAGACCTGCTGC	TGATCATGCCGCTTTGCTGGCA TCAG
LmxM.36.2230	To carry out a fusion PCR containing DNA fragment with the 5'UTR, ORF and cassette	AAACACACTTGTGCGTCG	TTCTTCTGGTAGCGAATGCG
LmxM.36.0460	To amplify the last 5'UTR of the gene	TCTGTCACGAGCGTCTGCA	TTTGTTGCCGTGTCGCAC
LmxM.36.0460	To amplify the first 500 bp of the ORF	ATGACTTCCGTATCCTCCTCG	TACGTGATGGGGTTCATGAACG
LmxM.36.0460	To amplify the cassette containing 3xmyc tags and drug resistance	CTCCGCTCCTCGTGCGACACGGCAACAAAGT ATAATGCAGACCTGCTGC	GTTTACCTTCGAGGAGGATACG GAAGTCATGCCCTTGCTCACAC TAGT
LmxM.36.0460	To carry out a fusion PCR containing	TCTGTCACGAGCGTCTGCA	TACGTGATGGGGTTCATGAACG

	DNA fragment with the 5'UTR, ORF and cassette		

2.3.1.2 Over-expression of Vps45

To examine the effects of over-expression on *Leishmania*, Vps45 over-expresser plasmids were generated using the pSSU plasmid in order to be transfected into *L. mexicana*. Firstly, primers with specific tags (Myc for LmxM.36.2230 and His for LmxM.36.0460) were designed to amplify both copies of Vps45 genes in PCR using Q5 DNA polymerase. Table 6 shows the primers used.

Table 6: List of primers and description of action to over-express Vps45.

Gene	Description	Forward sequence	Reverse sequence
LmxM.36.2230	To amplify gene with ClaI and StuI restriction site and 2xmyc tags	ATCGATATGGAACAAAAACTCATCTCAGAAGAGGATCTGATGAACCGCATCTTGCCGTGTG	AGGCCTTTACGATGGCGAGAGCTG
LmxM.36.2230	To amplify 3'UTR with stuI and SpeI restriction site	AGGCCTGAGAGGCGTGACGTGCTG	TGATCAGTGTCGGAACATCAAGCAGTCC
LmxM.36.0460	To amplify gene with TspMI and AvrII restriction site and 3xHis tags	CCCGGGATGCACCACCACCACCACCACATGACTTCCGTATCCTCCTCGA	GGATCCCTAGGAGGGCAGTGAGTGTATA
LmxM.36.0460	To amplify 3'UTR with AvrII and SbfI restriction site	CCTAGGAGGAAATCGAGGATTCGCCTG	CCTGCAGGGAGCCTGCGTGATGCGCAGT

2.3.1.3 Deletion of Vps45

Using the CRISPR-Cas9 gene editing tool, primers were designed to knockout both copies of Vps45 genes and replace it with a marker cassette (Figure 8). As LmxM.36.2230 knockout (KO) cell lines are currently available, LmxM.36.0460 KO and LmxM.36.2230/LmxM.36.0460 double KOs were attempted using specific primers (Table 7). In order to carry out the KO, drug

cassettes as well as the single guide RNAs (sgRNA) were amplified using PCR, mixed and transfected into *L. mexicana* (Figure 8).

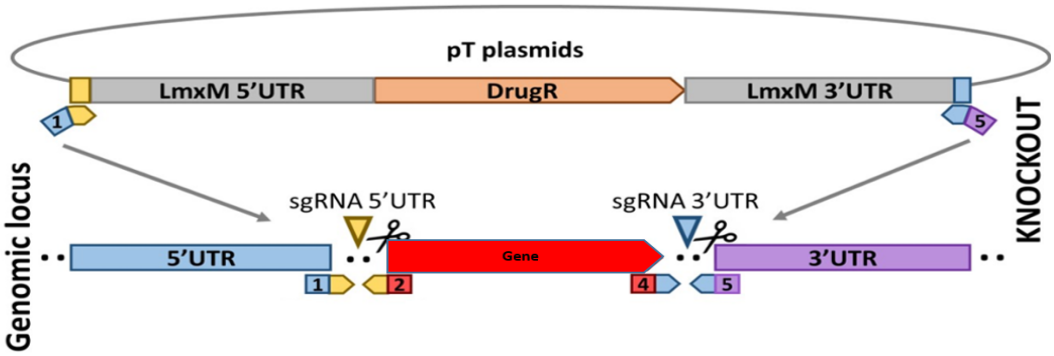

Figure 8: Schematic representation of the replacement of the Vps45 gene with the drug cassette. The CRISPR-Cas9 tool will cut the 5'UTR and 3'UTR of the gene and replace it with a cassette containing the resistance drug. Slightly adapted from LeishGEdit.

Table 7: List of primers used to knock the Vps45 gene.

Gene	Description	Forward sequence	Reverse sequence
LmxM.36.0460	To amplify the upstream of the cassette and gene	CGTGCGGGGGCGCTGTAAGCGTTCTTCATT gtataatgcagacctgctgc	ACACACACCTACTGGCCCCCACCTCCTCCCccaatttgagagacctgtgc
LmxM.36.0460	To amplify the 5' sgRNA	gaaattaatacgactcactataggTCTCCGCTCCTCGT GCGACAgttttagagctagaaatagc	OL6137 Oligos
LmxM.36.0460	To amplify the 3' sgRNA	gaaattaatacgactcactataggAGGAAATCG AGGATTCGCCTgttttagagctagaaatagc	OL6137 Oligos
LmxM.36.0460	To carry out a check after knocking-out the gene	TGCTTAATCTAGTGTCGCACG	TCTGGGTCGTCAATGACGAC

2.3.2 Agarose Gel Electrophoresis

PCR samples were separated on a 1% agarose gel in order to separate the DNA based on size. In order to make a 100ml agarose solution, 1g of Tris-Acetate-EDTA (TAE) was dissolved in 100 mL of sterile water and heated. This was then allowed to cool down to 50 °C. Ethidium bromide (0.5µg/mL) was then added to the solution, mixed and poured into a gel tray with a comb. This was then left to solidify for 30-40 minutes and TAE was added to the top of the gel. In order to load the DNA samples, loading dye (6x) was added to the samples and the

samples were loaded into the gel after removing the comb. The gel was then ran at 70 V for approximately half an hour and a ultraviolet radiation (UV) transilluminator was used to visualise the DNA.

2.3.3 DNA Extraction and Purification

DNA extraction from *Leishmania* was carried out using the PCRBIO Rapid Extract Lysis Kit (PCR Biosystems) as per manufacture instructions. DNA from agarose gel was then purified using the DNA, RNA, and protein purification Macherey-Nagel NuceloSpin Gel and PCR clean-up as per manufacturer's instructions.

2.3.4 Addition of A Overhangs

As a high-fidelity DNA polymerase (Q5) was used to amplify Vps45, the PCR products do not have overhangs that are compatible with the pSSU plasmid required to transfect *Leishmania*. Due to this, amplified Vps45 PCR samples need to be ligated with a pGEM-T plasmid firstly by adding 3'A overhangs. This was carried out by adding dATP (10mM), Taq DNA polymerase, Taq PCR buffer (10x) and sterile water to the PCR samples (10 – 100ng per 100bp). Samples were then incubated at 72 °C for 20 minutes.

2.3.5 Ligation and Restriction digest

In order to transfect *Leishmania* with over-expressor plasmids, PCR samples with added 3'A overhangs were ligated with pGEMT-T plasmid using the pGEM-T and pGEM-T Easy Vector Systems as per manufactures protocol. Products were then digested using restriction enzymes. The pSSU plasmid used to transfect *Leishmania* was also digested in order to have compatible ends with the Vps45 gene. Table 5 shows specific restriction enzymes used for each gene.

Using a 1.5mL tube, DNA, restriction enzymes, buffer and sterile water was mixed and incubated at 37 °C overnight. Table 8 shows a standard restriction digest mix.

Table 8: Products and amount needed to carry out a restriction digest.

Product	Amount
DNA	1 µg
10x CutSmart Buffer	5 µl (1x)
Restriction enzyme	1 µl
Restriction enzyme	1 µl
Sterile water	Up to 50 µl

After carrying out a restriction digest, samples were separated on an agarose gel and DNA product was purified. Samples were then ligated as Table 9 shows and incubated in 4 °C overnight. The ligated product was then transformed into *E. coli* cells.

Table 9: Products and amount needed to carry out a ligation.

Product	Amount
pSSU plasmid	50 ng
Insert DNA	37.5 ng
T4 DNA ligase bugger (10x)	2 µl
T4 DNA ligase	1 µl
Sterile water	Up to 20 µl

2.4 Transformation and Purification of *E. coli*

Competent *E. coli* bacterial cells were used to carry out transformations in order to extract plasmids used to over-express Vps45. Competent *E. coli* bacterial cells were readily available within the laboratory.

Competent *E. coli* cells were allowed to thaw on ice after being taken out of the -80 °C freezer. Approximately 100ng of DNA was mixed with 50 µl of *E. coli* and mixed gently. This was left to incubate on ice for 30 minutes. In order to transform the plasmid DNA into the bacterial cells, heat shock was used by inserting the DNA with *E. coli* sample in a 42 °C water bath for 45 seconds and place the tube back in ice for 2 minutes. 500 µl of LB was then added (with Ampicillin) and plated on LB agar plates. Agar plates were incubated at 37 °C overnight. Colonies from agar plates were then transferred into 5ml of LB and left in the shaking incubator overnight at 37 °C. In order to extract and purify the plasmid, NucleoSpin Plasmid kit from Macherey Nagel was used as per manufactures protocol.

2.5 Transfecting and Plating of *Leishmania*

2.5.1 Knockout of Vps45 Gene

In order to transfect *L. mexicana*, JM6571 cells were used. Cells need to be approximately 8.0×10^7 cells/ml. 8.0×10^7 cell at the log phase of growth were harvested and centrifuged for 10 minutes at 3500 RPM and the supernatant was removed. Cells were then resuspended in Phosphate-buffered saline (PBS) and centrifuged for 10 minutes at 3500 RPM. The supernatant was removed and appropriate DNA, cytomix and $CaCl_2$ was added, mixed and transferred to an Amaxa cuvette. *Leishmania* parasites were then transfected using the Amaxa Nucleofector II using U-033 programme. Transfected cells were then transferred into a flask containing 10 mL of M199 and incubated at 25 ^oC overnight.

2.6.2 Over-expression and Endogenous Tagging

In order to transfect *L. mexicana* M379, wild-type (WT) cells were used. Cells need to be approximately 1.0×10^7 cells/ml. 2.0×10^7 procyclic promastigotes were harvested and centrifuged for 10 minutes at 3500 RPM and the supernatant was removed. Cells were then resuspended in Phosphate-buffered saline (PBS) and centrifuged for 10 minutes at 3500 RPM. The supernatant was removed, and the pellet was resuspend in 100 µl of Tb-BSF (90mM sodium phosphate, 5mM potassium chloride, 0.15mM calcium chloride, 50mM HEPES at pH 7.3) and 10 µg of DNA was added. *Leishmania* parasites were then transfected using the

Amaxa Nucleofector II using U-033 programme. Transfected cells were then transferred into flask containing 10 mL of M199 and incubated at 25 ^{o}C overnight.

2.5.3 Plating of Transfected cells

To plate cells, 50 mL falcon tubes were used to prepare 1:8 dilutions. Antibiotics at their specific concentrations were added (Table 10). A control not containing antibiotics was also plated.

Table 10: List of antibiotics and the concentration used.

Antibiotic	Concentration
Hygromycin (HYG)	50 μg/mL
Puromycin (PAC)	50 μg/mL
Nourseothricin (SAT)	75 μg/mL
Blasticidin (BSD)	10 μg/mL
Neomycin (G418/NEO)	15 μg/mL

96-well plates were regularly checked for clones and after approximately 10 days, clones achieved were transferred into 10 mL flasks with antibiotics and incubated at 25 ^{o}C. When cells reach mid-log phase (7.0×10^{6} cells/ml), pellets were prepared for genomic DNA and protein extraction

2.6 Protein Extraction

In order to extract protein, procyclic promastigotes need to be approximately 1.0×10^{7} cells/ml. 1.0×10^{7} cells were harvested and centrifuged for 10 minutes at 3500 RPM and the supernatant was removed. Cells were then resuspended in Phosphate-buffered saline (PBS) and centrifuged for 10 minutes at 3500 RPM. The supernatant was removed and resuspend in 20 µl of Laemmli buffer (4% SDS, 10% 2-mercaptoehtanol, 20% glycerol, 0.004% bromophenol blue and 0.125 M Tris HC) and boiled at 95°C for 5-10 minutes.

2.7 Western Blotting

A western blot was carried out to test the expression of a fusion protein containing a tag. In order to carry out a western blot, a Tris-glycine sodium dodecyl sulfate (SDS) resolving gel (Table 11) was prepared and placed on a 1 mm thick plate. After letting it solidify, a 5% stacking gel (Table 12) was prepared and poured over the resolving gel. A 1 mm thick comb was inserted into the gel and allowed solidify. After approximately 1 hour, the comb was removed and the gel was placed on the gel chamber. 1x SDS (200ml 5XSDS-Tris and 800ml H_2O) was added to fill half of the chamber. Samples were loaded onto the gel and allowed to run at 150 V for 1 hour or until the blue dye can no longer be seen.

Table 11: List of products need to make a resolving gel.

Product	Amount
30% Acrylamide mix	6.7 mL
Tris-CL (1.5M, pH 8.8)	5 mL
SDS (10%)	200 µl

10% Ammonium persulfate	210 µl
TEMED	10 µl
Sterile water	Up to 20 mL

Table 12: List of products needed to make a stacking gel.

Product	Amount
30% Acrylamide mix	830 µl
Tris-CL (1.5M, pH 8.8)	630 µl
SDS (10%)	50 µl
10% Ammonium persulfate	60 µl
TEMED	8 µl
Sterile water	Up to 5 mL

The gel was then removed from the chamber, and a polyvinylidene fluoride (PVDF) membrane as well as filter pads were cut the same size as the gel. The PVDF membrane was placed in ethanol for 1 minute and then transferred into sterile water for 2 minutes, while 4 filter pads were soaked in transfer buffer (76ml H_2O, 4ml 25x transfer buffer and 20ml ethanol). Two filter pads were stacked, and the membrane was placed above. The gel was then transferred to the top of the membrane and two more filter pads were placed on top of the gel. This was transferred into an Electrophoretic Transfers machine and left to run for 1 hour at 20 V.

In order to detect the protein and the tag, the membrane was removed from filter pad and blocked with 15 mL of 5% milk in PBS buffer (2.5g of skimmed milk powder in 50ml of PBS Buffer) for 1 hour. The milk was then removed and the primary antibody (as per manufacture instructions or anti-tag used) in 1% milk was applied and left to agitate for 1 hour. The membrane was then washed 3 x 10 minutes in PBS and the secondary antibody (anti-mouse) was added and left to agitate for 1 hour. This was then washed 3 x 10 minutes in PBS and blotted with ECL detection reagent (GE Healthcare) and a UV machine was used to detect the protein.

2.8 Bioinformatics

In order to identify the correct Vps45 genes, a blast alignment was carried out using the TryTrypDB using the Vps45 sequence of the yeast, *S. cerevisiae*. National Centre for Biotechnology Information (NCBI) was used to identify Vps45 genes from *S. cerevisiae* and human. Genes identified were then aligned using Clustal W and Boxshade was used to identify similar sequences.

3. Results

The aim of this study was to determine the function of Vps45 in *L. mexicana* by the endogenous tagging, over-expression and the deletion of Vps45.

3.1 Blast alignment

Carrying out a blast alignment of the *S. cerevisiae* Vps45 gene, TryTrypDB identified two potential paralogs of Vps45 in *L. mexicana* known as LmxM.36.2230 (1680bp, 559aa, 65.66 kDa) and LmxM.36.0460 (1854bp, 617aa, 75.1 kDa). Table 13 shows the percentage similarity between the Vps45 copies in *S. cerevisiae, L. mexicana* and human. Vps45 copies in *Leishmania* have a percentage similarity of 31.93% and interestingly both copies of Vps45 have a high sequence similarity to the human Vps45 than *S. cerevisiae*.

Table 13: Percentage similarity of Vps45 in *S. cerevisiae*, *L. mexicana* and human.

	S. cerevisiae	Human	LmxM.36.2230	LmxM.36.0460
S. cerevisiae	100%	37.59%	28.87%	26.9%
Human	37.59%	100%	33.4%	29.89%
LmxM.36.2230	28.87%	33.4%	100%	31.93%
LmxM.36.0460	26.9%	29.89%	31.93%	100%

A sequence alignment using Clustal W was then used to examine the conservation of the Vps45 genes against human and *S. cerevisiae* Vps45. As Figure 9 shows, the N-peptide binding site pocket in yeast and humans is made up of five residues. The five residues in the human Vps45 gene have been conserved in LmxM.36.0460, while the five residues in yeast Vps45 gene have also been conserved in LmxM.36.2230.

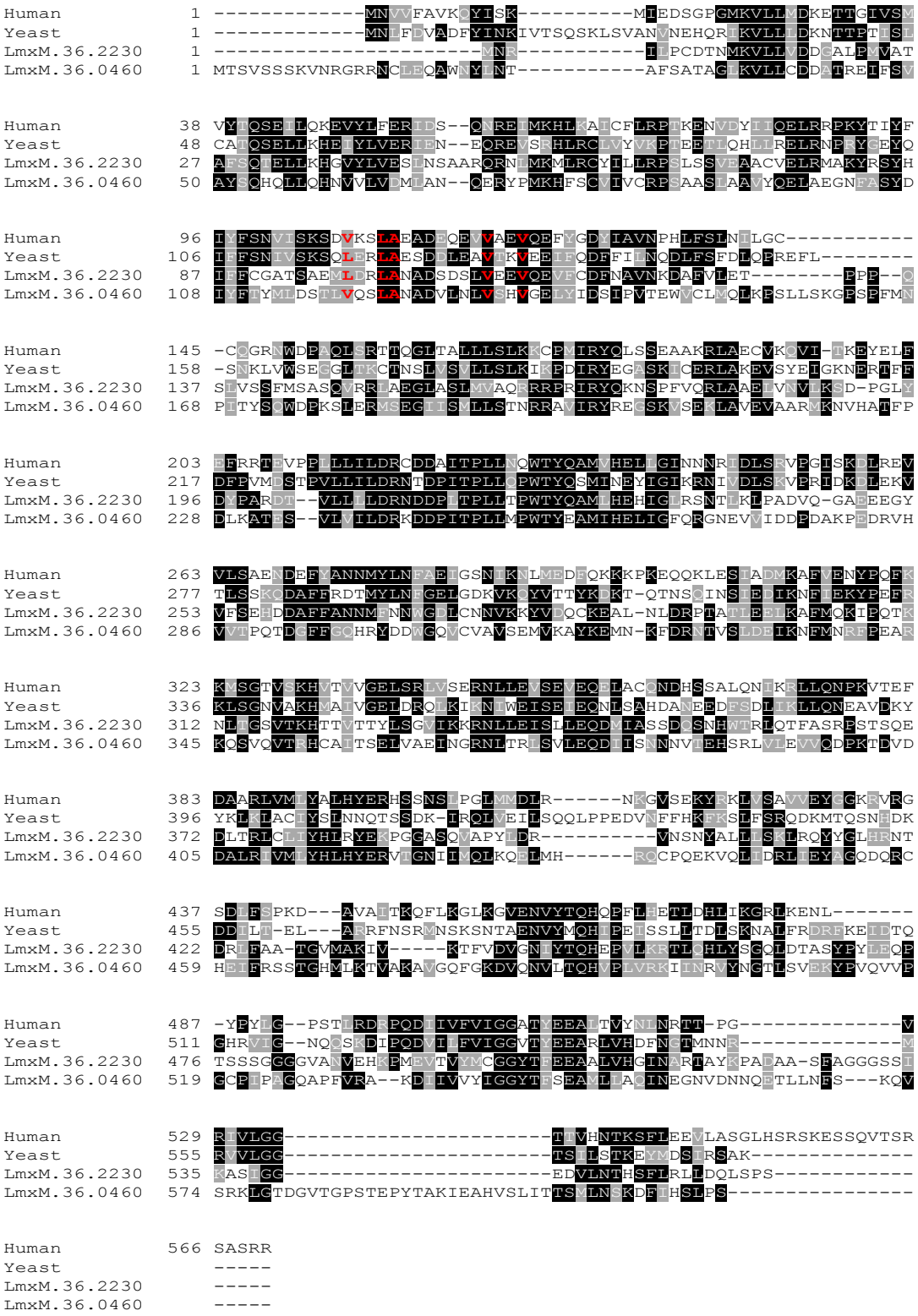

Figure 9: Alignment of human, yeast and *Leishmania* Vps45 using Clustal W followed by BOXSHADE 3.21. The black shades indicate conserved residues, the grey indicate conservative mutations and white show divergence. The red shades show the five residues which make up the N-peptide binding site in domain 1.

3.2 Endogenous Tagging of Vps45

In order to examine the function of Vps45, LmxM.36.2230 (Figure 10A) and LmxM.36.0460 (Figure 10B) were endogenously tagged through homologous recombination. 3xHA tags were used for LmxM.36.2230 and 6xMyc tag were used for LmxM.36.0460. As figure 10 shows, PCR reactions for amplifying the 5'UTR (500bp), ORF (500bp) and cassette (1000bp) from the pPOTv2 plasmid, all of which containing overlapping sequences were amplified successfully. A fusion PCR was then carried out to produce a fused product using the overlapping sequence in order to endogenously tag both copies of the Vps45 genes. PCR product was extracted using gel extraction. As the quantity of DNA was little, multiple PCR reaction were carried out as shown on Figure 10.

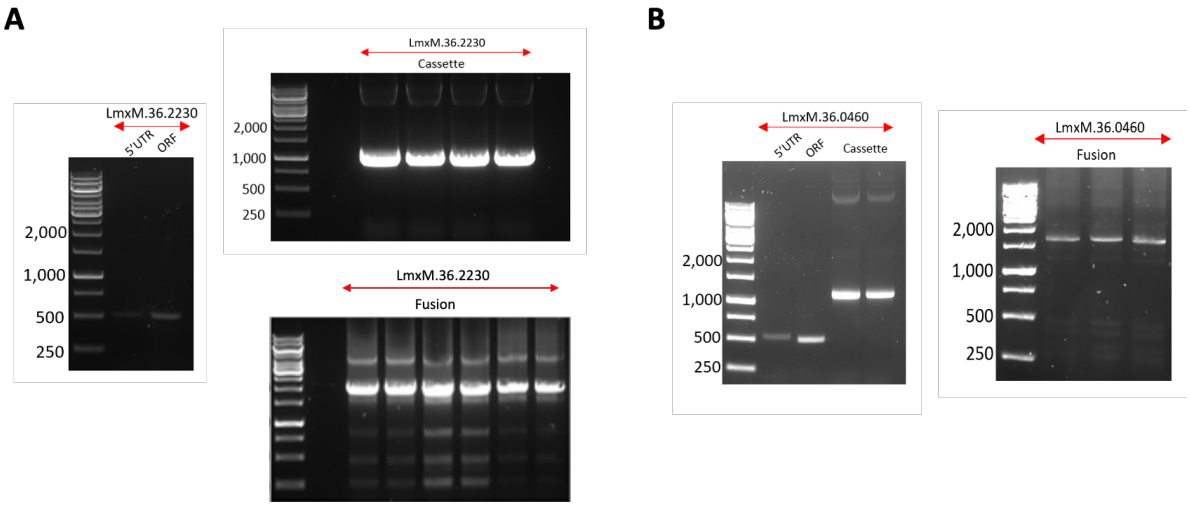

Figure 10: Endogenously tagging Vps45 in *L. mexicana* using homologous recombination. PCR was used to amplify 500bp 5'UTR and ORF as well as the 1000bp cassette in (A) LmxM.36.2230 (B) and LmxM.36.0460. PCR was then also used to integrate the three products into a fused DNA fragment and gel extraction was used to extract and purify the DNA. *L. mexicana*

L. mexicana was transfected with the PCR product encoding an epitope tag and a selective marker, to endogenously tag both copies of the Vps45 genes. Resistant clones grown in antibiotics were successfully achieved and were then expanded before cells were lysed and protein was extracted to carry out a western blot to assess whether the gene had been tagged. Figure 11A shows the western blot for LmxM.36.2230 HA tagged gene (65.66kDa), HA tagged Tlg2 (40kDa) as a positive control and *L. mexicana* WT. Western blot did not show any bands for LmxM.36.2230, indicating that the gene may have not been tagged or the protein is not highly expressed. Western blot for Myc tagged LmxM.36.0460 (Figure 11b) also did not show a band at 75.1kDa (the size of the gene) but showed the positive *L. mexicana* Myc positive control (100kDa). Interestingly, two bands were detected for LmxM.36.0460 and *L. mexiana* WT at 40kDa, that may potentially be a contaminant or a degraded product.

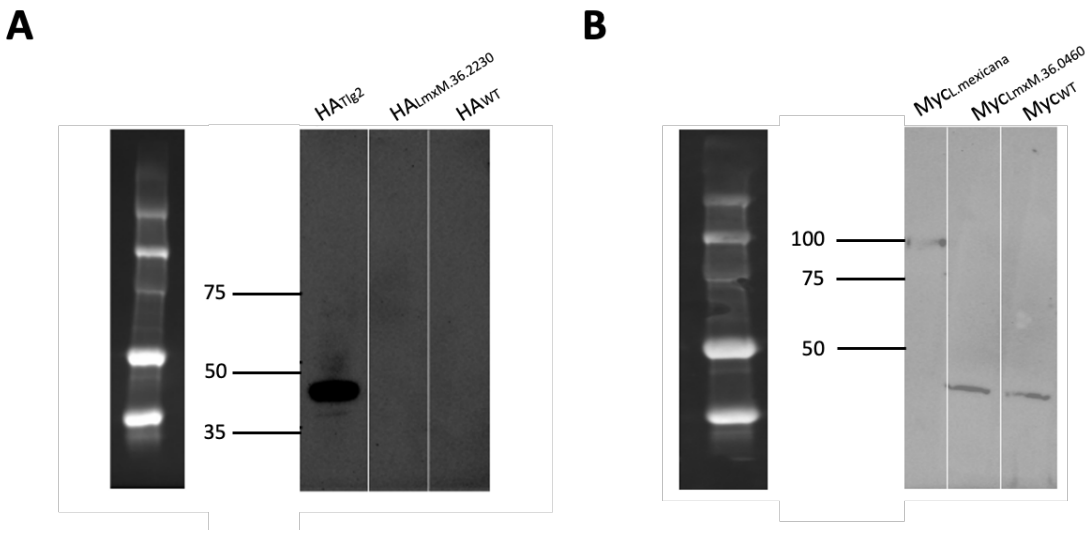

Figure 11: Western blot analysis of Vps45 in *L. mexicana* using homologous recombination. (A) Western blot analysis of LmxM.36.2230 tagged with HA (65.66 Kda), HA positive control and WT. No band was shown on LmxM.36.2230 lane. (B) Western blot analysis of LmxM.36.0460 tagged with Myc (75.1 Kda), Myc positive control and WT. A band was shown on LmxM.36.0460 lane as well as the WT control.

Vps45 antibody (Proteintech) species specific to human, mouse and rat was also purchased to examine whether it will interact with Vps45 copies in *L. mexicana.* The sequence of Vps45 antibody is human Vps45 and as figure 9 shows, the five residues that make up the N-peptide binding site in humans are conserved in LmxM.36.0460 and have an overall percentage similarity of 29.89%. Therefore, to test this, a western blot was carried out on WT *L. mexicana* containing both Vps45 genes, LmxM.36.2230 KO, LmxM.36.0460 KO and both copies of Vps45 knocked out. As Figure 12 shows, no product was detected by the Vps45 antibody in any of the samples which may be due to the antibody not being specific to *L. mexicana* or due to the low expression of the Vps45 protein as mentioned before.

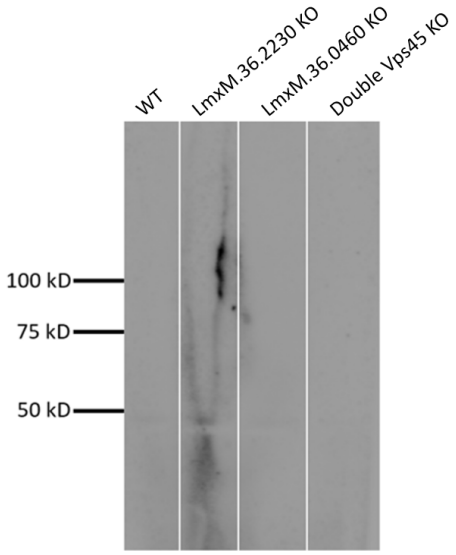

Figure 12: Western blot analysis of Vps45 commercial antibody against WT, LmxM.36.2230 KO, LmxM.36.0460 KO and LmxM.36.2230 and LmxM.36.0460 double KO. Western blot did not detect LmxM.36.2230 (62.41 kDa) or LmxM.36.0460 (69.58 kDa).

3.2 Over-expression of Vps45

Vps45 over-expresser plasmids were generated using the pSSU plasmid in order to be transfected into *L. mexicana*. The first vps45 gene LmxM36.36.2230 (1680bp) and its 3'UTR (1700bp) as well as the second vps45 gene LmxM.36.0460 (1800bp) and its 3'UTR (1000bp) were amplified (Figure 13A). A digestion of the PCR products was then carried out using restriction enzymes (Figure 13B) in order for the products to be ligated with the pSSU over-expressor plasmid. Ligated products were then transformed into *E. coli* cells and a colony PCR was carried out to determine the integration of the plasmid (Figure 13C). As Figure 13C shows, the colony PCR showed two plasmid clones (colony 8 and 11) containing LmxM.36.0460 (2800bp) and one plasmid (colony 4) containing LmxM.36.2230 gene and 3'UTR.

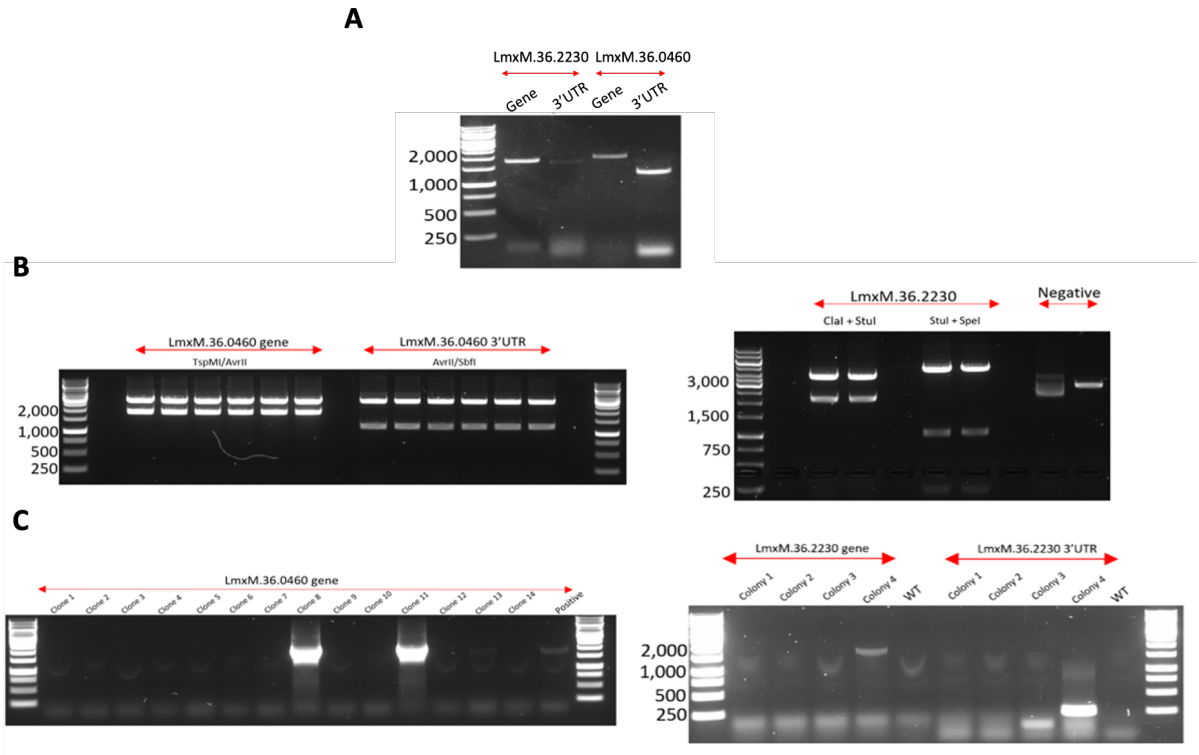

Figure 13: Gel images of over-expressing Vps45 in *L. mexicana*. (A) Vps45 gene and 3'UTR was amplified in both copies using Q5 DNA polymerase. (B) Both genes and the 3'UTR were digested with specific restriction enzymes to be ligated into the pSSU plasmid. For LmxM36.0460 gene, the enzymes TspMI/AvrII was used and AvrII/SbfI was used for the 3'UTR of the gene. ClaI/StuI was used for LmxM.36.2230 gene and StuI/SpeI was used for the 3'UTR of the gene. (C) A colony PCR was carried out on transformed *E. coli* cells and 14 colonies for LmxM.36.0460 were tested for the genes in comparison to 4 colonies for LmxM.36.2230 which were tested for the gene and 3'UTR.

To transfect *L. mexicana*, plasmid extraction from *E. coli* cells was carried out and the plasmids containing the gene and the 3'UTR were transfected for both vps45 copies. To determine if the plasmid has been integrated into the cells, antibiotics were added (as mentioned in the methodology) and *L. mexicana* were incubated. Transfection and addition of antibiotics only resulted in the growth of *L. mexicana* containing LmxM.36.0460 plasmids and the death of cells containing over-expressor LmxM.36.2230 plasmids. To further determine the integration of the LmxM.36.0460 plasmid, a western blot using an anti-His antibody (Figure 14) carried out showed no protein containing an anti-His tag was detected, indicating that plasmid may not have been taking up by the cells or its concentration is low.

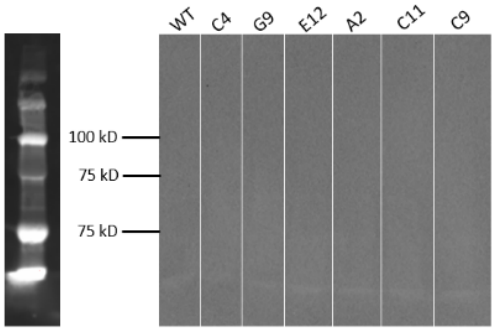

Figure 14: Western blot analysis of LmxM.36.0460 over-expressed clones for 6 different clones and a WT control. Anti-His primary antibody and an anti-mouse secondary antibody was used. No anti-His protein was detected.

3.4 Deletion of Vps45

For the purpose of this thesis, in this section, the gene LmxM.36.2230 will be identified as Vps45a and LmxM.36.0460 as Vps45b.

Using the CRISPR-Cas9 gene editing tool, both copies of Vps45 genes were knocked-out and replaced with a marker cassette (as shown in the methodology), as Vps45a knockout (KO) cell lines are currently available, Vps45b and Vps45a/b double KOs were attempted. In order to carry out this process, drug cassettes containing puromycin and blasticidin (2000bp) as well as the single guide RNAs (100bp) (sgRNA) were amplified using PCR as Figure 15 shows.

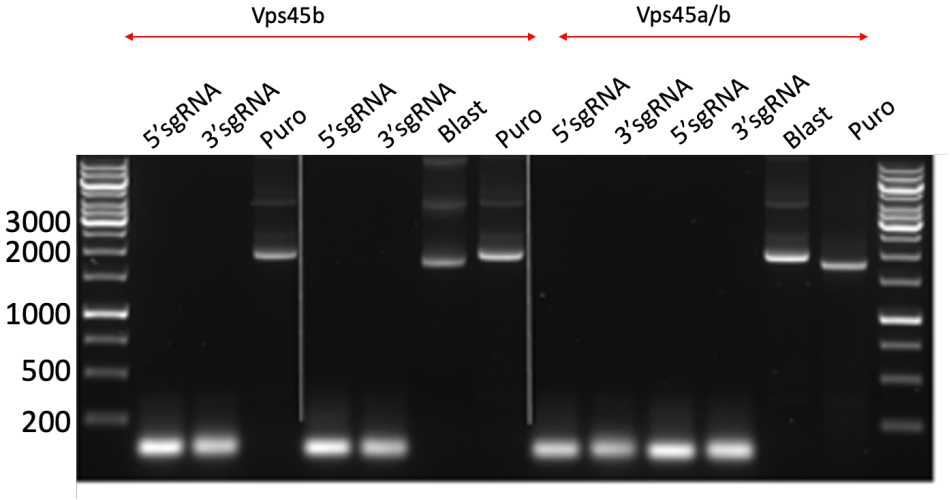

Figure 15: Gel images of amplification of drug cassettes (2000bp) and sgRNAs (100bp). The drug cassette puromycin (puro) and blasticidin (blast) were used to knockout both alleles of the vosp45b gene.

The PCR reaction was mixed and *L. mexicana* were transfected in order to knockout Vps45b and Vps45a/b. Addition of antibiotics caused the growth of 6 drug resistance *L. mexicana* clones with a Vps45b knockouts. To confirm the deletion of the gene, a PCR was carried out to validate the presence of the drug cassette and as Figure 16 shows, the cassette (500bp) was present and the gene was not amplified as the positive control WT shows.

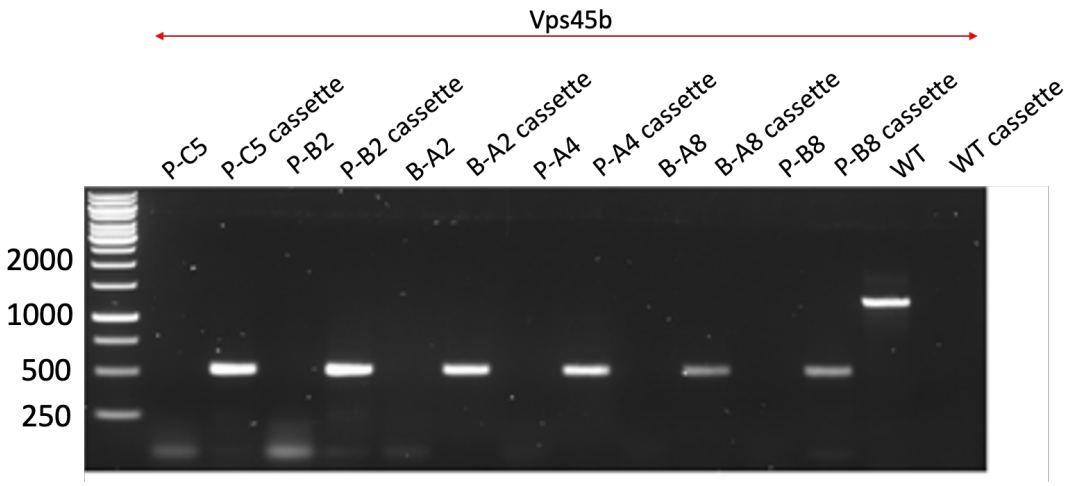

Figure 16: Gel images of validation of LmxM.36.0460 KO clones from DNA samples as well as WT control. Primers for the gene were used to amplify the first 500bp as well as the cassette (500bp). Gene primers as described in the methodology were used to amplify the WT gene. The gene was not present in 6 clones and the drug cassette was fully integrated.

In order to achieve knockout clones for both copies of Vps45, Vps45b KO cells were used to further knockout Vps45a in order to get a double knockout. As Figure 17 shows, one Vps45a/b KO was achieved (C12) heterozygote clone with one copy of the gene and one copy of the cassette (D5) was also achieved.

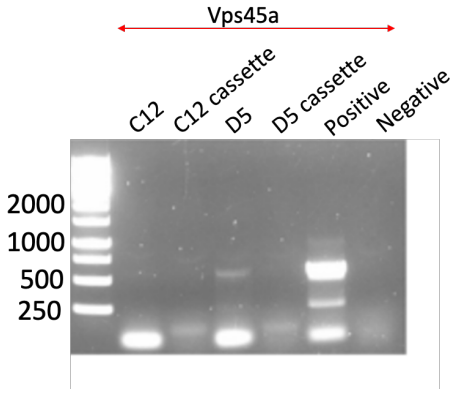

Figure 17: Gel images of validation of Vps45a KO clones using Vps45b KO from DNA samples as well as Vps45a positive and WT negative control. Primers for Vps45a specific cassettes (100bp) and Vps45a gene (500bp).

To further examine the effect of Vps45 absence on *L. mexicana*, a growth curve was carried out over 8 days on the different knockout *L. mexicana* cell lines as well as the WT cells. As Figure 18 shows, Vps45a KO and Vps45b KO are very similar to the WT cell. Cells start to gradually grow and reach a peak on day 4, with Vps45a KO growing a little slower than Vps45b and WT cells. Cells reach a stationary phase and start to reduce by day 8. In comparison, Figure 18 shows that Vps45a/b KO cells grow slightly slower than WT and reach a peak on day 5, a day later than WT and Vps45a and b KO. Interestingly, Vps45b knockout cells with a single copy of Vps45a allele seem to grow slower than other cell lines and seem to stabilise on day 6. To investigate this further, statistical analysis will need to be carried out to examine the significant difference.

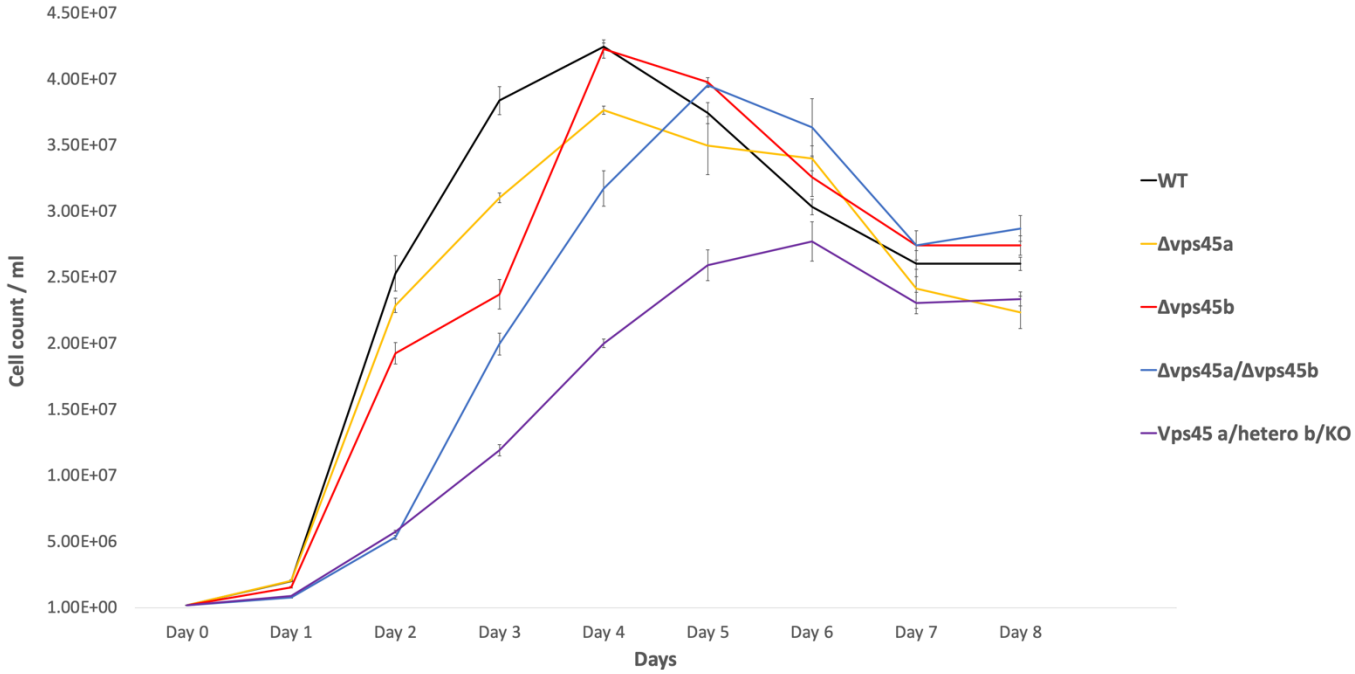

Figure 18: Growth curve of the different KO cell lines. CRISPR/Cas9 was used to knockout the genes. Figure shows WT *L. mexicana* (black), Vps45a KO (yellow), Vps45b KO (red), Vps45a/b KO (blue) Vps45b KO with a heterozygote Vps45a (purple).

4. Discussion

The regulation of SNARE proteins and complexes by SM proteins is an important process that ensures the regulation of membrane trafficking. Vps45 has been shown to have a role in regulating SNARE interactions and vesicle trafficking from the TGN into the endosomal pathway in the yeast *S. cerevisiae* (Carpp et al. 2006). Studies carried out in yeast have shown that Vps45 interacts and regulates the t-SNARE Tlg2 and the v-SNARE Snc2 (Carpp et al., 2006). The main aim of this project was to investigate the role of the SM protein Vps45 and examine whether its function has been conserved in *L. mexicana* as it is in *S. cerevisiae*.

4.1 Identification of Vps45 genes in *Leishmania*

TryTryDB was used to identify the Vps45 gene in *Leishmania* using the sequence of *S. cerevisiae* Vps45. As results above show, *L. mexicana* contains two paralog copies of the Vps45 gene found in chromosome 36 known as LmxM.36.2230 and LmxM.36.0460, unlike the presence of only one copy in yeast and humans. This is also seen in *L. major*, where two Vps45 genes are found (Besteiro, Coombs, and Mottram 2006). One reason for this can be that genome plasticity as well as high levels of gene duplication are known to occur in *Leishmania* species in order to ensure gene regulation and survival within the host (Fiebig, Kelly, and Gluenz 2015) (Rogers et al. 2011). For example, the gene Rad51 in *Leishmania infatum* known to be important in homologous recombination has three gene paralogs known as LiRad51-3, LiRad51-4 and LiRad51-6, with LiRad51-3 being essential for cell viability (Genois et al. 2015). This indicates that the presence of paralogous genes may be important for the survival of *Leishmania*. As this project identified two genes of Vps45 in *L. mexicana*, it will be interesting to investigate which gene is essential and if the roles of these genes are similar.

Although there are two Vps45 genes, the sequence similarity of the two genes is 31%. Both copies of the Vps45 gene have a high degree of similarity to the human Vps45 (33.4% for Lmxm.36.2230 and 29.89% for LmxM.36.0460) than *S. cerevisiae* (Table 13). However, although the Vps45 sequence in *Leishmania* are not very similar to the human or yeast Vps45, the N-peptide binding site pocket of the Vps45 protein where SNARE proteins bind is conserved. This indicates that the functions of these proteins within the different species may be similar.

4.2 Localisation and Interaction of Vps45 with SNARE Proteins

In order to examine the function of Vps45, attempts were made to endogenously tag both copies of the Vps45 gene using homologues recombination. Endogenously tagging Vps45 can be used to visualise the protein and examine where within the parasite the protein is localised as well as other proteins it may be interacting with using co-immunoprecipitation in order to understand whether its interaction to other proteins is conserved as it is in yeast. As Figure 11 shows, both copies of Vps45 have not been endogenously tagged as no bands were shown in the western blot. This may be due to the primers that have been used, as the ATG start codon is still present within the gene as well as the tag (Myc for LmxM.36.2230 and His for LmxM.36.0460) indicating that the initiation of translation may have occurred from the ATG

start codon within the gene and not the tag. As a result, this experiment will need to be repeated without the ATG start codon to ensure that translation occurs from the tag.

Although the start codon can be the issue, expression of the protein may have not been large enough for the detection to occur. Protein was extracted from procyclic promastigotes and according to studies carried out on autophagy, autophagy is highly upregulated during metacyclogenesis. To investigate this further, protein will need to be extracted from the different life-cycle stages (lag, log and stationary phase) and examine whether the expression of protein changes and therefore will be detected.

An attempt was also carried out to test human Vps45 antibody species specific to human, mouse and rat. Although it was assumed that the antibody may not work in *Leishmania*, bioinformatic analysis has shown that the five residues that makeup the N-terminal binding site have been conserved in human, yeast and *Leishmania* Vps45, as the alignment of Vps45 shows, indicating that Vps45 genes from *Leishmania* and humans are similar. However, as the western blot showed, no protein was detected in the different cell lines created. This again may be due to the expression of the protein as only cells in the log phase were harvested or due to the antibody not being specific for *Leishmania* Vps45. As a positive control was also not used, western blot techniques may have also been an issue. Nevertheless, further analysis will need to be carried out similar to the endogenous tagging. Protein will need to be extracted from the different life cycles and a human positive control will be used to visualise Vps45 to ensure that blotting techniques are not the issue.

Although endogenous tagging was not achieved in this study, many different studies have been carried out on the localisation and interactions of the Vps45 protein. According to a study a carried out by Nichols et al (1998) and Bryant and James (2001) on the localisation of Vps45 in *S. cerevisiae,* Vps45 was shown to be mainly localised in the cystol fraction and the membrane pellet of the cell, presumably due to the interaction with its SNARE proteins (Nichols, Holthuis, and Pelham 1998). As mentioned above, Vps45 has a role in regulating SNARE interactions and vesicle trafficking from the TGN into the endosomal pathway within the cytoplasm. Bryant and James (2001) has shown that in the presence of the t-SNARE Tlg2 (SNARE protein known to interact with Vps45), Vps45 is mainly located in the membrane pellet in comparison to the cystol fraction of the cell. The absence of Tlg2 causes the reduction and almost the absence of Vps45 within the membrane pellet indicating that without Tlg2, Vps45 is mainly present within the cystol. This may indicate that the localisation of Vps45 may also be found in the cystol fraction and the membrane pellet of the parasite, *L. mexicana*. To investigate this further, differential fractionation will be used to separate the endogenously tagged cells into cystol fraction and membrane pellet allowing the visualisation of where the protein is located through western blotting.

Bryant and James (2001) also analysed the N-terminal peptide in Tlg2 by constructing a mutated Tlg2 form lacking the N-terminal peptide. Results showed that Vps45 is not present within the membrane pellet signifying the important role of the N-terminal site for the interaction between Vps45 and Tlg2 and the localisation of Vps45 (Bryant and James, 2001). As the five residues that make-up the N-terminal binding site sequence have been conserved in *L. mexicana* in both copies of the Vps45 genes, it will be interesting to analyse this

interaction and examine whether the mutation of Tlg2 affects the localisation of Vps45 in *L. mexicana*. Furthermore, as the N-terminus seems to be highly important for the localisation and expression of Vps45, this may further explain the reasons as to why the protein was not detected in the western blot as figures above show. Although tagging was achieved as the validation by PCR showed, the addition of the tag to the N-terminus of the gene may have caused a disruption to the translated protein. In the future, this experiment will need to be repeated with tagging being carried within the C-terminus.

Both of these experiments carried out by Bryant and James (2001) indicate that the localisation of the Vps45 protein is important for its interaction with its t-SNARE Tlg2. To validate the function of the N-terminal peptide further, Carpp et al (2006) carried out a study looking a mutated form of Vps45 (Vps45L177R), which aims to prevent the binding of SNARE proteins to the N-terminal hydrophobic pocket by disrupting the arch-shaped structure on domain 1, without affecting membrane transport. Results showed that Tlg2 was unable to bind to Vps45, indicating that Tlg2 and Vps45 interact via the mode 1 N-terminal region found in Vps45 (Carpp et al. 2006). As the sequences of the N-peptide binding site pocket in Vps45 *S. cerevisiae* gene is conserved in *L. mexicana*, the interaction of Vps45 to Tlg2 may also act in the same manner in *L. mexicana*.

Although this study clearly shows that Vps45 binds to the t-SNARE Tlg2, further studies carried out by Carpp et al (2006) on the interaction of Vps45 with other SNARE proteins show that Vps45 is capable of directly binding to the v-SNARE Snc2. Carpp et al (2006) demonstrates that Vps45 was able to directly interact with both Tlg2 and Snc2 (Carpp et al*.,* (2006). However, unlike the interaction of Vps45 with Tlg2, the mechanism of interaction with Snc2 is not through the N-terminal peptide domain. Both wild type Vps45 and Vps45L177R were able to bind Snc2, indicating a different mode of interaction to Tlg2. Carrying out this experiment and constructing a mutated form of *L. mexicana* that prevents the binding of SNARE proteins to the N-terminal peptide will show whether Snc2 binds to Vps45 as *S. cerevisiae* or through a different mechanism. As Vps45, two genes of Tlg2 are also present in *Leishmania*, although research has not been carried out on them. To understand the interaction of the two proteins, Tlg2 will also need to be tagged in order to investigate which Vps45 protein interacts with Tlg2.

Further studies carried out on Vps45 and its interaction with proteins show that Vps45 is also capable of binding Tlg2 SNARE complexes Tlg1p-Tlg2p-Vtilep-Snc2p (Nia J. Bryant and James 2003). Sec18-1 is an ATPase that causes the disassembly of SNARE complexes in a temperature-controlled environment. Sec18-1 at 25˚C actively catalyses SNAREs, preventing the formation of SNARE complexes, in comparison to its restrictive temperature (37˚C) (Bryant and James, 2003). Results showed that Vps45 is present at 37˚C within the yeast cell, indicating its interaction with SNARE complexes, in comparison to the wild type and 25˚C cells (Bryant and James, 2003).

To further investigate the mode of interaction between Vps45 and Tlg2-complexes, Carpp et al (2006) examined wild type, mutated Vps45 and Tlg2 cells. Wild type Vps45, Vps45W244R (a dominant negative mutant that allows SNARE complexes to form (Carpp et al. 2007)) and Vps45L117R/W224R were able to bind Tlg2-complexes, indicating that the N-peptide region

is not required for this interaction. Figure 21B also shows that the absence of the N-terminal residue in Tlg2, prevents the binding of wild-type Vps45 and the mutant Vps45L117R. However, in comparison, Vps45W244R and Vps45L117R/W244R were able to bind with Tlg2-complexes indicating interaction may occur through mode 2.

Other than yeast, the research on Vps45 localisation is currently very limited in *Leishmania*. As previously mentioned, *L. major* also contain two copies of the Vps45 gene. Its localisation and interaction with other proteins within the cell has not been established. Therefore, it is highly important to carry further research on this topic in order to understand the relationship between Vps45 and autophagy. However, although Vps45 has not been studied in *Leishmania*, the presence of Vps45 in the parasite *Plasmodium falciparum* (Pf) has been investigated. Studies have found that like yeast, Pf Vps45 is mainly located within the cytoplasm of the cell (Jonscher et al. 2019). Interestingly the Pf Vps45 protein was highly upregulated in one life cycle stage known as the trophozoites stage while the protein was not largely detected in another stage (ring stage) as results from this project showed (Jonscher et al. 2019). This further indicates that further experiments will need to be repeated on the different life cycles of *Leishmania* in order to detect the protein.

4.3 Effect of the Over-expression and Deletion of Vps45

In order to examine the effects of over-expressing Vps45 in *L. mexicana*, Vps45 over-expressor plasmids were generated using the pSSU plasmids in order to be transfected into *L. mexicana* and investigate the effects on its SNARE protein partners, the t-SNARE Tlg2 and v-SNARE Snc2. Over-expressing Vps45 can be used to understand how the upregulation of Vps45 can affect vesicle and membrane trafficking. However, although the pSSU plasmids were generated for the Vps45 protein, no protein was detected on the western blot. The reason for this may be due to the low levels of protein expression within procyclic promastigotes. As protein was only extracted from this stage, its expression may have not been large enough to be detected as previously mentioned. In the future, different stages of *Leishmania* will be harvested in order to investigate whether there is a difference in expression. Furthermore, due to the low amplification of the gene using PCR, multiple amplifications were created as shown on Figure 13. The multiple bands of DNA that were produced and gel extracted in order to get a high quantity of DNA may have caused an issue due to the addition of agarose gel that may have contaminated the sample. A positive control was also not added to the western blot for the analysis of over-expression Vps45 protein, indicating that problems with western blot techniques may have also been an issue. Therefore, a positive control will be used to confirm western blotting techniques.

Although over-expressor plasmids were not achieved in this study, many different studies have carried out experiments looking at the over-expression of Vps45 in *S. cerevisiae.* In order to examine the role of Vps45 on the concentration of Tlg2 and Snc2, Shanks et al (2012), investigated the absence and the overproduction of Vps45 on its SNARE proteins. Results illustrates that the over-expression of Vps45 causes an increase in the cellular levels of Tlg2 and Snc2 (Shanks et al. 2012). Unfortunately, research on the overexpression of Vps45 in

other *Leishmania* species is very limited. This is the first project looking at Vps45 in *L. mexicana*.

As overexpression of the Vps45 gene failed, the deletion of Vps45 in *L. mexicana* was also attempted. Vps45b and both Vps45 copies (Vps45a/b) were successfully knocked-out and replaced with drug-resistance genes . As Vps45a KO copies are present, a growth curve was conducted in order to examine the effect of Vps45 deletion.

As shown on Figure 18, the growth rate between the WT, Vps45a knockout and Vps45b knockout does not seem considerably different. Both knockouts and WT increase steadily and peak on day 4 then gradually decrease. The minor difference may be due to the presence of a second copy of a Vps45 gene and as shown on Figure 18, although not largely, the Vps45a/b double knockout grew slower and showed a peak on day 5 in comparison to the WT and single knockouts. Interestingly, the growth rate of a Vps45a heterozygote (one copy of the allele and one copy of the cassette)/Vps45b knockout seem to grow drastically differently than other knockouts, although statistical analysis needs to be carried out to examine the differences more clearly. A reason for this may be due to the drastically low expression of the protein that may somehow alter the growth of the cell or this may be due to technical errors. To investigate this further, this experiment will need to be repeated by creating single allele knockouts for both genes and examining their growth. Although replicates were used from the same knockout cell line, knockout replicates will also be created to ensure that results are highly accurate.

Similarly to the over-expression and endogenous tagging of Vps45 in other *Leishmania* species, deletion of the gene has also not been carried out. However, research on other parasites has shown that the role of Vps45 is highly important for the growth of cells. In Pf, inactivation of Vps45 caused an increase in host cytosol-filled vesicles within the cytoplasm near the food vacuole (Jonscher et al. 2019). Host cell cytosol uptake (HCCU) is known to be important in the parasite as a source of haemoglobin uptake. The inactivation of Vps45 inhibited the transport of haemoglobin to the parasite digestive vacuole and also caused arrested growth (Jonscher et al. 2019) indicating that role of Vps45 is highly important for the growth of the parasite.

The deletion of Vps45 has also been carried out and researched in *S. cerevisiae*. Shanks et al (2012) showed that the absence of Vps45 causes a significant reduction on Tlg2 and Snc2, indicating that SNARE proteins are regulated by their respective SM proteins. As well as causing a significant reduction in the concentration of Tlg2 and Snc2, the deletion of Vps45 in yeast has been shown to cause growth defects and temperature sensitivity (Shanks et al. 2012). Yeast cells have a longer doubling time in comparison to other SM mutants as well as the inability to grow on 39˚C in comparison to 30˚C. This indicates that Vps45 is required for the survival of yeast cells at elevated temperatures. The absence of Vps45 in *L. mexicana* did not a show a significant growth defect as *S. cerevisiae*, however more studies will need to be carried out to examine any other effects it may have on the membrane and vesicle trafficking. As this project only looked at life cycle stages that are within the fly (promastigotes), examining the effect of Vps45 deletion on amastigotes may show the important of Vps45

within the host. In the future, metacyclic promastigotes that differentiate into amastigotes will be incubated at 37˚C and a growth curve will be carried out.

Vps45 is mainly localised in the cystol and interact with SNARE proteins found within the TGN into the endosomal pathway, therefore, its absence may have a large impact on the morphology of the cell. Cowles et al (1994) carried out a study on the absence of Vps45 on the morphology of yeast cell. Mutated Vps45 contain an accumulation of vesicle clusters which are not observed in wild type cells and other Vps mutant cells (Cowles, Emr, and Horazdovsky 1994). This indicates that the absence of Vps45 has a major effect on vesicle sorting of yeast and illustrates an important role of Vps45. It would be highly interesting to examine this on *L. mexicana* and investigate whether the morphology changes in the absence of Vps45. To test this, samples will be harvested, and an electron microscope will be used to identify the cells and examine whether there is an accumulation of vesicles.

As previously mentioned, Tlg2 has been shown to be highly important in the regulation of the autophagy like protein Atg9, required for autophagosome formation in yeast. Studies carried out by Nair et al (2011) found that the presence of Tlg2 is required for autophagy, as the deletion of the SNARE protein caused a significant reduction in activity of the knockout cells when compared to WT cells. Results also showed that Tlg2 is required for the transport of Atg9 (Nair et al., 2011). As the deletion of Vps45 has been shown to cause a significant reduction in Tlg2, the levels of Atg9 may also be influenced, therefore affecting the regulation of autophagy. As Tlg2 genes are found in *Leishmania*, their expression can be monitored and Atg9 can be tagged in order to understand the effects of Vps45 deletion on *Leishmania*.

4.4 Conclusion and Future Research

Autophagy is an important process that allows the degradation and recycling of the cytoplasm, proteins and damaged organelles to occur. Autophagy has been shown to be highly important in the regulation of the parasite *Leishmania* during cellular differentiation and starvation conditions. Previous studies have shown autophagy related genes to be highly upregulated during metacyclogenesis. The over-expression of Atg8, the protein required for the formation of autophagosomes has been shown to increase the ability of the parasite to cause infection as well as become resistant to stress indicating that autophagy is an important process that must be controlled within the cell. The process of autophagy occurs through vesicle trafficking driven by SNARE protein complex formation that are tightly regulated by SM proteins. The main aim of this project was to investigate the SM protein Vps45 and examine its role within autophagy.

Although many experiments were attempted in this project, working with *Leishmania* can be extremely difficult. Over-expressor plasmids and endogenous tagging were attempted but not achieved. However, the deletion of Vps45 was successfully carried out and showed interesting results in regard to the Vps45a heterozygote/Vps45b knockout.

In the future, different life cycles stages will be harvested to detect the protein as this may have been an issue due to the presumed small protein expression. Tagging will be repeated

again without the ATG start codon to ensure that protein translation occurs from the tag. As homologous recombination was used to endogenously tag and over-express the Vps45 gene in WT cells, these will be repeated by using JM6571 cells and the CRISPR-Cas9 gene editing tool. To investigate the knockout cell lines further and validate them, DNA will need to be extracted and sequencing will need to be carried out to accurately investigate the Vps45 sequence present and understand the cause of the slow growth in comparison to the other knockout cell lines. Electron microscopy will also be used to examine whether there are any accumulation of vesicles present as seen on Pf and yeast. To investigate the role of autophagy and Vps45, Atg8 markers will be used to look at the expression of autophagosome and therefore autophagy.

Current research for *Leishmania* Vps45 and autophagy is very limited, therefore investigating this topic will be highly interesting in understanding the interaction of *Leishmania* with its host as well as understanding its survival mechanisms through autophagy. This will all be highly beneficial in the production of improved diagnostics methods as well as treatments. As only one year was spent working on this project, understanding the connection between autophagy and Vps45 was not achieved.

Reference

Abeliovich, Hagai, Tamara Darsow, and Scott D. Emr. 1999. “Cytoplasm to Vacuole Trafficking of Aminopeptidase I Requires a T-SNARE-Sec1p Complex Composed of Tlg2p and Vps45p.” *EMBO Journal*. https://doi.org/10.1093/emboj/18.21.6005.

Akhoundi, Mohammad, Katrin Kuhls, Arnaud Cannet, Jan Votýpka, Pierre Marty, Pascal Delaunay, and Denis Sereno. 2016. “A Historical Overview of the Classification, Evolution, and Dispersion of Leishmania Parasites and Sandflies.” *PLoS Neglected Tropical Diseases*. https://doi.org/10.1371/journal.pntd.0004349.

Alves, Fabiana, Graeme Bilbe, Séverine Blesson, Vishal Goyal, Séverine Monnerat, Charles Mowbray, Gina Muthoni Ouattara, et al. 2018. “Recent Development of Visceral Leishmaniasis Treatments: Successes, Pitfalls, and Perspectives.” *Clinical Microbiology Reviews*. https://doi.org/10.1128/CMR.00048-18.

Ambit, Audrey, Kerry L. Woods, Benjamin Cull, Graham H. Coombs, and Jeremy C. Mottram. 2011. “Morphological Events during the Cell Cycle of Leishmania Major.” *Eukaryotic Cell*. https://doi.org/10.1128/EC.05118-11.

Archbold, Julia K., Andrew E. Whitten, Shu Hong Hu, Brett M. Collins, and Jennifer L. Martin. 2014. “SNARE-Ing the Structures of Sec1/Munc18 Proteins.” *Current Opinion in Structural Biology*. https://doi.org/10.1016/j.sbi.2014.09.003.

Aronson, Naomi, Barbara L. Herwaldt, Michael Libman, Richard Pearson, Rogelio Lopez-Velez, Peter Weina, Edgar Carvalho, Moshe Ephros, Selma Jeronimo, and Alan Magill. 2017a. “Diagnosis and Treatment of Leishmaniasis: Clinical Practice Guidelines by the Infectious Diseases Society of America (IDSA) and the American Society of Tropical Medicine and Hygiene (ASTMH).” *American Journal of Tropical Medicine and Hygiene*. https://doi.org/10.4269/ajtmh.16-84256.

Basselin, Mireille, Hubert Denise, Graham H. Coombs, and Michael P. Barrett. 2002. “Resistance to Pentamidine in Leishmania Mexicana Involves Exclusion of the Drug from the Mitochondrion.” *Antimicrobial Agents and Chemotherapy*. https://doi.org/10.1128/AAC.46.12.3731-3738.2002.

Bates, Paul A. 2007. “Transmission of Leishmania Metacyclic Promastigotes by Phlebotomine Sand Flies.” *International Journal for Parasitology*. https://doi.org/10.1016/j.ijpara.2007.04.003.

Besteiro, Sébastien, Graham H. Coombs, and Jeremy C. Mottram. 2006. “The SNARE Protein Family of Leishmania Major.” *BMC Genomics*. https://doi.org/10.1186/1471-2164-7-250.

Besteiro, Sébastien, R. A M Williams, Lesley S. Morrison, Graham H. Coombs, and Jeremy C. Mottram. 2006. “Endosome Sorting and Autophagy Are Essential for Differentiation and Virulence of Leishmania Major.” *Journal of Biological Chemistry*. https://doi.org/10.1074/jbc.M512307200.

Besteiro, Sébastien, Roderick A M Williams, Graham H. Coombs, and Jeremy C. Mottram. 2007. “Protein Turnover and Differentiation in *Leishmania*.” *International Journal for Parasitology*. https://doi.org/10.1016/j.ijpara.2007.03.008.

Bowers, Katherine, and Tom H. Stevens. 2005. “Protein Transport from the Late Golgi to the Vacuole in the Yeast Saccharomyces Cerevisiae.” *Biochimica et Biophysica Acta - Molecular Cell Research*. https://doi.org/10.1016/j.bbamcr.2005.04.004.

Bryant, N. J., and D. E. James. 2001. “Vps45p Stabilizes the Syntaxin Homologue Tlg2p and Positively Regulates SNARE Complex Formation.” *EMBO Journal*. https://doi.org/10.1093/emboj/20.13.3380.

Bryant, Nia J., and David E. James. 2003. “The Sec1p/Munc18 (SM) Protein, Vps45p, Cycles on and off Membranes during Vesicle Transport.” *Journal of Cell Biology*. https://doi.org/10.1083/jcb.200212078.

Carneiro, Pedro Paulo, Jacilara Conceição, Michael Macedo, Viviane Magalhães, Edgar M. Carvalho, and Olivia Bacellar. 2016. “The Role of Nitric Oxide and Reactive Oxygen Species in the Killing of Leishmania Braziliensis by Monocytes from Patients with Cutaneous Leishmaniasis.” *PLoS ONE*. https://doi.org/10.1371/journal.pone.0148084.

Carpp, Lindsay N., Leonora F. Ciufo, Scott G. Shanks, Alan Boyd, and Nia J. Bryant. 2006. “The Sec1p/Munc18 Protein Vps45p Binds Its Cognate SNARE Proteins via Two Distinct Modes.” *Journal of Cell Biology* 173 (6): 927–36. https://doi.org/10.1083/jcb.200512024.

Carpp, Lindsay N., Scott G. Shanks, Marion S. Struthers, and Nia J. Bryant. 2007. “Cellular Levels of the Syntaxin Tlg2p Are Regulated by a Single Mode of Binding to Vps45p.” *Biochemical and Biophysical Research Communications*. https://doi.org/10.1016/j.bbrc.2007.09.067.

Chakravarty, Jaya, and Shyam Sundar. 2019. “Current and Emerging Medications for the Treatment of Leishmaniasis.” *Expert Opinion on Pharmacotherapy*. https://doi.org/10.1080/14656566.2019.1609940.

Cowles, C, S Emr, and B Horazdovsky. 1994. “Mutations in the VPS45 Gene, a SEC1 Homologue, Result in Vacuolar Protein Sorting Defects and Accumulation of Membrane Vesicles.” *Journal of Cell Science*.

Dagger, Francehuli, Camila Bengio, Angel Martinez, and Carlos Ayesta. 2018. “Leishmania Mexicana Differentiation Involves a Selective Plasma Membrane Autophagic-like Process.” *Cell Stress and Chaperones*. https://doi.org/10.1007/s12192-017-0864-z.

Dean, Samuel, Jack Sunter, Richard J. Wheeler, Ian Hodkinson, Eva Gluenz, and Keith Gull. 2015. “A Toolkit Enabling Efficient, Scalable and Reproducible Gene Tagging in Trypanosomatids.” *Open Biology*. https://doi.org/10.1098/rsob.140197.

Dorlo, Thomas P.C., Manica Balasegaram, Jos H. Beijnen, and Peter J. de vries. 2012. “Miltefosine: A Review of Its Pharmacology and Therapeutic Efficacy in the Treatment of Leishmaniasis.” *Journal of Antimicrobial Chemotherapy*. https://doi.org/10.1093/jac/dks275.

Dorlo, Thomas P.C., Manica Balasegaram, María Angeles Lima, Peter J. De Vries, Jos H. Beijnen, and Alwin D.R. Huitema. 2012. “Translational Pharmacokinetic Modelling and Simulation for the Assessment of Duration of Contraceptive Use after Treatment with Miltefosine.” *Journal of Antimicrobial Chemotherapy*. https://doi.org/10.1093/jac/dks164.

Dostálová, Anna, and Petr Volf. 2012. “Leishmania Development in Sand Flies: Parasite-Vector Interactions Overview.” *Parasites and Vectors*. https://doi.org/10.1186/1756-3305-5-276.

Dulubova, Irina, Tomohiro Yamaguchi, Yan Gao, Sang Won Min, Iryna Huryeva, Thomas C. Südhof, and Josep Rizo. 2002. “How Tlg2p/Syntaxin 16 ‘snares’ Vps45.” *EMBO Journal*. https://doi.org/10.1093/emboj/cdf381.

Eisemann, Travis J., Frederick Allen, Kelly Lau, Gregory R. Shimamura, Philip D. Jeffrey, and Frederick M. Hughson. 2020. “The Sec1/Munc18 Protein Vps45 Holds the Qa-Snare Tlg2 in an Open Conformation.” *ELife*. https://doi.org/10.7554/ELIFE.60724.

Feng, Yuchen, and Daniel J. Klionsky. 2017. “Autophagic Membrane Delivery through ATG9.” *Cell Research*. https://doi.org/10.1038/cr.2017.4.

Fiebig, Michael, Steven Kelly, and Eva Gluenz. 2015. “Comparative Life Cycle Transcriptomics Revises Leishmania Mexicana Genome Annotation and Links a Chromosome Duplication with Parasitism of Vertebrates.” *PLoS Pathogens*. https://doi.org/10.1371/journal.ppat.1005186.

Frank, Benjamin, Ana Marcu, Antonio Luis De Oliveira Almeida Petersen, Heike Weber, Christian Stigloher, Jeremy C. Mottram, Claus Juergen Scholz, and Uta Schurigt. 2015. “Autophagic Digestion of Leishmania Major by Host Macrophages Is Associated with Differential Expression of BNIP3, CTSE, and the MiRNAs MiR-101c, MiR-129, and MiR-210.” *Parasites and Vectors*. https://doi.org/10.1186/s13071-015-0974-3.

Furgason, M. L. M., C. MacDonald, S. G. Shanks, S. P. Ryder, N. J. Bryant, and M. Munson. 2009. “The N-Terminal Peptide of the Syntaxin Tlg2p Modulates Binding of Its Closed Conformation to Vps45p.” *Proceedings of the National Academy of Sciences*. https://doi.org/10.1073/pnas.0902976106.

Genois, Marie Michelle, Marie Plourde, Chantal Éthier, Gaétan Roy, Guy G. Poirier, Marc Ouellette, and Jean Yves Masson. 2015. “Roles of Rad51 Paralogs for Promoting Homologous Recombination in Leishmania Infantum.” *Nucleic Acids Research*. https://doi.org/10.1093/nar/gkv118.

Ghosh, Rajeshwary, and J. Scott Pattison. 2018. “Macroautophagy and Chaperone-Mediated Autophagy in Heart Failure: The Known and the Unknown.” *Oxidative Medicine and Cellular Longevity*. https://doi.org/10.1155/2018/8602041.

Giri, Sagnik, and Chandrima Shaha. 2019. “Leishmania Donovani Parasite Requires Atg8 Protein for Infectivity and Survival under Stress.” *Cell Death and Disease*. https://doi.org/10.1038/s41419-019-2038-7.

Gossage, Sharon M., Matthew E. Rogers, and Paul A. Bates. 2003. “Two Separate Growth Phases during the Development of Leishmania in Sand Flies: Implications for Understanding the Life Cycle.” *International Journal for Parasitology*. https://doi.org/10.1016/S0020-7519(03)00142-5.

Goto, Hiro, and José Angelo Lauletta Lindoso. 2010. “Current Diagnosis and Treatment of Cutaneous and Mucocutaneous Leishmaniasis.” *Expert Review of Anti-Infective Therapy*. https://doi.org/10.1586/eri.10.19.

Halachmi, Naomi, and Zeev Lev. 1996. “The Sec1 Family: A Novel Family of Proteins Involved in Synaptic Transmission and General Secretion.” *Journal of Neurochemistry*. https://doi.org/10.1046/j.1471-4159.1996.66030889.x.

Haldar, Arun Kumar, Pradip Sen, and Syamal Roy. 2011. “Use of Antimony in the Treatment of Leishmaniasis: Current Status and Future Directions.” *Molecular Biology International*. https://doi.org/10.4061/2011/571242.

Inbar, Ehud, V. Keith Hughitt, Laura A.L. Dillon, Kashinath Ghosh, Najib M. El-Sayed, and David L. Sacks. 2017. “The Transcriptome of Leishmania Major Developmental Stages in Their Natural Sand Fly Vector.” *MBio*. https://doi.org/10.1128/mBio.00029-17.

Jonscher, Ernst, Sven Flemming, Marius Schmitt, Ricarda Sabitzki, Nick Reichard, Jakob Birnbaum, Bärbel Bergmann, Katharina Höhn, and Tobias Spielmann. 2019. “PfVPS45 Is Required for Host Cell Cytosol Uptake by Malaria Blood Stage Parasites.” *Cell Host and Microbe*. https://doi.org/10.1016/j.chom.2018.11.010.

Lamb, Christopher A., Hannah C. Dooley, and Sharon A. Tooze. 2013. “Endocytosis and Autophagy: Shared Machinery for Degradation.” *BioEssays*. https://doi.org/10.1002/bies.201200130.

Lamb, Christopher A., Tamotsu Yoshimori, and Sharon A. Tooze. 2013. “The Autophagosome: Origins Unknown, Biogenesis Complex.” *Nature Reviews Molecular Cell Biology*. https://doi.org/10.1038/nrm3696.

Levine, Beth, and Daniel J. Klionsky. 2004. “Development by Self-Digestion: Molecular Mechanisms and Biological Functions of Autophagy.” *Developmental Cell*. https://doi.org/10.1016/S1534-5807(04)00099-1.

Liu, Dong, and Jude E. Uzonna. 2012. “The Early Interaction of Leishmania with Macrophages and Dendritic Cells and Its Influence on the Host Immune Response.” *Frontiers in Cellular and Infection Microbiology*. https://doi.org/10.3389/fcimb.2012.00083.

Nair, Usha, Anjali Jotwani, Jiefei Geng, Noor Gammoh, Diana Richerson, Wei Lien Yen, Janice Griffith, et al. 2011a. “SNARE Proteins Are Required for Macroautophagy.” *Cell*. https://doi.org/10.1016/j.cell.2011.06.022.

Nichols, B. J., J. C.M. Holthuis, and H. R.B. Pelham. 1998. “The Sec1p Homologue Vps45p Binds to the Syntaxin Tlg2p.” *European Journal of Cell Biology*. https://doi.org/10.1016/S0171-9335(98)80084-8.

Novais, Fernanda O., Ba T. Nguyen, Daniel P. Beiting, Lucas P. Carvalho, Nelson D. Glennie, Sara Passos, Edgar M. Carvalho, and Phillip Scott. 2014. “Human Classical Monocytes Control the Intracellular Stage of Leishmania Braziliensis by Reactive Oxygen Species.” *Journal of Infectious Diseases*. https://doi.org/10.1093/infdis/jiu013.

Pace, David. 2014. “Leishmaniasis.” *Journal of Infection* 69 (November): S10–18. https://doi.org/10.1016/J.JINF.2014.07.016.

Parzych, Katherine R., and Daniel J. Klionsky. 2014. “An Overview of Autophagy: Morphology, Mechanism, and Regulation.” *Antioxidants and Redox Signaling*. https://doi.org/10.1089/ars.2013.5371.

Quiroga, Cristina, Varsovia Cevallos, Diego Morales, Manuel E. Baldeón, Paúl Cárdenas, Patricio Rojas-Silva, and Patricio Ponce. 2017. "Molecular Identification of Leishmania Spp. in Sand Flies (Diptera: Psychodidae, Phlebotominae) From Ecuador." *Journal of Medical Entomology*. https://doi.org/10.1093/jme/tjx122.

Ramalho-Ortigao, Marcelo. 2010. "Sand Fly-Leishmania Interactions: Long Relationships Are Not Necessarily Easy." *The Open Parasitology Journal*. https://doi.org/10.2174/1874421401004010195.

Ramírez, Juan David, Carolina Hernández, Cielo M. León, Martha S. Ayala, Carolina Flórez, and Camila González. 2016. "Taxonomy, Diversity, Temporal and Geographical Distribution of Cutaneous Leishmaniasis in Colombia: A Retrospective Study." *Scientific Reports*. https://doi.org/10.1038/srep28266.

Reithinger, Richard, Jean-Claude Dujardin, Hechmi Louzir, Claude Pirmez, Bruce Alexander, and Simon Brooker. 2007. "Cutaneous Leishmaniasis." *The Lancet Infectious Diseases* 7 (9): 581–96. https://doi.org/10.1016/S1473-3099(07)70209-8.

Rogers, Matthew B., James D. Hilley, Nicholas J. Dickens, Jon Wilkes, Paul A. Bates, Daniel P. Depledge, David Harris, et al. 2011. "Chromosome and Gene Copy Number Variation Allow Major Structural Change between Species and Strains of Leishmania." *Genome Research*. https://doi.org/10.1101/gr.122945.111.

Shanks, Scott G., Lindsay N. Carpp, Marion S. Struthers, Rebecca K. McCann, and Nia J. Bryant. 2012. "The Sec1/Munc18 Protein Vps45 Regulates Cellular Levels of Its SNARE Binding Partners Tlg2 and Snc2 in Saccharomyces Cerevisiae." *PLoS ONE*. https://doi.org/10.1371/journal.pone.0049628.

Stepp, J. David, Kristen Huang, and Sandra K. Lemmon. 1997. "The Yeast Adaptor Protein Complex, AP-3, Is Essential for the Efficient Delivery of Alkaline Phosphatase by the Alternate Pathway to the Vacuole." *Journal of Cell Biology*. https://doi.org/10.1083/jcb.139.7.1761.

Steverding, Dietmar. 2017. "The History of Leishmaniasis." *Parasites and Vectors*. https://doi.org/10.1186/s13071-017-2028-5.

Stockdale, Lisa, and Robert Newton. 2013. "A Review of Preventative Methods against Human Leishmaniasis Infection." *PLoS Neglected Tropical Diseases*. https://doi.org/10.1371/journal.pntd.0002278.

Sundar, Shyam, and M. Rai. 2002. "Laboratory Diagnosis of Visceral Leishmaniasis." *Clinical and Diagnostic Laboratory Immunology*. https://doi.org/10.1128/CDLI.9.5.951-958.2002.

Sunter, Jack, and Keith Gull. 2017. "Shape, Form, Function and Leishmania Pathogenicity: From Textbook Descriptions to Biological Understanding." *Open Biology*. https://doi.org/10.1098/rsob.170165.

Sunyoto, Temmy, Julien Potet, and Marleen Boelaert. 2018. "Why Miltefosine - A Life-Saving Drug for Leishmaniasis-Is Unavailable to People Who Need It the Most." *BMJ Global Health*. https://doi.org/10.1136/bmjgh-2018-000709.

Thumm, M., R. Egner, B. Koch, M. Schlumpberger, M. Straub, M. Veenhuis, and D. H. Wolf.

1994. “Isolation of Autophagocytosis Mutants of Saccharomyces Cerevisiae.” *FEBS Letters*. https://doi.org/10.1016/0014-5793(94)00672-5.

Toonen, Ruud F.G., and Matthijs Verhage. 2003. “Vesicle Trafficking: Pleasure and Pain from SM Genes.” *Trends in Cell Biology*. https://doi.org/10.1016/S0962-8924(03)00031-X.

Torres-Guerrero, Edoardo, Marco Romano Quintanilla-Cedillo, Julieta Ruiz-Esmenjaud, and Roberto Arenas. 2017. “Leishmaniasis: A Review.” *F1000Research*. https://doi.org/10.12688/f1000research.11120.1.

Tsukada, Miki, and Yoshinori Ohsumi. 1993. “Isolation and Characterization of Autophagy-Defective Mutants of Saccharomyces Cerevisiae.” *FEBS Letters*. https://doi.org/10.1016/0014-5793(93)80398-E.

Walker, Dawn M., Steve Oghumu, Gaurav Gupta, Bradford S. McGwire, Mark E. Drew, and Abhay R. Satoskar. 2013. “Mechanisms of Cellular Invasion by Intracellular Parasites.” *Cellular and Molecular Life Sciences*. https://doi.org/10.1007/s00018-013-1491-1.

Wiese, Martin, Daniela Kuhn, and Christoph G. Grünfelder. 2003. “Protein Kinase Involved in Flagellar-Length Control.” *Eukaryotic Cell*. https://doi.org/10.1128/EC.2.4.769-777.2003.

Williams, Roderick A., Laurence Tetley, Jeremy C. Mottram, and Graham H. Coombs. 2006. “Cysteine Peptidases CPA and CPB Are Vital for Autophagy and Differentiation in Leishmania Mexicana.” *Molecular Microbiology*. https://doi.org/10.1111/j.1365-2958.2006.05274.x.

Xie, Zhiping, and Daniel J. Klionsky. 2007. “Autophagosome Formation: Core Machinery and Adaptations.” *Nature Cell Biology*. https://doi.org/10.1038/ncb1007-1102.

Zhuang, Xiaohong, Kin Pan Chung, Yong Cui, Weili Lin, Caiji Gao, Byung Ho Kang, Liwen Jiang, and Diane C. Bassham. 2017. “ATG9 Regulates Autophagosome Progression from the Endoplasmic Reticulum in Arabidopsis.” *Proceedings of the National Academy of Sciences of the United States of America*. https://doi.org/10.1073/pnas.1616299114.