Development of an inducible system for *Leishmania* gene deletion; application to the cell cycle protein kinase CRK3

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Abstract

Leishmania spp. are protozoan parasites that infect humans and other vertebrates to cause a spectrum of disease, ranging from cutaneous ulceration to visceral dissemination dependent on the species. Leishmaniasis is prevalent across the developing world and is a major global health issue, yet difficulties in the efficacy and administration route of current anti-leishmanial treatments means the existing drug repertoire is inadequate. To address this, further research and development measures are necessary to identify *Leishmania* proteins representing useful targets for drug inhibition. Essential genes encode proteins that are necessary for parasite survival and therefore represent suitable drug targets, but the study of such genes is limited by the absence of a conditional deletion system. A family of proteins which has previously been shown to regulate crucial aspects of *Leishmania* biology are the protein kinases. Protein kinases have been validated in mammalian systems as drug targets in cancer therapy, therefore they represent a promising avenue for research into anti-leishmanial drugs. The cdc-related kinases CRK3 has been studied in particular depth in *Leishmania,* and current reverse genetic techniques have implicated expression of CRK3 as essential to promastigote survival. CRK3 regulates the cell cycle as demonstrated by treatment of cdc2 inhibitors, but a lack of a system to regulate expression prevents more specific phenotypic dissection of the role of CRK3. In addition the validation of CRK3 as a drug target has been limited by an absence of a conditional genetic system to ablate the gene in mammalian infective amastigotes.

To regulate CRK3 expression in a conditional manner to assess its function in the cell cycle of promastigotes and validate it as essential for amastigotes, we have implemented an inducible gene deletion system based on a dimerised Cre recombinase (diCre) for use in *L. mexicana*. Cre recombinase mediates the excision of DNA sequences flanked by 34bp loxP sites ('floxed'). diCre is encoded as two separate subunits each linked to rapamycin binding domains (FRB and FKBP12); therefore recombinase activity is induced by rapamycin treatment which causes dimerisation of the subunits. Our method involves replacing both *CRK3* alleles with a 'floxed' *CRK3* open reading frame and the diCre coding sequence through promastigote transfection and homologous recombination.

Induction of diCre through rapamycin treatment of promastigotes results in highly efficient deletion of *CRK3* and a distinct growth arrest phenotype corresponding to a block in G2/M. Induced loss of *CRK3* can be complemented by expression of a *CRK3* transgene but not by expression of an inactive site (T178E) *CRK3* mutant, showing that protein kinase activity is crucial for CRK3 function. Significantly, inducible deletion of *CRK3* in stationary phase promastigotes prevents the establishment of murine infection, thereby demonstrating an essential role in the amastigote cell cycle to further validate CRK3 as a drug target.

Promisingly, inducible deletion is functional in lesion-derived amastigotes and will enable direct phenotypic assessment following essential gene loss in this life cycle stage. To establish a basis for future *in vivo* application of diCre in *Leishmania*, a murine infection model was developed with which to track bioluminescent parasite burden by *in vivo* imaging and assess innate immune cell recruitment to the site of infection by flow cytometry analysis. The combination of functional gene regulation in amastigotes and measures of parasite burden and immune response will yield a powerful tool for the further study of *Leishmania* genes encoding suitable drug targets.

The application of the diCre technique to *Leishmania* would be greatly benefitted by targeting genes where there is evidence of a regulatory role of orthologous genes in model organisms. The utilisation of genome or protein family-wide RNAi screens in *Trypanosoma brucei* has identified a number of protein kinases which regulate the differentiation of the parasite between life cycle stages. The repressor of differentiation (RDK1) protein regulates bloodstream form to procyclic form differentiation in *T. brucei*, and the identification of a protein in *L. mexicana* with high sequence identity suggested a potentially analogous role in preventing *Leishmania* from undergoing amastigote to promastigote differentiation *in vivo*. To assess this, a cell line was generated deficient in RDK1 but no effect on differentiation was identified, as parasites were able to maintain murine infection and differentiate between life cycle stages.

This study represents an important addition to the reverse genetic toolkit to study aspects of cell cycle regulation *in vitro,* and further assess essential genes

as drug targets by deletion in amastigotes. The application of the diCre conditional deletion method will enhance the discovery and evaluation of suitable drug targets in *Leishmania* by phenotypic analysis.

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"THE POSSIBILITY OF PHYSICAL AND MENTAL COLLAPSE IS NOW VERY REAL. NO SYMPATHY FOR THE DEVIL, KEEP THAT IN MIND. BUY THE TICKET, TAKE THE RIDE."- HUNTER S. THOMPSON, FEAR AND LOATHING IN LAS VEGAS

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Author's Declaration

The results stated in this thesis are my own work, except where otherwise stated.

Samuel Martin Duncan

Abbreviations

aa	amino acid
AGO1	argonaute protein 1
APC	Antigen presenting cell
ATP	adenosine triphosphate
BLA	blasticidin deaminase
BLEO	bleomycin / phleomycin
bp	base pair
BSA	bovine serum albumin
BSD	blasticidin
BSF	bloodstream form *T. brucei*
cAMP	cyclic adenosine monophosphate
CCL	chemokine ligand
CCR	chemokine receptor
CDK	cyclin dependent kinase
CDS	coding sequence
CL	cutaneous leishmaniasis
CPB	cysteine peptidase B
CRK	cdc2 related kinase
CYC	cyclin
DAPI	4,6-diamidino-2-phenylindole (nucleic acid stain)
DC	Dendritic cell
DCL	diffuse cutaneous leishmaniasis
dd	destabilisation domain
DDC	Dermal dendritic cell
DDT	dichlorodiphenyltrichloroethane
DFMO	α-difluoromethylornithine
DHFR-TS	dihydrofolate reductase-thymidylate synthase
dLN	draining lymph node
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DNDi	Drugs for neglected diseases initiative
dsRNA	double stranded RNA
DTT	dithiothreitol
EDTA	ethylene diamine tetra acetic acid
EF-1α	elongation factor 1 a
ePK	eukaryotic protein kinase
ER	estrogen receptor
FACS	fluorescence activated cell sorting

FAZ	flagellar attachment zone
FCS	fetal calf serum
FR	flanking region
GCV	ganciclovir
gDNA	genomic DNA
GFP	green fluorescent protein
HA	human influenza hemagglutinin
HIS	histidine
HRP	horseradish peroxidase
HYG	hygromycin B
iC3b	Inactive C3b
IL	interleukin
iNOS	Inducible nitric oxide synthase
IVIS	in vivo imaging system
K	kinetoplast
Kb	kilo base
kDa	kilo Dalton
LB	Luria bertani medium
LBD	ligand binding domain
LCL	localised cutaneous leishmaniasis
loxP	locus of crossover of bacteriophage P1
LPG	lipophosphoglycan
LUC2	firefly luciferase
m	milli / metre
M	molar
MAC	membrane attack complex
MCL	mucocutaneous leishmaniasis
Mo-DC	monocyte derived dendritic cell
Mo-Mϕ	monocyte derived macrophage
mRNA	messenger ribonucleic acid
n	nano
N	nucleus
NEO	neomycin phosphotransferase
NETs	neutrophil extracellular traps
NLS	nuclear localisation signal
nt	nucleotide
ORF	open reading frame
PAC	puromycin acetyltransferase
PAS	polyadenylation site
PBS	phosphate buffered saline

PCF	procyclic form T. brucei
PCR	polymerase chain reaction
PK	protein kinase
PM	peritrophic matrix
PPG	proteophosphoglycan
qPCR	quantitave PCR
RAP	rapamycin
RE9H	red-shifted luciferase
RNA	ribonucleic acid
RNAi	ribonucleic acid interference
rRNA	ribosomal ribonucleic acid
SAS	splice acceptor site
SAT	streptothricin acetyltransferase
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SSG	sodium stibogluconate
SSU	ribosomal small subunit
TGF-β	tumour growth factor beta
Th	T helper cell
TK	thymidine kinase
TLR	toll-like receptor
TMP	trimethroprim
TS	targeting sequence
UTR	untranslated region
UV	ultra violet
V	volts
v/v	volume to volume
VL	visceral leishmaniasis
VSG	variant surface glycoprotein
w/v	weight to volume
WHO	World Health Organisation
µ	micro

1 GENERAL INTRODUCTION

1.1 *Leishmania* sp.

1.1.1 Disease prevalence

Leishmania are parasitic protozoa causing a spectrum of disease in 98 countries, ranging from localised cutaneous lesions to visceral infections of the liver and spleen. Approximately 1.3 million new cases occur each year (Alvar et al. 2012), and an estimated 20,000 to 30,000 deaths annually. Leishmaniasis manifests as a spectrum of disease which ranges from Old World cutaneous infections in endemic areas such as North Africa, the Mediterranean, the Middle East, India and Central Asia to deadly visceral infections in East Africa and the Sudan *(L. donovani*) (WHO Committee 2010). Cutaneous infections can arise from anthroponotic transmission mainly in urban areas (*L. tropica*) resulting in skin lesions that usually heal within 6-15 months or zoonotic transmission in more rural areas (*L. major*) which also causes lesions which heal within 2-4 months but can persist for up to five years. In New World leishmaniasis, the main incidences occur in Central and South America, and mainly manifest as cutaneous lesions (*L. mexicana, L. braziliensis, L. panamensis*, *L. guyanensis*) or chronic mucocutaneous infections where infections spread to mucosal membranes in the throat, mouth and nose. Such infections cause severe damage and disfiguration to these areas and are caused by species such as *L. braziliensis,* and *L. panamensis*, whilst visceral leishmaniasis is generally caused by *L. infantum*, also referred to as *L. chagasi* in South America (**Figure** 1:1).

The WHO highlights the fact that the major contributor to the wide-spread prevalence and risk of infection with leishmaniasis is poverty (WHO Committee 2010). Poor socioeconomic conditions makes those at risk more susceptible to infection. For example, malnutrition increases the risk of the disease becoming visceral, and is compounded by the increased risk of transmission already associated with poverty. Environmental factors exacerbate the risk of infection by increasing human interactions with the sand fly vectors that are attracted to areas of overcrowding which yield a good source of blood-meals. Urbanisation exacerbates infection risk by increasing the concentration of hosts and therefore disease reservoir, but also through the expansion of cities into previously

forested areas where sand fly populations are established. Migration of non-immune people to endemic areas acts as a reservoir of susceptible hosts for the parasite can also lead to an accelerate incidence of infection. Much of the industry and therefore available jobs in countries where leishmaniasis is prevalent involve agriculture and mining which take place in previously forested areas, bringing labourers in contact with sand fly populations and increasing the rate of transmission (Ramdas 2012). Compounding this increased transmission rate, access to primary health care in such regions may also be difficult.

To tackle the global health issue associated with leishmaniasis, a number of programmes have been established. The London Declaration was drawn up in 2012 as a collaborative effort between funding bodies such as the Bill and Melinda Gates Foundation and pharmaceutical companies such as Novartis to tackle the neglected tropical diseases (NTDs). A chief implementation of this strategy is the expansion of existing drug access programmes to increase the availability of anti-leishmanial treatments, with a further provision of funds to advance research and development into new treatments, improved access to diagnosis and interventions for visceral leishmaniasis. The aim of the declaration is to achieve control of visceral forms of the disease by 2020, and an increased availability and reduced cost of anti-leishmanials. The publishing of the third progress report of the London Declaration (2015) indicates a reduced rate of visceral leishmaniasis between 2011-2013, however the number of drug donations to treat the disease are low relative to other NTDs such as lymphatic filariasis and onchocerciasis.

The Drugs for Neglected Disease initiative (DNDi) was established in 2003 as a collaborative effort between researchers across the world to expand the current repertoire of anti-leishmanial drugs. Development of compounds through combination of existing therapies and further assessment of existing lead compounds are their short and medium term approaches which have yielded success in the field (DNDi, 2015). A long term approach is the focus on the development of safe, cheap and efficacious medicines with a simple administration route to enhance the cure rate of visceral and cutaneous leishmaniasis. The emphasis on improved drug research and development

demonstrates that the potential for this field of research is critical for helping treat the leishmaniasis.

1.1.2 Leishmaniasis: recent classification of an ancient disease

The diseases caused by *Leishmania* have been documented over the course of recorded human history, from descriptions of cutaneous lesions from texts owned by the Assyrian King Ashurbanipal dating back as far as 2500BC (Cox 2002), to the representation of mucocutaneous lesions by intricate, anthropomorphic pottery called 'huacos' from pre-Hispanic Peru and Bolivia in AD200-1000 (Gade 1979). More recently, rounded bodies were observed by light microscopy from lesion biopsies and implicated as the causative agents of 'tropical ulcers' by many including Henry Wright in 1903 (Wright 1903), leading to the subsequent classification as *L. tropica* in 1906 (Lainson 2010). However, it was the Scottish pathologist and army medical officer William Leishman who first classified these parasites as the causative agents of the visceral disease 'kala-azar' after being stationed in colonial India. In 1903, he published an account of the discovery of amastigotes in the British Medical Journal by staining infected spleen samples with 'Leishman's stain', a methylene blue and eosin mixture. The parallel discovery of *Leishmania* parasites by light microscopy of biopsies obtained from patients presenting with visceral disease by Charles Donovan in India led to the first species being named *Leishmania donovani*. The identification of a distinct, New World species was conducted by the Brazilian clinican Gaspar Vianna, who identified the causative agent of American cutaneous Leishmaniasis in 1911, naming it *L. braziliensis* (Lainson 2010). The distinction of this species from *L. donovani* and *L. tropica* was an early indication of their diversity, and over the course of the 20th century our increased understanding of the variety of *Leishmania* species has been aided by advancing genetic techniques to phenotype and categorise the genus (Lye et al. 2010, Fraga et al. 2013). To date, over 40 species are deemed as *Leishmania*, and all are parasitic in lifestyle. There are two sub genus within *Leishmania* broadly corresponding to Old World, *L.* (*Leishmania*) and New World, *L.* (*Viannia*), however some species such as those in the *L. mexicana* complex and *L. chagasi* do not fit this generalisation because they can be grouped into the *L.* (*Leishmania*) sub genus despite presenting as New World infections. Interestingly, *L. chagasi* is not distinct from *L. infantum* and likely represents

the same species but in South America as opposed to the Old World. In terms of the human disease which each species cause, these can be broadly categorized in terms of four main clinical manifestations; local cutaneous or diffuse cutaneous, mucocutaneous, and visceral (**Figure** 1:1). A further complication with categorising the variety of *Leishmania* species was first documented by Ravel et al in 2006, where hybrids between *L. major* and *L. infantum* were isolated from immunocompromised patients in Portugal. These genetic chimeras are evidence that cross-species genetic exchange could be driven by selective pressure, and further complicates the classification of *Leishmania* genus. In addition, such selection could drive the generation of drug resistance in the field, and signifies the adaptability of *Leishmania* sp.

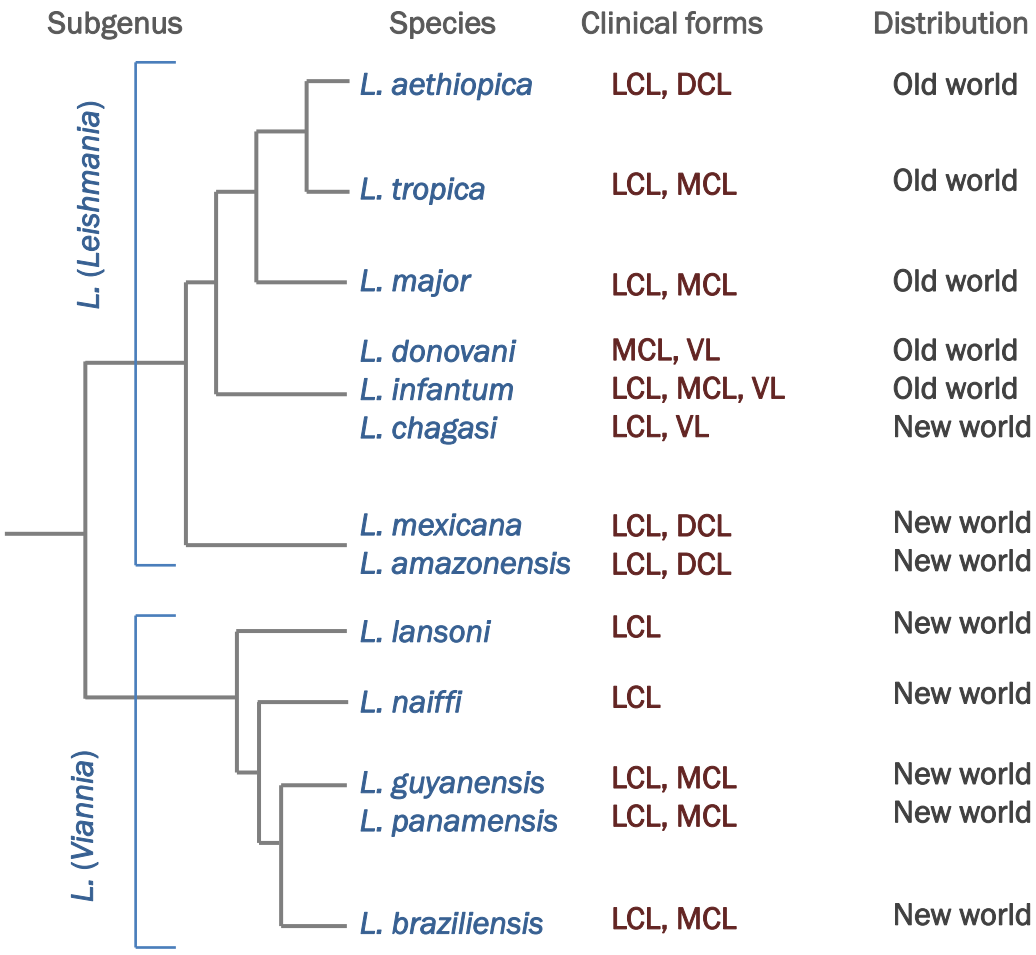


Figure 1:1- A phylogenetic representation of the key human infective *Leishmania* species, their clinical forms and geographical distributions. LCL, localised cutaneous leishmaniasis; DCL, diffuse cutaneous leishmaniasis; MCL, mucocutaneous leishmaniasis; VL, visceral leishmaniasis. Adapted from Fraga et al. (2013).

1.1.3 Available treatments for leishmaniasis

Unlike the accelerated rate of species discovery, methods for treatment and prevention of leishmaniasis have remained comparatively stagnant over the course of the 20th century. There are no prophylactic drugs, nor vaccines available to confer resistance to infection. This is mainly due to the complexity of the immune response to leishmania infection, with research into this area uncovering ever expanding complexities into the host-parasite interactions which mediate immunity and drive infection (Kaye & Scott 2011). The practice of deliberate infection with leishmania (termed 'leishmaniasation') at an obscured location on the body such as the buttocks has been a method to prevent subsequent infection and establishment of lesions on a more visible site such as the face. Although highly undesirable, the concomitant immunity conferred by leishmanisation highlights the potential of vaccine strategies, with the use of attenuated parasites a possible route for canine vaccination if not possible for humans. Instead, recombinant protein formulations yielding some success and the application of DNA vaccination (Dunning 2009) may yield useful preparations for the induction of an appropriate anti-leishmanial response. The development of such vaccines is comprehensively reviewed by (Kumar & Engwerda 2014).

In contrast to an available vaccine, there is a repertoire of drugs with which to treat leishmaniasis (**Table** 1-1). The first use of urea stibamine by Sir Upendranath Brahmachari over 100 years ago marks the start of drug strategies against leishmaniasis. The use of this pentavalent antimonial was instrumental in saving the lives of millions of people infected with visceral leishmaniasis during the 1922 epidemic in India. In such a severe circumstance the efficacy of the drug was preferable to the morbidity caused by such a widespread epidemic, however the toxic side effects of the drug rendered it a dangerous treatment for routine use. The compound was engineered into a less toxic form during the Second World War, and established as the gold standard for anti-leishmanial treatment for over 60 subsequent years. Pentavalent antimonials are still available today as Pentosam® in the UK, Glucantime® in France and a more affordable sodium stibogluconate (SSG) formulation (Croft & Olliaro 2011). Treatment with these compounds remains far from ideal due to their cardiotoxicity, relative expense and the necessity to be administered by a healthcare professional by intramuscular or intravenous injection over the course of four weeks. In

addition, significant levels of antimony resistance have been reported in epidemic areas of India from as early as 1970, with a more recent case study from the Northern state of Bihar showed that treatment of visceral patients with antimony resulted in a high failure rate of 65% (Sundar et al. 2000). This compares with a failure rate of 14% when treating patients from the Uttar Pradesh region, an area also epidemic for visceral leishmaniasis shows an inherent resistance to the drug in Bihar. Since this study, resistance has become more widespread and more than 60% of visceral leishmaniasis patients do not respond to front-line treatment (Bhandari et al. 2012). The molecular mechanisms of resistance in *L. donovani* is now a significant field of study.

A key aim of the 2007 WHO report on the control of the leishmaniasis is the development of new medicines for orally and topically administered treatments which rely on a shorter administration cycle (WHO 2007). Promisingly, a number of initiatives now have established programmes to accelerate research and development into novel cures for leishmaniasis, such as the Drugs for Neglected Disease initiative (DNDi) who have separate strategies for the treatment of CL and VL. In addition, ongoing funding from the Gates Foundation and increased collaborative efforts with industry to facilitate anti-leishmanial compound screens are accelerating the process of drug discovery. A number of alternative anti-leishmanial compounds have been identified over the past two decades, such as Amphotericin B, Miltefosine, Paromomycin and Pentamidine (**Table** 1-1).

Amphotericin B was originally developed as an antifungal agent, but treatment of *Leishmania* results in the formation of complexes with sterols, leading to the permeabilisation of the *Leishmania* cell membrane by pore formation and a resulting lethal cell lysis (Saha et al. 1986). Amphotericin B is an effective anti-leishmanial agent and utilised for the treatment of all manifestations of the disease; however side effects of treatment do occur. To address this, a less toxic but equally efficacious formulation was developed to treat fungal infection of immuno-compromised patients by the incorporation of amphotericin B into liposomes. The reduced toxicity was associated with the preferential binding to high density lipoproteins (Wasan et al. 1994). This lipid preparation termed AmBisome is a potent anti-leishmanial, but despite efforts from the WHO to negotiate a reduced price of $18/50mg vial, the total cost of treatment remains

unaffordable for many. Recent efforts aim to make AmBiosome more affordable to enable widespread distribution, and in 2011 the WHO secured a partnership deal with the suppliers Gilead to donate 445,000 vials over five years (2015).

Miltefosine is an incredibly useful compound in regards to efficacy and oral route of administration (Croft & Olliaro 2011). This route is particularly important for the distribution of anti-leishmanial compounds to areas where primary healthcare is not accessible, therefore intravenous and intramuscular injections cannot be conducted appropriately. The successful treatment of VL with this compound makes it a powerful addition to the current drug repertoire, particularly in overcoming antimonial resistance; yet there can be variation in regards to the efficacy of this compound resulting from the 28 day course of oral administration, opening up the possibility of improper or irregular dosing by the patient (Croft & Olliaro 2011). The use of paromomycin has been instrumental in tackling the drug resistance observed in VL infections in India by its high efficacy of 94% in phase III clinical trials (Sundar et al. 2007). By intramuscular injection of this aminoglycoside the use of paromomycin is an alternative treatment to antimonials and the course of treatment is inexpensive at 15$ for a 15 day course. Antoher alternative to antimonial treatment is pentamadine isethionate, however a case study in Suriname demonstrates that the high cost of $90 for 3 rounds of injections either intralesional or into the buttocks is unaffordable to most (Ramdas 2012). In addition, the administration of pentamidine is viewed as exceptionally painful, and is partially responsible for a cultural move against seeking treatment for New World CL, with those infected utilising harsh, alternative ‘treatments’ such as battery acid and pesticide to treat their lesions. Topical paromomycin for CL has been developed which would be viewed as less painful, but are not available in this region. Compounding this socio-psychological aspect, those studied were at high risk of infection by working in densely forested areas as labourers due to constant interaction with infected sand fly, and who are poorly paid so are unable to afford treatment.

To address the issues with efficacy and resistance to individual drugs, DNDi have prioritised the implementation of combination therapies (2015). The utilisation of SSG and paromomycin combination treatment has been in effect since 2010 to enhance cure rates and prevent visceral leishmaniasis resistance in East Africa,

whilst pilot trials to test combination therapies utilising AmBisome, Miltefosine and Paromomocyin have yielded >97.5% cure rates and are extremely important to address antimony resistance in India. Despite the efficacy of these therapies, existing issues with cost and administration remain. In a bid to make more effective therapy for cutaneous leishmaniasis, DNDi aim to combine chemotherapy with immune-moduation. By this method, the majority of parasites will be eliminated by drug treatment whilst persistent or subsequent infection resistance will be driven by vaccine or adjuvant treatment to enhance the immune response against the parasites. Modulators currently utilised in cancer treatment may have efficacy in this strategy. The range of treatments available against the various forms of leishmaniasis have variable efficacy and problems, however the recent increase in research and funding efforts aim to accelerate the development of better treatments and address such issues.

Drug	Property and administration route	Disease treatment
Pentavalent antimonials; (Pentostam, Glucantime, WHO approved SSG)	Organo-metal complexes; intravenous and intramuscular	VL and CL
Amphoterecin B (Fungizone)	Polyene antibiotic; intravenous	VL, CL and MCL
Liposomal amphotericin B (AmBisome)	Unilamellar liposome; intravenous	VL, CL, MCL and PKDL
Miltefosine (Impavido)	Hexadecylphosphocholine; oral	CL (variable) and VL
Paromomycin	Aminoglycoside, intramuscular (VL) or topical (CL)	VL and CL
Pentamidine	Diamidine, intramuscular	CL

Table 1-1- The available drug repertoire for treatment of leishamaniasis. Current treatments available for visceral (VL), cutaneous (CL), mucocutaneous (MCL) and post-kalazar dermal (PKDL) leishmaniasis. Adapted from (Croft & Olliaro 2011).

1.1.4 Life cycle in the vector

The nature of *Leishmania* transmission was unknown until the Edouard and Etienne Sergent published experimental proof of the transmission of *Leishmania* to humans by sandflies of the genus *Phlebotomus* in 1921, with direct demonstration of such transmission in 1941 (Cox 2002). This genus is responsible

for the spread of Old World leishmaniasis, and in 1922 the vector spreading the New World disease was identified as the *Lutzomyia* genus. *Leishmania* have a complex, dimorphic life-cycle in order to transmit from mammalian host to sandfly vector. Transmission from one mammalian host to another is facilitated by the uptake of intracellular amastigotes during a blood meal. Upon release into the ambient temperature of the sand fly gut these amastigotes undergo differentiation to replicative, extracellular procyclic promastigotes. By examination of *L. braziliensis* and *L. mexicana* infections of sand fly, the original classification of *Leishmania* into two distinct subgenus of *L.* (*Viannia*) and *L.* (*Leishmania*) was defined by the differential adherence of promastigotes to structures within the gut (Lainson et al. 1977). In the context of discussing promastigote differentiation within sand fly in this introduction, most references will concern the latter subgenus to which *L. mexicana* belongs, and which has been studied more in depth. Differences in establishment of infection in sand fly by different *Leishmania* species is permissive only when the two organisms reside in the same geographical location, with such specificity resulting from the expression of lipophosphoglycan (LPG) (Kamhawi et al. 2004). LPG is necessary to prevent removal of the parasites by fluid flow over the gut, and interestingly, this epithelial anchoring by attachment of LPG to *Phlebotomus* PpGalect, an abundant galectin expressed in abundance on the surface of the midgut. Galectin expression is a key mediator of the species-specificity of sand fly infection by *Leishmania*. Environment factors such as temperature also influence the establishment of sand fly infection, as seen by the difference in optimal growth temperatures between even closely related species both of which infect the same *L. longipalpis* vector (Hlavacova et al. 2013).

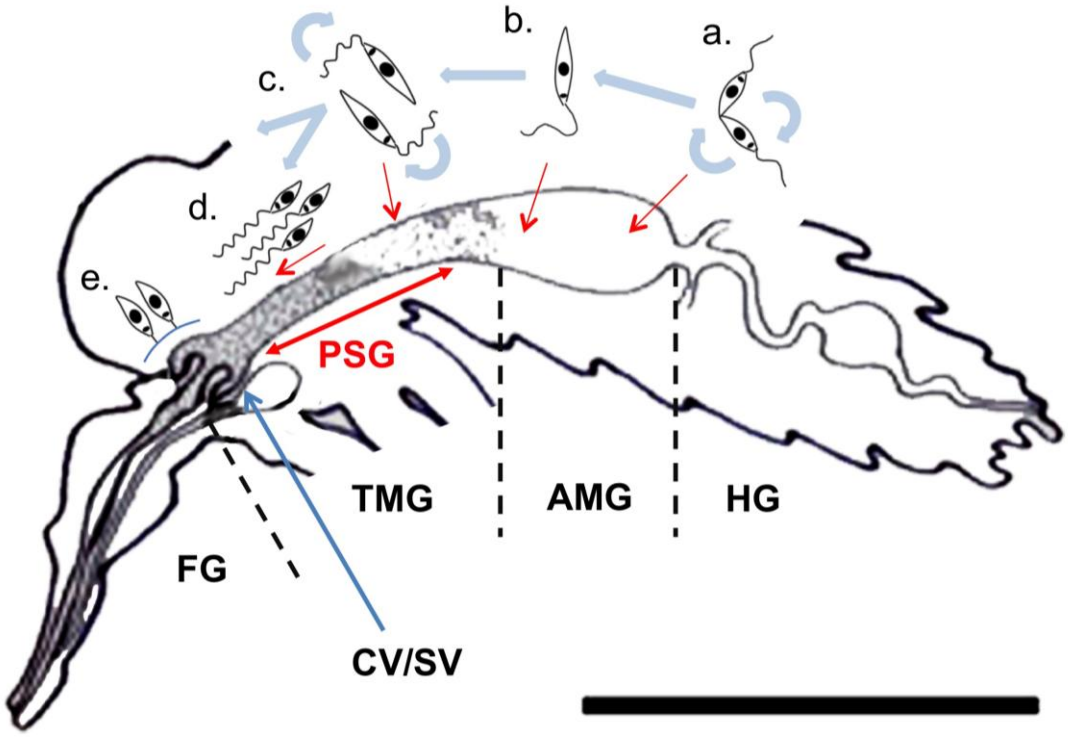


Figure 1:2- The *Leishmania* life cycle within the sand fly vector. *Leishmania* undergo multiple differentiation steps from amastigotes ingested in blood meal to the hindgut (HG). Differentiation to a. replicative, procyclic forms occurs in the anterior midgut (AMG), to b. non-dividing nectomonads which migrate upwards towards the thoracic mid gut (TMG). Differentiation to replicative c. leptomonads in the TMG is followed by differentiation to infectious, non-dividing d. metacyclics and also to haptomonads which attach to the cardiac or stromodeal valve (CV/SV). Heavy infection results in the accumulation of promastigote salivary gel (PSG) in the TMG and some metacyclic promastigotes may infiltrate the foregut (FG). Scale bar: 1mm, adapted from Rogers (2012).

This vector stage of the life cycle represents a huge challenge to the parasites as they must also resist proteolytic attack by digestive enzymes in the gut which are secreted by epithelial cells following the extraction of a blood meal and uptake of intracellular amastigotes into a peritrophic matrix (PM) in the posterior midgut. In this context, the expression of LPG on the promastigote cell surface and the release and accumulation on the surface of a secreted proteophosphoglycan (PPG) are implicated in the evasion of proteolytic lysis (Secundino et al. 2010). The expression of such factors facilitates the establishment of infection and the differentiation to a number of distinct, intermediate forms occurs as the parasites migrate up towards the mouthparts in order to establish transmissible levels of infectious metacyclics to facilitate re-infection (**Figure** 1:2). Differentiation to replicative procyclic promastigotes occurs after 24-48 hours post blood meal to establish colonisation of the posterior midgut, with the differentiation to longer, non-dividing nectomonads which then escape from the PM and migrate to the anterior midgut 48-72 hours

post blood meal feeding. Subsequent differentiation to replicative leptomonads which establish colonisation of the thoracic midgut occurs after 4-7 days, at which time the differentiation of two distinct stages play a role in subsequent infection; the first is the differentiation to haptomonads, a static stage which adheres to the lining of the stomodeal valve and to neighbouring parasites, thereby blocking the opening of the valve (Kamhawi 2006). The second is the differentiation of infectious, metacyclic promastigote.

The study of this pathologically significant cell type has been amenable for around forty years through axenic *in vitro* culture (Berens et al. 1976). A model for differentiation in the sand fly uses cultures seeded with promastigotes at a low cell density, which multiply as procyclic promastigotes until they undergo growth arrest and differentiation to the virulent, stationary metacyclic stage begins. A seminal study by Da Silva & Sacks (1987) demonstrated the infectivity of metacyclic forms compared with procyclic promastigotes, whereby infection of BALB/c mice with *in vitro* replicative, or *Leishmania* purified from the midgut of recently infected *Lutzomyia anthophora* sand fly was not established. In contrast, infection with promastigotes derived from stationary cultures or from the midguts of *L. anthophora* infected for twice the time as previously was robust. These findings were in agreement with others, and now metacyclic forms are routinely purified from stationary phase culture. This pre-adaptation of the metacyclic form for infectivity in the mammalian host is a crucial stage in the life cycle, and the differentiation from procyclic to metacyclic promastigotes is termed metacyclogenesis. The production of a promastigote salivary gel (PSG), a proteophosphoglycan gel secreted by the leptomonad promastigotes in thoracic mid-gut is implicated in the establishment of a dense, protective niche within the sand fly thoracic midgut. The production of PSG thereby enables the enrichment of metacyclic cells and subsequent infectivity (Rogers 2012). In addition, the differentiation of ‘pear shaped’ haptomonad parasites and their adherence to the stromodeal valve by way of a hemidesmosome (Walters et al. 1989) establishes a ‘plug’, thereby acting in conjunction with PSG to enrich the thoracic midgut with metacyclics and prevent their clearance by the sand fly. Haptomonad attachment can result in cellular damage to the valve, forming openings which facilitate the entry of metacyclics into the foregut and proboscis (Kamhawi 2006). Finally, the PSG obstruction results in more frequent feeding as

the sand fly is unable to procure an adequate blood meal (Stierhof et al. 1999), potentially enhancing the transmission into mammalian hosts.

1.1.5 Development in the mammalian host

The establishment of infection by the inoculation of around 600 metacyclic promastigotes into the host by sand fly inoculation (Kimblin et al. 2008). Once in the skin, these parasites are internalised by innate, skin derived phagocytes such as dendritic cells (DC) and macrophages. For metacyclic forms, establishing intracellular infection and avoiding phagocyte mediated killing is facilitated by the expression of the metalloprotease gp63 and lipophosphoglycan (LPG) on the surface of metacyclic promastigotes. LPG is expressed in abundance on the surface of procyclic promastigotes to facilitate binding to the sand fly midgut, but is elongated in metacyclic promastigotes by the addition of phosphorylated disaccharide repeat units (Sacks et al. 1995). LPG elongation functions in the establishment of infection by two mechanisms; binding to the mid gut epithelial cells no longer occurs, allowing transmission of free, infectious metacyclic promastigotes during bloodmeal feeding. In addition, elongated LPG results in a thickened glycocalyx to prevent complement mediated lysis. The establishment of intracellular infection is facilitated by the release of additional components derived from the sand fly vector into the infection site. These include the PSG, which is regurgitated at the bite site, leading to the active recruitment of macrophage and the synthesis of compounds essential for intracellular growth (Rogers et al. 2009). The role of sand fly saliva in enhancing virulence is reviewed by Gomes & Oliveira (2012), with vasodilation and anti-inflammatory properties of salivary peptides such as maxadilan contributing to the establishment of disease. In contrast, pre-exposure to sand fly saliva derived protein has been cited as a potential mediator for induction of host immunity to *L. chagasi*, whereby immunisation of dogs with recombinant salivary gland proteins induced a Th1 response, leading to enhanced lymphocyte mediated parasite killing (Collin et al. 2009). Once intracellular infection is established, the differentiation to immotile amastigotes occurs inside the acidic phagolysosomal compartment, with decreased pH and increased temperature the external cues for this event (Alexander et al. 1999). Replication of this cell cycle stage and lack of an appropriate host immune response results in the

manifestation of lesions or dissemination to visceral organs, and the maintenance of infection.

1.2 An understanding of the immune response to *Leishmania* infection; implications on therapeutic design

The survival of *Leishmania* as an intracellular pathogen is dependent on its ability to modulate and disrupt an appropriate host immune response. The immense complexity of the host-parasite interactions taking place during infection have become explained in more detail by the use of murine models of infection, with ever increasingly sophisticated methods to image, manipulate and analyse the resulting immune response (Peters 2008; Ng et al. 2008; Hurrell et al. 2015a). Such studies have been amenable by the genetic manipulation of *Leishmania* to generate transgenic reporter lines, whilst virulence factor deficient mutants enable the dissection of how the parasites directly modulate the immune response (Beattie et al. 2008). All these efforts are conducted to identify the crucial factors in mediating immunity to *Leishmania* infection. As the immune response is so closely linked to disease outcome, the development of appropriate chemotherapy to treat leishmaniasis necessitates an evaluation of the influence on the immune response.

1.2.1 Murine models for studying cutaneous leishmaniasis *in vivo*

1.2.1.1 Establishing the Th1/Th2 paradigm

Animal models of human leishmaniasis have been utilised since the infection of *L. infantum* in dogs in 1909 by Nicolle and Comte, with subsequent use of hamster, mouse, Guinea-pig and even monkey models throughout the 20th century (Bryceson et al. 1970). Such studies showed the necessity of lymphocyte transformation and the release of lymphokine factors in the resolution of *Leishmania* infection. It was not until 1987 that Locksley et al. showed that resistance was correlated to the expansion of phenotypically distinct CD4+ T cells. This conclusion was drawn from a series of experiments using murine models of *L. major* infection, and today the majority of studies at present utilise experimental mouse models. The use of murine models of *L. major* infection has facilitated in depth analysis of the T helper (Th) cell paradigm, revealing that

infection resolution is dependent on the activation and differentiation of CD4+ T cells to inflammatory, Th1 producers of interleukin-12 (IL-12) and interferon-gamma (IFN-γ) to drive cell-mediated killing of intracellular amastigotes by iNOS production (Constantinescu et al. 1998). In this Th1 context, comparisons between protective immune responses in C57BL/6 or C3H mice, and susceptible, chronically infected BALB/c infected mice have enabled dissection of the importance of particular cellular subsets in mediating an appropriate immune response. The susceptibility or resistance to infection in these models is related to their cytokine profiles; resistant C57BL/6 and C3H mice express cytokines such as IL-2, IL-12 and IFN-Y which drive a cell mediated immune response and are implicated in disease resolution (Heinzel et al. 1991). The timings of such expression can have an impact on disease resolution, as IL-12 production appears crucial only during the early development of a Th1 response whereas IFN-Y is necessary for parasite clearance throughout (Constantinescu et al. 1998). In contrast, BALB/c mice produce a Th2 response upon infection with expression of IL-4, IL-5 and IL-10 (Heinzel et al. 1991). C57BL/6 mice can become immunocomprimised by physiological manipulation as exemplified by C57BL/6 mice deficient in skin draining lymph nodes being unable to develop a Th1 response to *L. major* infection (Ehrchen et al. 2008). Further use of mice deficient in inflammatory cytokines has established their importance for disease resolution (Belkaid et al. 2000),. By direct comparisons in these animal models, the development of immunity to *Leishmania* reveals important stages in an adaptive response.

1.2.1.2 Advanced *in vivo* imaging techniques

Murine models are desirable for the study of host-parasite interactions *in vivo* as the complexity of the host parasite interface is difficult to reproduce using reductionist *in vitro* methods (Filipe-Santos et al. 2009). Sophisticated methods to track fluorescent immune cell migration by intravital multi-photon laser scanning microscopy (MPLSM) imaging at both the ear and draining lymph node (Gibson et al. 2012), or the use of transgenic, photo-switchable 'Kaede' mice (Tomura et al. 2008) enable the dissection of the immune response in a relevant, *in vivo* context. MPLSM has enabled the study of interactions of *Leishmania* with cells such as neutrophils or dermal dendritic cells which represent intracellular niches for the establishment of infection (Ng et al. 2008;

Peters & Sacks 2009; Hurrell et al. 2015a). The resolution of *Leishmania* infection is understood in the context of cytokine expression as a result of T cell differentiation; yet the induction of this response is ill-defined in regards to immune cell motility, clustering behaviour and the formation of immunological synapse between T cells and DC. Evidence for impaired immunity resulting from reduced DC-T cell interactions and co-stimulation has been presented during malaria infection of mice (Millington et al. 2007), with the application to *Leishmania* having important implications for the future.

Such analyses are dependent on murine models, yet there exist important differences between humans and mice such as T helper cell differentiation, cytokine types and their receptors (Mestas & Hughes 2004) which must be considered when translating the results obtained by murine infection into useful data for human disease resolution. Despite this, mouse models for *Leishmania* infections are an important tool for studying both the immune response in ever increasing detail. The application of *in vivo* models are indispensable for the testing of effective therapeutics for translation into the treatment of human leishmaniasis. A limitation of *in vivo* approaches has always been the use of live animals as test subjects, however the adoption of new methods to facilitate more efficacious, longitudinal study is easing this burden. In this regard, the generation of bioluminescent reporter lines represents a sophisticated method to measure the outcome of treatment whilst reducing the number of necessary test subjects (Millington et al. 2010), thereby facilitating the study of host-parasite interactions.

1.2.2 The immune response to *Leishmania* infection

The use of murine infection models has advanced the study of *Leishmania* infection from the simple Th1/Th2 paradigm, to identifying how distinct cell subsets contribute to the resolution or susceptibility to infection site. The major immune cell subsets and immune mediators which have been studied in the context of *Leishmania* infection are summarised below.

1.2.2.1 The complement system

Immediately following inoculation into the mammalian host, extracellular metacyclic promastigotes must evade complement mediated lysis. Opsonins are soluble peptides which bind to microorganisms and represent the main arm of the complement system. The opsonin C3 which is cleaved to C3b and mediates phagocytosis by binding to the cell surface of extracellular microorganisms, which are subsequently recognised by the complement receptors, such as CR1 and CR3 present on the surface of phagocytes (Da Silva et al. 1989). *Leishmania* metacyclic promastigotes are bound by C3b, but the expression of gp63 on their surface catalyses the cleavage of C3b to inactive C3b (iC3b) (**Figure** 1:3); iC3b is still recognised by CR1 to induce phagocytosis results in an impaired cytotoxic activity (Brittingham et al. 1995). In addition, the LPG coat expressed on the surface of metacyclic promastiogtes is elongated by the addition of phosphorylated disaccharide repeat units (Sacks et al. 1995), acting as a barrier to prevent the insertion of a C3b attack complex. LPG has been identified as a virulence factor by generation of LPG *null* mutants though gene replacement (Späth et al. 2003) and by protein destabilisation (Madeira da Silva et al. 2009). Expression of LPG is necessary to prevent complement mediated lysis by a C5b-C9 membrane attack complex (MAC) which forms pores in the microbial cell surface. By expression of these virulence factors, *Leishmania* mediate entry into phagocytes without eliciting an appropriate response.

1.2.2.2 Skin resident cells; keratinocytes and dermal dendritic cells

The interaction between *Leishmania* parasites and host immune cells occurs immediately upon taking a blood meal as the proboscis of the sand fly damages the tissue surrounding the site of injection causing an inflammatory response (Peters *et al*, 2008). As the metacyclic promastigotes are regurgitated into the host they move through the skin and primarily encounter a variety of resident immune cells such as dermal macrophages and non-immune epithelial keratinocytes. Experimentation with cutaneous *L. major* infections of experimental mice by Ehrchen et al. (2010) revealed that keratinocytes in particular may be particularly important at releasing immunomodulatory cytokines such as IL-4 and IL-6 early in infection (**Figure** 1:3). This initial release was shown to be crucial at driving the direction of T helper cell differentiation

to a Type 1 response which has long been shown to help confer resistance to *Leishmania* infection (Locksley et al. 1987).

At this early stage of infection, dermal dendritic cells (DDC) also play an important defensive role by actively capturing *Leishmania* cells. This was only discovered by the use of *in vivo* 2-photon imaging of such cells by Ng et al. (2008), who observed in real-time that DDC act as gatekeepers, patrolling the dermal interstitial space until encountering *L. major* at which point they become sessile, conventionally DC shaped and actively capture and internalise the parasites with elongated pseudopodia.

1.2.2.3 The interaction of *Leishmania* and neutrophils

Neutrophils are the most rapid responders to the tissue damage caused by feeding, with substantial accumulation occurring a few hours following inoculation (Peters et al. 2008). Neutrophils circulate in the blood vasculature and extravasate in response to skin inflammation through dermal vessels. Extravasation is driven by the release of chemokine (C-X-C motif) ligands 1 and 2 (CXCL1, CXCL2) by macrophages (Racoosin & Beverley 1997), and interleukin 4 and 6 release by keratinocytes (Ehrchen et al. 2010), in addition to parasite factors. Neutrophils play a key role in the killing of microorganisms by phagocytosis and subsequent lysis by the production of radical oxygen species, such as inducible nitric oxide synthase (iNOS) and the release of cytotoxic, antimicrobial granules (Faurschou & Borregaard 2003). However, *Leishmania* evade such intracellular lysis, remaining viable for extended periods within neutrophils *in vivo* (Laufs et al. 2002). As neutrophils compose the majority population of responding cells at the infection site (Peters et al. 2008), these represent an abundant intracellular initial niche for metacyclic parasites. *Leishmania* are primed to mediate an immunologically 'silent' entry into neutrophil before a blood meal is taken through the build up of apoptic promastigotes in the sand fly vector. When immune cells such as neutrophils encounter apoptic markers on the promastigotes cell surfaces, they release anti-inflammatory cytokine TGF-β as apoptotic cells pose no infection risk. The result of this is that the infective, live parasites are shielded from an inflammatory response by oxidative burst and degranulation (Ritter et al. 2009). The presence of PSG is important at this stage by enhancing neutrophil recruitment to the bite

site, thereby allowing parasite uptake. Infected neutrophils undergo delayed apoptosis, resulting in their uptake along with intracellular parasites by macrophage and dendritic cells in the absence of an inflammatory response (van Zandbergen et al. 2004). To further avoid cell death once inside the neutrophils, *L. major* prevent the fusion of lysosomal vesicles with the parasitophorous vacuole (Mollinedo et al. 2010). Neutrophil mediated *L. donovani* lysis is shown to be avoided by the lipophosphoglycan (LPG) which massively inhibits the fusion of lysosomal vesicles to parasitophorous vacuoles containing Wt parasites compared to mutants with reduced levels of LPG forming enzymes (Gueirard et al. 2008). It is important to note that the role of LPG in neutrophils differ between species of *Leishmania* (Beattie et al. 2008). *Leishmania* parasites appear to manipulate neutrophil uptake by macrophage as an immunologically silent route of entry, using neutrophils as 'Trojan Horses'.

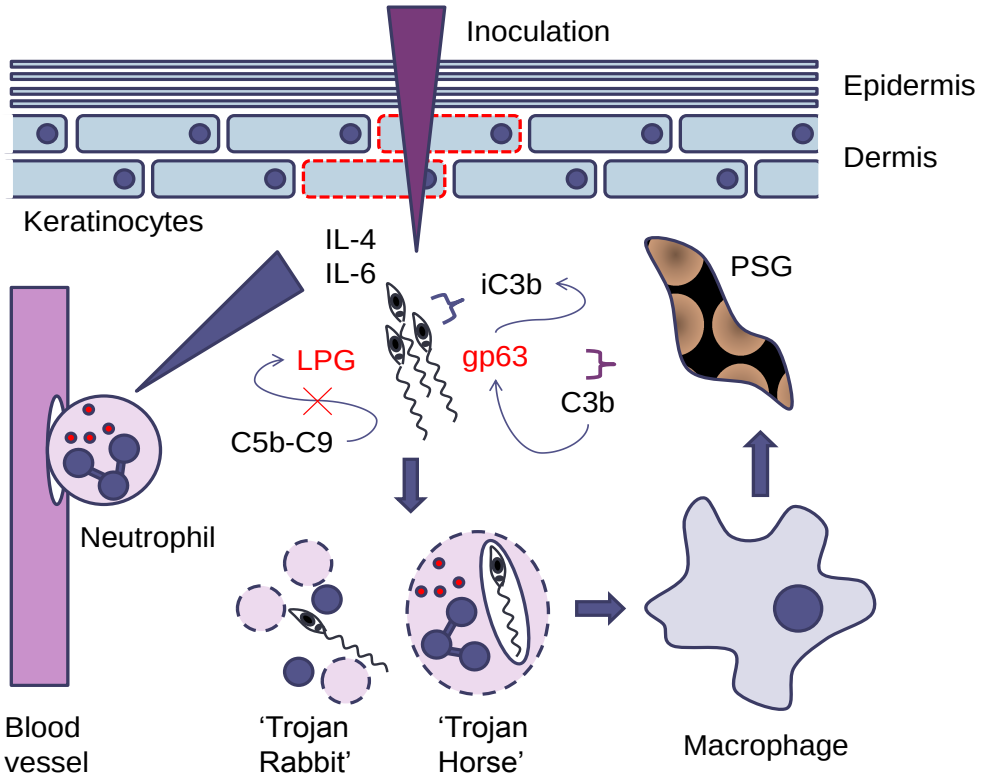


Figure 1:3- The early immune response to *Leishmania* infection. Tissue damage caused by inoculation through the epidermis and dermis causes the release of IL-4 and IL-6 from keratinocytes to drive the extravasation of neutrophil from the circulating blood to the infection site. These are the first host cells for *Leishmania* metacyclic promastigotes, which avoid complement mediated lysis by the C5b-C9 attack complex through LPG expression or C3b complement binding through cleavage of C3b to inactive C3b by gp63. The promastigote secretory gel (PSG) induces increased recruitment of macrophage to the bite site, which subsequently take up apoptopic neutrophil containing viable Leishmania ('Trojan Horse') or by uptake of live promastigotes alongside neutrophil particulate ('Trojan Rabbit').

To establish the dynamics of neutrophil recruitment and parasite entry into these cells, the infection site was imaged *in vivo* using 2-photon intravital microscopy (Peters et al. 2008). Following ear infection with transgenic RFP reporter *L. major*, extracellular promastigotes were immotile in the dermis, resulting in sequestration by increasing numbers of neutrophils. Despite the presence of viable, intracellular *Leishmania,* neutrophils became less motile and underwent apoptosis, with some parasites escaping the neutrophils before complete apoptosis had occurred (Peters et al. 2008). Such a situation has been hypothesised by Ritter et al. (2009) as a potential 'Trojan rabbit' route of entry, whereby macrophage engulf apoptic neutrophils and free promastigotes at the same time (**Figure** 1:3). By imaging the process *in vivo*, (Peters et al. 2008) confirmed active entry of *L. major* into neutrophils in real time, the subsequent reduction of infected neutrophils and importantly an increase in the number of infected macrophages over time. By this method they established this entry mechanism manipulation of the host immune cells as a 'Trojan' route of infection. They also demonstrated that reduction in the number of viable parasites and therefore virulence could be achieved by inhibiting neutrophils binding via antibodies, further demonstrating the key role of neutrophils in initiating *Leishmania* infection. A more recent study by Hurrell et al. (2015) provides further evidence of the inhibitory role neutrophils play in the resolution of *Leishmania* infections. *L. mexicana* infections of *Genista* mice deficient in mature neutrophils resolve lesions by 8 weeks by the induction of an inflammatory, Th1 response. By 2-photon imaging, an increased recruitment of DC to the infection site in neutrophil depleted mice was observed, resulting in the subsequent recruitment of monocytes and monocyte-derived DC to the infection site, and migration to the draining lymph node. This comprehensive study provides compelling evidence for neutrophils as mediating an inappropriate immune response, and serves as an example of how by viewing interactions *in vivo*, *in vitro* studies can be confirmed, new dynamics discovered and the groundwork required for development of therapeutics can be laid.

Neutrophils have recently been demonstrated as eliciting a more complex method of targeting invading *Leishmania* compared with phagocytosis. Inflammatory neutrophils recruited to the site of infection release DNA, elastase and histone to produce extracellular 'traps' (NETs) to ensnare and kill

amastigotes. DNAse treatment of NETs formed in response to *L. amazonensis* infection causes increased pathogenicity, suggestive of an inhibitory role of these structures (Guimarães-Costa et al. 2009). NETs are also formed in response to *L. mexicana* infection, but in contrast the parasites are able to survive NET mediated killing both *in vivo* and *in vitro* (Hurrell et al. 2015a). The absence of *L. mexicana* to NET-mediated killing is suggestive of a differential mechanism of immune-evasion, and the role of virulence factors such as inhibitors of serine peptidases (ISP) in conferring resistance is an important area of research (Eschenlauer et al. 2009), particularly because ISP1 has been shown to inhibit neutrophil elastase, a chief component of NETs (Morrison et al. 2012).

1.2.2.4 *Leishmania* interactions with macrophage

Macrophages play a crucial role in the completion of the *Leishmania* life cycle as they are the predominant intracellular niche for long term parasite growth (Kaye & Scott 2011). Recruitment of monocyte derived macrophage (mo-MΦ) is driven by the expression of CCL3 and CCL4 by infiltrating neutrophils, with *Leishmania* infection resulting in substantial levels of MΦ recruitment (van Zandbergen et al. 2004). Entry of MΦ by promastigotes via the 'Trojan Horse', 'Trojan Rabbit' or by direct uptake results in the formation of a parasitophorous vacuole (PV) (**Figure** 1:3). This is formed by the fusion of a phagosome with endosomes and lysosomes containing antimicrobial hydrolases. In addition the PV is acidic, yet intracellular *Leishmania* survive in this environment by inhibiting the production of reactive oxygen species and impairing the development of oxidative enzymes (Moradin & Descoteaux 2012). Upon macrophage uptake, many species of *Leishmania* have evolved the means to manipulate macrophage cytokine signalling. Cleavage of the NF-κB subunit $p65^{RelA}$, a potent transcriptional activator to $p35^{RelA}$ by gp63 alters macrophage transcriptional regulation, resulting in altered cytokine expression (Gregory et al. 2008). *L. mexicana* parasites have also been shown to use cysteine peptidase B (CPB) to inhibit the release of IL-12 by modulating Nf-κB signalling, thereby preventing a T helper type 1 response (Cameron et al. 2004). Interestingly, the recruitment of macrophages can also be enhanced at the bite site via PSG, and the responding macrophages are enhanced in their production of polyamines which are essential to intracellular parasitic growth (Rogers et al. 2009). Once differentiated to amastigotes, subsequent internalisation by macrophage is driven by the binding

of IgG opsonisation of *L. mexicana* and subsequent uptake by macrophage via the invariant Fcγ receptors. Uptake of parasites by this process results in the release of IL-10 and IL-12. Infection of mice lacking antibody production results in reduced lesion development, indicating the necessity of antibody to exacerbate disease (Kima et al. 2000). By such immune modulation, the parasites establish an amenable intracellular niche.

1.2.2.5 Dendritic cells are important mediators of *Leishmania* resistance

The impact of dendritic cell signalling and antigen presentation is being studied in increasing depth. DCs are 'professional' antigen presenting cells (APC) which drive T cell priming and development of an appropriate adaptive immune response at the draining lymph node, as evidenced by the depletion of DCs during immunisation abrogates subsequent LN hypertrophy (Webster et al. 2006). By driving an adaptive immune response by this mechanism, DCs have a crucial role as mediators between the innate and adaptive arms of the immune response, and as a consequence are implicated as important intermediaries of immunity to *Leishmania* infection. Key to this is the importance of an appropriate Th1 mediated response originating from the draining lymph node. This is evidenced by the work of Ehrchen et al. (2008), where the absence of cutaneous lymph nodes draining the site of *L. major* infection prevented the development of an appropriate Th1 response in the resistant C57BL/6 infection model. Therefore clearance of *Leishmania* at the infection site is dependent on the migration of DC to the draining LN (**Figure** 1:4a).

DC migration from the skin is driven by the increased expression of the chemokine receptor 7 (CCR7) on the cell surface, as demonstrated by loss of lymph node migration in *CCR7* null mice (Tal et al. 2011). Once DCs are activated, the increased expression of CCR7 on their cell surface enables binding of chemokine ligand 21 (CCL21) at localised regions on lymphatic vessels. DCs rapidly enter lymphatic vessels by passing through endothelial flap valves located on the basement membrane and into the lumen (Pflicke & Sixt 2009), where they subsequently migrate to the draining lymph node through the lymphatics. DCs induce lymph node expansion by initiating vascular growth to increase the influx of circulating cells (Webster et al. 2006), whilst also presenting parasite antigen to in fluxing naïve CD4+ T cells to induce an

appropriate T cell response. In *L. major* infection of resistant C57BL/6 mice, the CCR7 dependent migration of DCs to the dLN is regulated by the expression of toll-like receptor 9 (TLR9) on the cell surface of non-infected, 'bystander' DC (Carvalho et al. 2012). It is not known how these uninfected cells detect the presence of parasites to up regulate TLR9, but loss of TLR9 expression on these DCs prevents subsequent upregulation of CCR7, thereby inhibiting migration to the dLN and resulting in non-resolving *L. major* infection.

The role of infiltrating, monocyte-derived dendritic cells (mo-DC) in mediating resistance to *Leishmania* infection is an expanding area of research (Ribeiro-Gomes et al. 2012; Petritus et al. 2012; Hurrell et al. 2015a). Blood circulating monocytes are formed in the bone marrow and are recruited to sites of inflammation in particular foci such as infected skin, or expanded lymphoid organs. During *L. major* skin infections of C57BL/6 mice, infiltrating monocytes undergo *de novo* differentiation to mo-DC in both the dermis and dLN (León et al. 2007) (**Figure** 1:4a). In resolving C57BL/6 infections with *L. major*, mo-DC differentiation occurs at the same rate as in susceptible *L. mexicana* infections, but the overall increased recruitment of monocytes to *L. major* infections results in a significant population of these cells at the infection site early in infection (Petritus et al. 2012). The increased uptake of *L. major* into mo-DC results in enhanced cellular migration to the dLN resulting in LN hypertrophy. A significantly lower production of iNOS is indicative of an impaired inflammatory response; recruitment of monocytes and iNOS production can be significantly increased by ablating the anti-inflammatory cytokine IL-10 from *L. mexicana* infected ears (Petritus et al. 2012). The impaired recruitment of monocytes may result from an early impaired neutrophil response. In the resistant C57BL/6 model, neutrophils express high levels of CCL3 24 hours after infection to recruit mo-DC to the infection site (Charmoy et al. 2010). Reduced mo-DC recruitment is induced by the blocking of CCL3 with Evasin-1 or using *CCL3* deficient mice, resulting in a delayed onset of an appropriate Th1 response. The significant reduction of recruited DCs is indicative of the role neutrophils play in mediating a response, but also implicate mo-DC as important cells for driving the resolution of *Leishmania* infection.

1.2.2.6 CD4+ T Cells: Drivers of inflammation

CD4+ T Cells play a crucial role in the ultimate clearing of *Leishmania* infections, in particular type 1 T helper cells (Th1) which release cytokines that cause a cell mediated immune response to activate phagocytosis by macrophage and clear invading microorganisms. In contrast, Th2 cells enable the intracellular pathogens to establish a chronic infection (Filipe-Santos *et al,* 2009). T cell differentiation is affected by many of the early immune interactions detailed above, therefore it is important to understand the effect of the early response as it dictates the resulting infection dynamics in terms of memory and effector functions (Garside and Brewer, 2010). An appropriate inflammatory response is the accepted prerequisite for clearance of *Leishmania*, with the hypertrophy of the draining lymph node (dLN) facilitating antigen presentation and the maturation of effector T cells (Carvalho et al. 2012). This process is driven by dendritic cells (DC), which stimulate the differentiation of CD4+ T helper 1 (Th1) cells by antigen presentation and release of IL-12 (Schwarz et al. 2013), which in turn induces expression IFN-γ by these effector T cells to mediate disease resolution and drive phagocyte-mediated killing of intracellular amastigotes (Hsu & Scott 2007). *L. mexicana* directly inhibits dLN hypertrophy and differentiation of such cells by immune mediation, as exemplified by mutants deficient in the multi-copy, cysteine protease B (CPB) genes unable to establish long term infection due to the development of a Th1 response in susceptible C3H mice (Buxbaum et al. 2003a). Inhibition of an appropriate inflammatory response to *L. mexicana* infection in another susceptible strain, the C57BL/6 mouse appears directly influenced by the production of IL-10, with its absence resulting in disease resolution by gene deletion (Buxbaum & Scott 2005), or by blocking with an αIL-10 antibody (Petritus et al. 2012). Interestingly, a recent study dissects this phenomenon further, showing that loss of Il-10 expression from CD4+ and CD8+ T cells results in an appropriate immune response, whereas loss of expression from macrophage and granulocytes has no effect (Buxbaum 2015). In agreement with this, adoptive transfer of naïve CD3+ T cells into IL-10 deficient mice and subsequent *L. mexicana* infection did not result in increased CD4/8 T cell differentiation, IFN- γ production or LN expansion by 4 weeks post infection (**Figure** 1:4b), demonstrating that loss of IL-10 early in infection is not sufficient to induce a Th1 response (Hsu & Scott 2007). LN hypertrophy and an appropriate Th1 response can be initiated by inoculation of *L. mexicana* in combination with

the immuno-adjuvant CpG, a ligand for toll-like receptors 9 (TLR9) expressed on dendritic cells (DC) (Hsu & Scott 2007), which is necessary for the early resistance to *L. major* in C57BL/6 mice (Carvalho et al. 2012). These data implicate the activation and migration of professional antigen presenting cells such as DC in priming a CD4+ Th1 response to *Leishmania* infection. By comparing resolving *L. major* infections with chronic *L. mexicana* infections of C57BL/6 mice, the influence of these cells has been studied further.

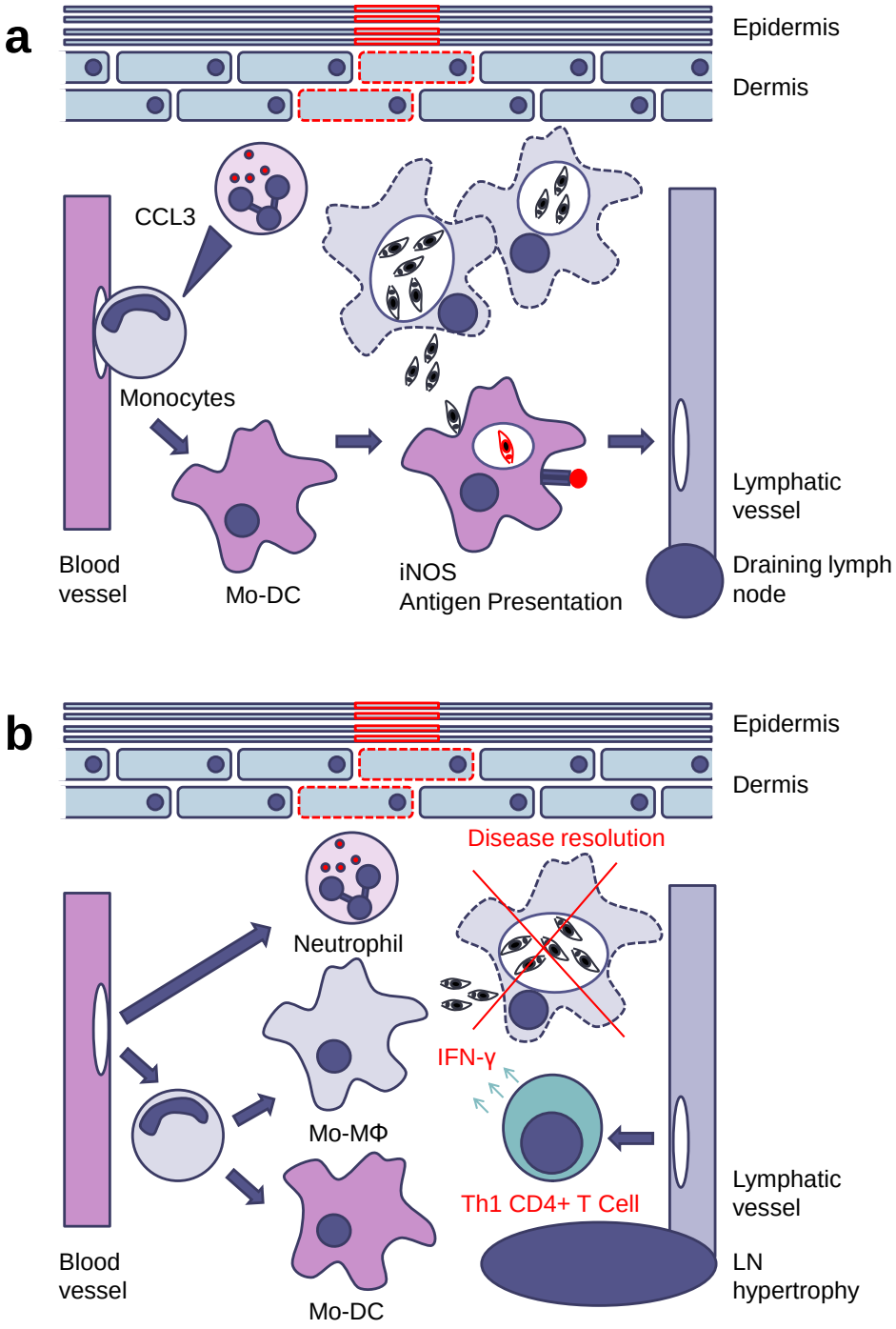


Figure 1:4- The resolution of *Leishmania* infection by an inflammatory immune response. a. Between 24 to 48 hours post infection the infiltration of monocytes in response to CCL3 released by neutrophils results in the *de novo* differentiation of monocyte-derived dendritic cells (mo-DC). Phagocytosed amastigotes are killed by the release of iNOS and the mo-DC migrates to the draining lymph node (LN) via the lymphatic vessel to present antigen to naïve T cells. **b**. Following LN hypertrophy around 1 week post infection, antigen specific Th1 CD4+ T cells differentiate and migrate to the infection site where they release inflammatory cytokines such as IFN-Y. This adaptive immune response results in a subsequent accumulation of leukocytes such as neutrophil and monocytes which differentiate to mo-DC and monocyte derived macrophage (mo-MΦ). By Th1 cell mediation, these activated phagocytes are able to induce sufficient levels of iNOS to kill the resident amastigotes.

1.3 Genetic manipulation of *Leishmania*

1.3.1 *Leishmania*: cultured parasites

Leishmania have many established models to study their biology and the disease they cause. The axenic propagation of the sand fly infective promastigotes has been amenable in cell culture medium for 40 years (Berens et al. 1976), whilst the ability to infect murine and hamster models have facilitated the studies of *in vivo* disease maintenance. Such methods have allowed the exploration of a variety of biological aspects of *Leishmania* as they move through their complex life cycle, such as the regulation of gene and protein expression, altered metabolism between host and vector and the differentiation to multiple life-cycle stages. Given the debilitating spectrum of disease caused by these parasites, the founding of such robust culture methods and subsequent disease models paved the way for studying the basic principles of disease establishment with the aim of identifying novel methods to confer resistance to infection or appropriate disease resolution by appropriate therapeutic treatment.

1.3.2 Homologous recombination and drug resistance selection

Despite the established culture and disease models, the manipulation of *Leishmania* to study aspects of disease maintenance was limited. It was not until 1990 that a method for genetically engineering *Leishmania* was established (Cruz & Beverley 1990) by exploiting the inherent propensity of *Leishmania* to undergo homologous recombination. This method relies on the cloning of gene replacement vectors containing a gene encoding a protein which confers resistance to an antibiotic, flanked by homologous regions for a target gene amplified from genomic DNA by PCR. Following restriction digestion and purification from the plasmid backbone, transfection of replicative promastigotes with an individual drug resistance marker allows replacement of the target gene (Figure 1:5a). The polycistronic expression of RNA precursors in *Leishmania* negates the need for inclusion of a promoter, and instead the expression of the encoded drug resistance protein by a *trans*-splicing reaction requires transgenic flanks containing a splice acceptor site (SAS) and a poly-adenylation site (PAS) (Cruz & Beverley 1990). Such regulatory sequences are commonly derived from flanks amplified from the sequences surrounding the

tubulin, actin or *dhfr* genes. RNA polymerase II mediates the transcription of long, pre-mRNAs which become individually capped at the 5' mini-exon 'splice leader' sequence derived from the SAS, and then poly-adenylated at the 3' PAS to generate transcribed, messenger RNA (Parsons et al. 1986). Selection of drug resistant clones which constitutively express the resistance marker are checked for integration, and subsequently transfected to replace the second endogenous gene copy to generate *null* mutants for phenotypic analysis. By *in vitro* drug selection, gene replacement by integration of drug selectable markers into the genome results in their vertical transfer over subsequent generations. There exists a relatively large repertoire of drug resistance genes for application to clonal selection of *Leishmania* (Table 1-2).

Encoded selectable marker		Antibiotic	Reference
Hygromycin phosphotransferase	*HYG*	Hygromycin B	Cruz et al. 1991
Neomycin phosphotransferase	*NEO*	G418	Cruz et al. 1991
N-acetylglucosamine-1-phosphate transferase	*NAGT*	Tunicamycin	Liu & Chang 1992
Puromycin acetyltransferase	*PAC*	Puromycin	Freedman & Beverley 1993
Dihhydrofolate reductase-thymidylate synthase	*DHFR-TS*	Methotrexate	Arrebola et al. 1994
Phleomycin resistance	*PHLEO*	Phleomycin/ Bleomycin	Freedman & Beverley 1993
Streptothricin acetyltransferase	*SAT*	Nourseothricin	Joshi et al. 1995
Blasticidin deaminase	*BLA*	Blasticidin	Goyard & Beverley 2000

Table 1-2- Available drug selectable markers for *Leishmania* genetic manipulation.

Leishmania have been shown to undergo intra and inter-specific genetic exchange in extracellular promastigotes by the crossing of heterologous drug resistant lines to form double drug resistant progeny in sandfly (Inbar et al. 2013; Romano et al. 2014). In addition, the isolation of *L. major* and *L. infantum* hybrids provides further evidence for the propensity of *Leishmania* to undergo sexual recombination (Ravel et al. 2006). Despite this, recombination during *in vitro* culturing is infrequent and clones can be maintained by drug selective pressure. By this method, a vast array of genes have been investigated by *null* mutant generation to investigate a multiplicity of aspects of *Leishmania* biology, with an expanded repertoire of drug resistance markers facilitating multiple rounds of transfection (Table 1-2). A powerful outcome of this technique is the inoculation of viable, *null* mutant metacyclic promastigotes into murine hosts to establish the outcome of gene deletion in the context of virulence of the amastigote form. By this method, a number of encoded proteins have been identified as essential to survival *in vivo*, or which directly alter the immune response to *Leishmania* infection (Buxbaum et al. 2003a; Wang et al. 2005; Eschenlauer et al. 2009; Faria et al. 2011). An original proposal of this method was the generation of such cells whereby gene deletion of virulence factors (CPB) or essential components of metabolism rendered the parasites avirulent, thereby validating their use as prophylactics to confer resistance to subsequent infection; however despite their possible efficacy the safety implications of such a 'live' vaccine negate their use in humans (Beattie et al. 2008).

Despite the efficacy of this method, the approach is limited to a 'proof of principle' approach when applied to ablate the expression genes encoding proteins which are essential to the viability of promastigotes. For such genes, replacement and selection of double drug resistant clones results in the generation of cells with dramatic changes in their genome structure, resulting in aneuploidy or tetraploidy (Cruz et al. 1993). Such genotypic plasticity in *Leishmania* has been described in ever increasing detail, with strong selection on the retention of the gene indicative of its essentiality. To expand this genetic proof further, endogenous gene replacement in the absence of altered ploidy is amenable by expression of an ectopic target transgene as an episome or from an exogenous gene locus (Figure 1:5b). This method has been applied to study a range of genes encoding proteins with crucial regulatory aspects of cell cycle

control, life cycle differentiation and organelle segregation (Wiese 1998; Paul Hassan et al. 2001; Wang et al. 2005; Ambit et al. 2008). This method can be applied further by murine infection, whereby the purification of parasites retaining the gene as an episome in the absence of drug selective pressure establishes essentiality for amastigote viability *in vivo*, and where conversely episome loss is indicative of a non-essential gene (Wang et al. 2005). The limitations of essential gene targeting are discussed further in section 4.1.

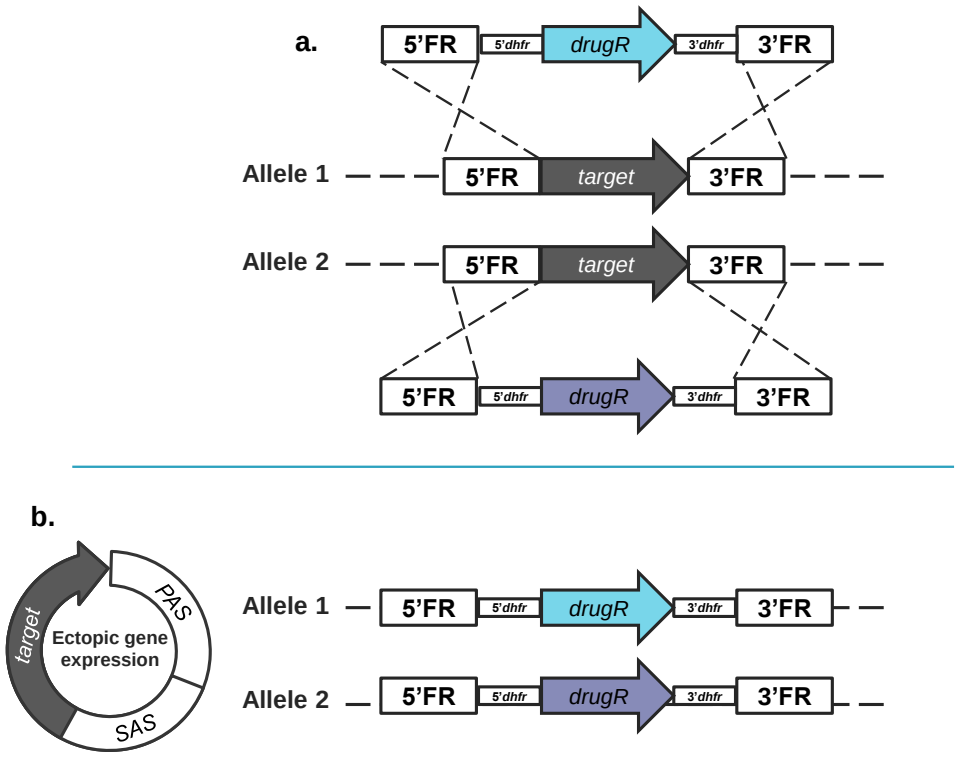


Figure 1:5-Gene replacement by homologous recombination of drug resistance markers. a. Replacement of both gene loci by genes encoding drug resistance proteins (drugR) with appropriate 5' splice (SAS) and 3' polyadenylation sites (PAS); in this case conferred from the *dhfr* locus. FR are flanking regions homologous to the target gene locus. b. Replacement of essential genes necessitates the expression of an episomal copy on a plasmid before endogenous gene replacement.

1.3.3 Exploiting homologous recombination for manipulating *Leishmania*; reporter line generation

The expression of transgenes by the transfection of episomal or integrative vectors and subsequent drug resistance selection represents a robust and reproducible method for the generation of transgenic *Leishmania*. In particular, the development of integrative expression constructs for uniform, high levels of transgene expression have formed the basis for such work (Misslitz et al. 2000). The rationale for the generation of such lines depends on the biological question

being addressed; for example the generation of clones expressing high levels of fluorescent protein enables multi-photon laser scanning microscopy (MPLSM) of infection sites allows detection of the invasion of particular subsets of host immune cells by metacyclic promastigotes (Peters et al. 2008; Ng et al. 2008), by fluorescence detection of intracellular amastigotes, an appropriate fluorochrome-conjugated antibody panel permits phenotyping of infected immune cells by flow cytometry (Ribeiro-Gomes et al. 2012; Hurrell et al. 2015a). Such studies implicate particular cell subsets in mediating the immune response to *Leishmania* infection, whereas the generation of parasites expressing bioluminescent protein enables the tracking of infection in the context of parasite burden and dissemination within the whole host (Michel et al. 2011). Assessing parasite burden by detection of the light signal emitting from the infected mouse is a powerful approach for testing of novel chemical entities for anti-parasitic activity *in vivo*. This method circumvents the need to assess parasite burden by the lengthy, labour intensive approach of limiting dilution until a 'hit' has first being validated by loss of emitted light. These distinct approaches have their own limitations in regards to the limitations of i*n vivo* signal detection, therefore the generation of reporter lines has a range of advantageous applications in the assessment of leishmaniasis, as reviewed by Millington et al. (2010).

1.3.4 Exploiting homologous recombination for manipulating *Leishmania*; advanced molecular tools

The process of transgene transfection and selection of drug resistant parasites has been greatly enhanced by an expanded repertoire of drug resistance markers and increasingly efficient transfection techniques. In parallel, methods to manipulate the expression of *Leishmania* genes to probe their function have also advanced from the original gene replacement strategy (Cruz & Beverley 1990). The main limitation of this approach is the inability to regulate gene expression in a conditional manner, with a general aim of more recently developed molecular approaches to regulate the expression of the target gene, transcript or protein. Conditional regulation of the molecular target is a desirable tool to probe the function of said target by its inducible loss or gain of expression, permitting analysis of the resulting phenotype by appropriate assays. The implications of such a tool and the development of a method to conditionally

delete essential genes in *Leishmania* is discussed in more depth in chapters 4 and 5. This introduction will focus on discussing the existing molecular toolkit for regulating gene and protein expression in *Leishmania*.

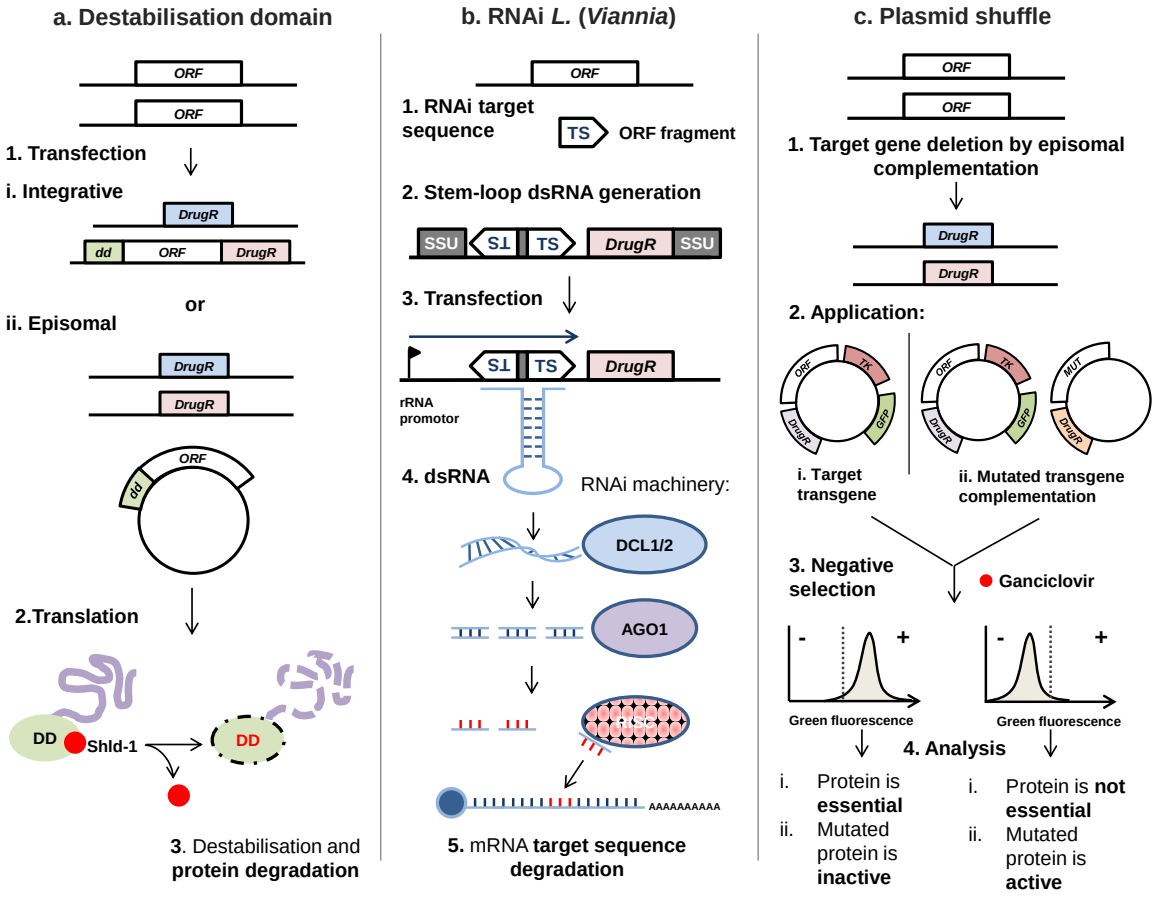


Figure 1:6- The current molecular toolkit for regulation of *Leishmania* gene, transcript and protein expression. a. Destabilisation domain enables expression of a dd-conjugated protein expressed by 1. open reading frame (*ORF*) transgene integration and replacement at the endogenous locus or 2. episomal expression and subsequent endogenous gene deletion. The translated protein is regulated by the stabilising ligand Shield-1 (Shld-1). **b.** Functional RNAi pathway in *L.* (*Viannia*) enables targeting of mRNA by integration of an RNAi targeting sequence (TS) into a ribosomal small subunit (SSU) integrative construct. Transfection of the stem-loop construct generates dsRNA by transcription from the ribosomal RNA (rRNA) promotor. Dicer-like (DCL) enzymes process the TS dsRNA into siRNA duplexes that are processed into single stranded siRNA by Argonaute (AGO1) and loaded to form the RNA-induced silencing complex (RISC). **c.** Plasmid shuffle utilises episomal expression of a target gene in array with the negative selectable thymidine kinase (TK) and positive markers for green fluorescent protein (GFP) and drug resistance (drugR). Transgene complementation (i) enables deletion of endogenous gene copies and ganciclovir (GCV) treatment induces selection for plasmid retention and the expression of GFP if essential or loss of expression if non-essential. A fourth round of complementation with a mutated version of the target gene ORF (MUT) (ii) enables the identification of active residues within the encoded protein. By GCV negative selection, retention of the ORF and GFP expression is indicative of a deleterious mutation which prevents active protein expression. In contrast, loss of the non-mutated ORF by inducible complementation the MUT gene identifies a functional mutated protein.

The development of molecular tools in *Leishmania* as alternatives to gene replacement have had variable success. Some of the main approaches which

have contributed to the expanding toolkit will be discussed below, summarised in Table 1 and depicted in **Figure** 1:6.

1.3.4.1 Destabilisation domain

An approach to regulate protein expression by conferring inducible stability involves the linkage of a target protein to a regulatory, destabilisation domain (DD) derived and modified from an FKBP domain (Banaszynski et al. 2006). Protein stability and therefore activity is conferred by Shld-1 addition. This approach was utilized effectively in *L. braziliensis* promastigotes by Madeira da Silva et al. (2009) to stabilise a variety of target proteins such as yellow fluorescent protein (YFP), luciferase (LUC) and components of the C1 carbon metabolism pathway formate tetrahyrofolate ligase (FTL) and 5,10-methenyltetrahydrofolate cyclohydrolase/5,10-methylene tetrahydrofolate dehydrogenase (DHCH). These manipulations were as proof of principle to establish the efficacy of ligand mediated protein stabilisation, with expansion of the technique facilitating the regulation of the key surface glycoprotein LPG to be regulated. By integration of a *ddGLF* construct into a homozygous, *glf*$^{-}$ null mutant *L. major* line, stability of a dd conjugated UDP-galactopyranose mutase (ddUGM) was conferrable by Shld-1 treatment, with removal of Shld-1 rapidly resulting in the inducible susceptibility to complement mediated lysis by expression of LPG truncated in the glycan region. Attempts to complement *Leishmania* with dd-conjugated proteins and generate essential gene *null* mutants has proven difficult. Personal communication suggests that the use of this technique can result in regulation of the stability of essential genes such as FTL and DHCH, yet a copy of the endogenous gene remains by selective pressure to retain expression of the endogenous protein lacking a dd domain. Such issues may arise by the relatively large size of the dd fusion tag, as protein tagging with even small epitopes such as 6xHis can result in altered biochemical properties (Wu & Filutowicz 1999), thereby repressing enzymatic activity at key residues at either protein terminus.

In addition, one could speculate that the process of 20S proteosomal degradation or endoplasmic reticule dependent turnover in *Leishmania* necessitates the target protein being trafficked to the cytoplasm (Robertson 1999), however if a protein functions as part of a complex inside a distinct cellular compartment,

such as the nuclear membrane, then the degradative process following destabilisation would be delayed. An absence of published studies where the dd is utilised to functionally assess even non-essential factors is a likely outcome of the inherent difficulties in manipulating *Leishmania,* particularly when regulating the expression of proteins which are essential to their survival.

Molecular method	Molecular target(s)	Application	Reference
Protein destabilization domain (dd)	**UGM**, YFP, LUC, FTL, DHCH1	Conditional stabilisation of the precursor required for biosynthesis of the virulence factor LPG *in L. major*	(Madeira da Silva et al. 2009)
Small inhibitory RNA interference (RNAi)	**PFR1**, **PFR2**, **AGO1**, GFP**,** LPG1, LPG2, LPG3, HGPRT, LUC	Efficient knockdown of paraflagellar rod proteins and an essential component of the RNA pathway in *L. braziliensis*	(Lye et al. 2010)
Plasmid Shuffle	**DHCH1**	Confirming essentiality in *L. major*	(Murta et al. 2009)
	STI1	Confirming essentiality in *L. donovani*, application to generate partial null mutants to probe essential phosphorylation sites	(M. a. Morales et al. 2010)
	MPK4	Confirming essentiality in *L. major*; application to generate partial null mutants with altered differentiation phenotypes and impaired infectivity	(Dacher et al. 2014)
	CYP51	Confirming essentiality in *L. donovani*; application to generate enhance sensitivity to inhibitors of the sterol biosynthesis pathway by *CYP51* deficiency	(McCall et al. 2015)
	NMT	Establishment of essentiality by murine infection of *L. donovani*	(Paape, unpublished data)

Table 1-3- Development and application of the expanding *Leishmania* molecular toolkit. Bold font represents the key target(s) being manipulated, transgenic reporter for proof of principle analysis are underlined and normal font represents targets which were not fully assessed or were inefficiently knocked down**.**

1.3.4.2 RNA interference

RNA interference (RNAi) is an established and useful tool for regulating the expression of protein at the transcript level which was rapidly utilized in the kinetoplastid *T. brucei* following its discovery over 17 years ago (Ngô et al. 1998). The RNAi pathway functions by the expression of Dicer-like enzymes DCL1 and 2 which process long dsRNA into small, double stranded duplexes. dsRNA is 'sliced' into single stranded siRNA and loaded as a guide sequence by Argonaute enzyme mediated processing, forming the RNA-inducing silencing complex (RISC) to degrade full length transcript (Kolev et al. 2011). Exploitation of this inherent biological pathway has enabled conditional transcript knockdown by inducible stem loop formation, resulting in its application to powerful, high-throughput studies of gene function in a variety of *T. brucei* screens (discussed further in chapters 3 and 5). However, knockdown by a non-inducible RNAi strategy in both *L. major* and *L. donovani* has no effect (Robinson & Beverley 2003) due to the absence of the RNAi pathway in the parasites of the *Leishmania* sub genus by the evolutionary loss of Dicer-like and Argonaute proteins (Lye et al. 2010). In contrast, the retention of such cellular machinery in the *L.* (*Viannia*) subgenus holds promise for the utilization of RNAi as a method for functional analysis of genes in parasite species such as *L. braziliensis*. RNAi was functionally assessed in this species by Lye et al. (2010) by electroporation of promastigotes in the presence of dsRNA, yielding variability in the efficacy of this method by low levels of knockdown against mediators of LPG expression (*LPG1, LPG2, LPG3*) but efficient reduction of the paraflagellar rod proteins (PFR1 and PFR2). An elegant assay was utilized to demonstrate the efficacy of RNAi; a LUC reporter line containing a stem loop construct to degrade LUC transcript was transfected with a second stem loop construct targeting Argonaute, the protein mediating the 'slicing' of dsRNA to siRNA (Figure 2ii), thereby recovering the bioluminescent signal due to loss of Argonaute mediated RISC formation. Despite this assay, the highly variable levels of RNAi knockdown elicited for non-essential transcript is challenging, whilst the absence of an inducible method for dsRNA generation limits the approach to non-essential targets. In addition, data presented at the recent Kinetoplastid Molecular Cell Biology meeting (Lye et al, unpublished data) show that efficient RNAi relies on large (>250nt) stem loop constructs that are necessary for efficient knockdown, with such large fragments generated increasing the potential for 'off target' effects, whilst the observed

accumulation of extremely high levels of target siRNA may also contribute to such off-target effects. Yet there holds some promise for this tool, as the author also presents the reduced activity of quinonoid-dihydropteridine reductase (QDPR) by transfection with a targeting stem loop construct, a previously challenging factor to genetically regulate due to its genomic location between two encoded genes in a multiple, tandem array (Lye et al. 2002). The limited application of RNAi to the *L.* (*Viannia*) subgenus, in addition to its variability in knockdown and poor utilization since 2009 necessitates its optimization, and additional approaches for regulation of endogenous *Leishmania* factors are necessary.

1.3.4.3 Plasmid shuffle

Complementation of an essential gene with a transgene is necessary to replace the endogenous gene copies without altering ploidy, thereby implicating the encoded product as essential. This criteria was deemed suitable for assessing a gene as essential, however a more stringent approach to test this has been adapted from fission yeast (Kiely et al. 2000) for use in *Leishmania*. This 'plasmid shuffle' methodology expands on episomal complementation to confer negative selection against expression of the transgene; expression in array with a thymidine kinase (TK) 'suicide' cassette confers sensitivity to ganclicovir (GCV) (Murta et al. 2009). Active TK synthesises GCV into the toxic metabolite GCV triphosphate which inhibits DNA synthesis (Barese et al. 2012) leading to the *Leishmania* cellular stress (LeBowitz et al. 1992), thereby exerting strong selective pressure on the loss of the plasmid. The inclusion of positive marker cassettes for drug resistance and green fluorescent protein (GFP) facilitate clonal selection and downstream gene expression analysis (**Figure** 1:6). By this method, a negative selection line can be generated by episomal complementation and subsequent endogenous gene replacement. Murta et al. (2009) validated the essentiality of the *DHCH* gene encoding a dual-function protein involved in 10-Formyl-THF metabolism which was previously targeted by dd-tagging (Madeira da Silva et al. 2009) and implicated as necessary for survival. Negative selection was induced by 24 hour GCV treatment, followed by clonal selection in 96 well plates and subsequent FACs analysis, leading to a substantial proportion of resulting populations (478 GFP+ out of 672 measured) retaining high levels of GFP expression, and therefore expression of the DHCH

protein. A benefit of this strategy was the high number of individual populations analysed in this manner, thereby providing a robust measure of DHCH retention.

Further application of the plasmid shuffle technique enabled the *L. donovoni* CYP51 to be identified as essential to promastigote survival by retention of the gene on a *TK* expression plasmid in the presence of GCV treatment (McCall et al. 2015). In addition, the generation of a cell line absent in endogenous CYP51 copies enabled the treatment of these deficient lines with an inhibitor developed against the *T. cruzi* CYP51 homologue. The increased sensitivity to treatment with this compound by deficient promastigotes implicates *CYP51* and its activity in the regulation of sterol biosynthesis, thereby identifying this pathway as a potential mechanism by which parasite growth may be chemically attenuated.

Despite the use of negative selection to confirm essentiality, this application is limited in respect to exploring gene function. A further development of this technique enables the generation of 'partial' essential gene *null* mutants for functional analysis of protein domains by inducible complementation (**Figure** 1:6 c. ii) by loss of the TK plasmid and subsequent retention of a second plasmid encoding the gene yet lacking the suicide cassette (Morales et al. 2010; Dacher et al. 2014). The identification of active site residues encoded within *Leishmania* essential gene sequences was previously limited to yeast complementation and recombinant protein functional assays, therefore the application of plasmid shuffle for the identification of key residues within viable *Leishmania* represents an important advancement. In the first of these studies conducted by Morales et al. (2010), phosphoproteomic analysis identified a complex implicated in the regulation of the heat-shock response elicited when promastigotes are inoculated into their warm-blooded mammalian host. An amastigote specific chaperone complex containing STI1/HOP was identified and subsequently manipulated by endogenous *STI1* deletion in the presence of an episomal copy in array with the TK and GFP cassettes. By negative selection with GCV, the plasmid was retained and the encoded protein established as essential, however the authors expanded on this method to identify phosphorylation sites essential to the activity of STI1. This elegant approach involved the further complementation of the plasmid shuffle line with a second episome containing

an *STI1* open reading frame which had undergone mutagenesis at potential active sites. Negative selection following complementation with five such mutated sequences enabled the identification of two phosphoserine residues essential for the function of the encoded chaperone.

Plasmid shuffle was also applied to identify the essentiality of the gene encoding *L. major* map kinase 4 (MPK4), a protein kinase which is implicated in mediating differentiation through the life cycle (discussed further in chapter 6). By further complementation of this line with *MPK4* ATP binding site mutants, partial *null* mutants were generated by GCV treatment to establish clones which replicate in the presence of negative selection, but have altered protein kinase activity in subsequent differentiation assays. Inducible complementation allowed the identification of K59 residue *MPK4* expressing mutants which undergo increased metacyclic differentiation *in vitro* but have reduce intracellular macrophage survival. The development of a potentially more robust approach for such inducible complementation by diCre mediated inducible complementation will also facilitate such *in situ* study is discussed in Chapter 5, however Dacher et al. (2014) here demonstrate the further potential of plasmid shuffle.

A final application of the plasmid shuffle approach is the *in vivo* confirmation of the essentiality of N-Myristoyltransferase (NMT) for the maintenance of murine infection by *L. donovani* (Paape, manuscript in production). This currently unpublished study utilises plasmid shuffle to establish the essentiality in amastigotes during murine infection by applying GCV treatment to *L. donovani* expressing the *NMT* transgene in array with TK to confer selective pressure during infection. NMT was previously established as essential to *L. major* promastigotes by the inability to replace all endogenous gene copies (Price et al. 2003), therefore this important work establishes that the *NMT* gene is retained in the presence of negative selection *in vivo*, establishing an essential role for active NMT in the survival of amastigotes. This work further implicates NMT as a suitable drug target against the replicative amastigote stage.

The rapid adoption of plasmid shuffle is testament to the powerful biological insights that can be uncovered for a target gene (Table 1-3). Its application to a variety of genes in different contexts represents the flexibility of plasmid shuffle to probe gene function.

1.3.5 The future of genome engineering: CRISPR/Cas9

The CRISPR/Cas9 DNA editing methodology has been rapidly adopted by the scientific community since its demonstration in early 2013, with the comprehensive review by Sternberg & Doudna (2015) identifying 1000 publications since that time. This huge number of studies performed in a diverse array of organisms opens up the potential for CRISPR/Cas9 mediated editing of the *Leishmania* genome, and the recent online publication of its application to the paraflagellar rod 2 (PFR2) locus to replace both loci by one round of transfection (Sollelis et al. 2015). The efficacy of CRISPR/Cas9 mediated integration was variable, however clones were obtained which were absent in PFR2 expression by limited dilution. The potential to ablate both copies of a gene in one round of selection has important implications on the study of *Leishmania*, particularly as many potentially important genes are encoded on supernumerary chromosomes (Rogers et al. 2011) rendering their regulation difficult by current methods. The study of gene dosage, a phenomenon in which *Leishmania* alter gene expression by copy number variation is implicated in drug resistance (Leprohon et al. 2015), virulence (Dickens, personal communication) and the rapid adaptation of promastigotes and amastigotes to survival in culture media or *in vivo* (Spaeth, unpublished data). The investigation of this phenomenon would be greatly enhanced by the potential for CRISPR/Cas9 mediated manipulation to reduce overrepresented genes to investigate dosage on such significant aspects of infection.

1.4 Protein kinases as drug targets in *Leishmania*

1.4.1 Protein kinases: validated drug targets

Protein kinases in eukaroytes (ePKs) belong to an extensive superfamily of homologous proteins which modulate a target substrate by the phosphorylation of key residues. This process of phosphorylation elicits a functional change in the target protein, leading to altered activity, localisation or interactions with other proteins. All ePKs mediate this process and share a conserved catalytic core involved in ATP binding and phosphoryl transfer. This ATP binding site is located between the N- and C-terminal domains in a deep cleft. It is composed of a hinge region which connects the two domains, a large, hydrophobic ‘gatekeeper’

residue which controls the size of a hydrophobic, purine binding cavity. There is an additional activation loop or T-loop which activates protein kinase function following phosphorylation and translocation (Jänne et al. 2009). Activity of the PK is also dependent on the orientation and binding to the target substrate, as mediated by the recognition of a specific distal docking motif (Cheng et al. 2011). The γ-phosphate is derived from a nuclear triphosphate, most commonly ATP which is covalently linked to an amino acid residue on the target protein. The specificity of the phosphorylated substrate is based on the protein kinase catalytic domain, and is a useful criteria for the categorisation of protein kinases; they are classified as either dual specificity serine/threonine kinases or protein tyrosine kinases (Hanks & Hunter 1995). The protein kinases are further classified into various sub-groups and families based on their catalytic domain sequence similarity, with the main groups being composed of AGC, CAMK, TK, TKL, GMGC, STE and others (Hanks & Hunter 1995).

The human genome contains 518 predicted typical eukaryotic protein kinases (ePKs), composing around 2% of protein encoding genes in humans and around 2-2.5% in yeast (Hanks 2003). Other protein kinases include the diverse, atypical protein kinases, which have no structural similarity to ePKs, and the lipid and sugar kinases that phosphorylate small molecules. ePKs mediate many aspects of cellular function such as proliferation and survival by phosphorylation of other proteins, therefore deregulation of substrate phosphorylation can impair a wide variety of biological functions (Cicenas & Valius 2011). Cancer therapy by protein kinase inhibition was therefore a desirable outcome, and competitive binding of small molecules to the ATP binding pocket was deemed a potential mechanism for ablating kinase function. However, such inhibition was deemed impossible due to the conservation of this active site on all protein kinases as an inhibitor of one ePK would lead to inhibition that of all other ePKs. In 1996, Buchdunger et al. challenged this dogma by the dual inhibition of an BCR-ABL protein-tyrosine kinase complex and the platelet-derived growth factor (PDGF) receptor tyrosine kinase by small molecule inhibition. Previous studies had established the formation of a *brc-abl* oncogene by chromosomal translocation, resulting in the expression of a constitutively active ABL tyrosine-kinase. Substrate phosphorylation by this PK induces the onset of chronic myelogenous leukemia (CML) unless activity is inhibited by treatment with the compound CGP 57148.

This crucial work established the basis for the first clinical trials of the kinase inhibitor (later termed Imatinib) in 1998 (reviewed by Druker 2004). To date, the use of high-throughput screening of potential kinase inhibitors, protein kinase crystallography and nuclear magnetic resonance (NMR) spectroscopy have enabled the design of specific kinase inhibitors which sit within the ATP binding pocket of the target PK or at allosteric sites (Jänne et al. 2009).

This structural based approach to the design of inhibitors represents a rational method to specifically inhibit target PKs. To date, there are 28 inhibitors against single or multiple PKs which have been approved for clinical use as cancer therapies, with the application of targeted PK inhibitors yielding a better success rate than conventional chemotherapeutics (Knapp & Sundström 2014). In addition, the development of kinase inhibition has important implications on the treatment of auto-inflammatory disorders such as rheumatoid arthritis, psoriasis, inflammatory bowel syndrome and Crohn's disease (Patterson et al. 2014). Structural studies have enabled the classification of inhibitors based on their binding to different regions of the ePK; type I are ATP competitive and bind the region occupied by the adenine ring of ATP, whilst forming hydrogen bonds with the hinge region. In addition, some inhibitors can occupy one of two distinct hydrophobic regions, the ribose region or the phosphate binding region to fix ePK in its active confirmation (**Figure** 1:7). Despite always occupying the adenine ring, the various regions which type I inhibitors also occupy forms the basis for increased selectivity over other ePKs (Liu & Gray 2006). Type II inhibitors (such as Imatinib) bind adjacent to the active site when the PK is inactive, thereby preventing subsequent conformational changes to the active form (Liu & Gray 2006). Type III and IV inhibitors bind away from the active site to cause allosteric changes to the protein that directly inactivates the protein or prevents it from becoming activated. Such 'target based' drug development has yielded potent inhibitors and a wealth of information on the specific binding moieties of inhibitors, however a possible criticism of the use of such screens is focussed on biochemical selectivity assays as opposed to cellular assays. Inhibition of a recombinant or purified protein complex may identify the compound as a potent inhibitor, however this negates crucial biological aspects of drug inhibition such as cell membrane permeability and the abundance of ATP within the cell. A dual

approach is therefore desirable to ascertain both selectivity of an inhibitor, in addition to its efficacy *in vivo*.

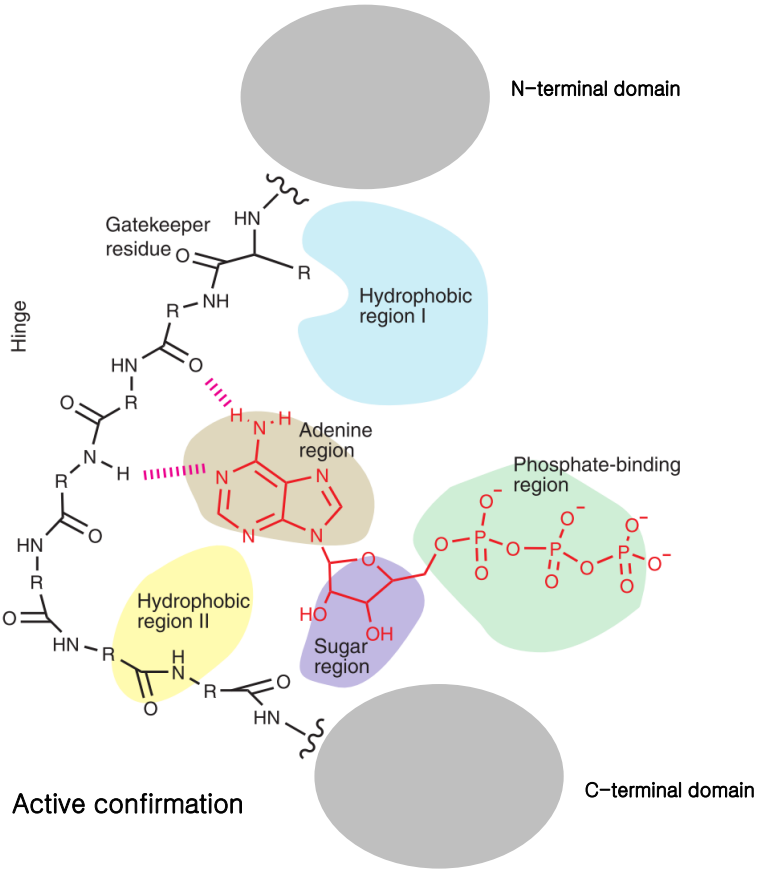


Figure 1:7-The mechanism of Type I ePK inhibition. The protein kinase is fixed in an active confirmation by Type I inhibition. Adapted from (Liu & Gray 2006).

1.4.2 The *Leishmania* kinome

The use of PK inhibitors against mammalian kinases establishes the application of therapeutic kinase inhibitors in other organisms. The ability to inhibit a specific ePK in humans opens up the possibility for the development of protein kinase inhibitors against parasite PKs. This represents an important area of research to identify lead compounds with anti-leishmanial properties by *Leishmania* specific protein kinase inhibition, thereby expanding the poor drug repertoire against leishmaniasis. A number of important protein kinases encoded by *Leishmania* were identified by polymerase chain reagent (PCR) amplification and subsequently identified as essential for parasite survival. These include members of the CMGC group; mitogen activated protein kinases (MAPK) and cyclin-dependent protein kinases (CDK). The former are implicated in the transduction of cellular signals to react to environmental cues such as temperature and pH changes (Wiese 2007; Morales et al. 2010; Cayla et al.

2014), whereas the latter are homologues of the mammalian cdc2 kinase and are essential for mediating the transition through the G1 and G2/M cell cycle stages in procyclic promastigotes (Mottram et al. 1996; Paul Hassan et al. 2001). In particular, the CDK related (CRK) 3 protein has been studied extensively in terms of function and small molecule inhibition by recombinant protein activity investigation and inhibitor screens (as discussed in depth in chapter 5) (Grant et al. 2004; Cleghorn et al. 2011; Walker et al. 2011). Despite being a chemically and validated drug target in procyclic promastigotes, genetic validation has only been indirect by the inability to remove all copies of the gene in promastigotes, whilst such testing has not been applied to probe the function of this protein kinase during the amastigote stage of *L. mexicana*.

The identification of a few PKs and much of the functional assessment was conducted prior to the publication of the genome for *L. major* in 2005 (Ivens et al.). This work enabled the identification of 179 ePKs and 16 atypical PKs (aPKs) composing the *Leishmania* kinome based on bioinformatic analysis and comparisons between the *L. major*, *T. brucei* and *T. cruzi* genomes (Parsons et al. 2005). The kinome comprises 2% of the genome as in humans, however there are fewer PKs encoded in the *Leishmania* genome and many of the human ePK groups are completely unrepresented in *L. major* (**Figure** 1:8), particularly the tyrosine kinase (TK) and tyrosine kinase-like (TKL) groups. Interestingly, the most abundant ePK group is the GMGC, which comprises those *Leishmania* PKs classified as CDKs and MAPKs which were studied prior to the genome publication alongside the glycogen synthase kinases (GSK) and CDK-like kinases (CLKs).

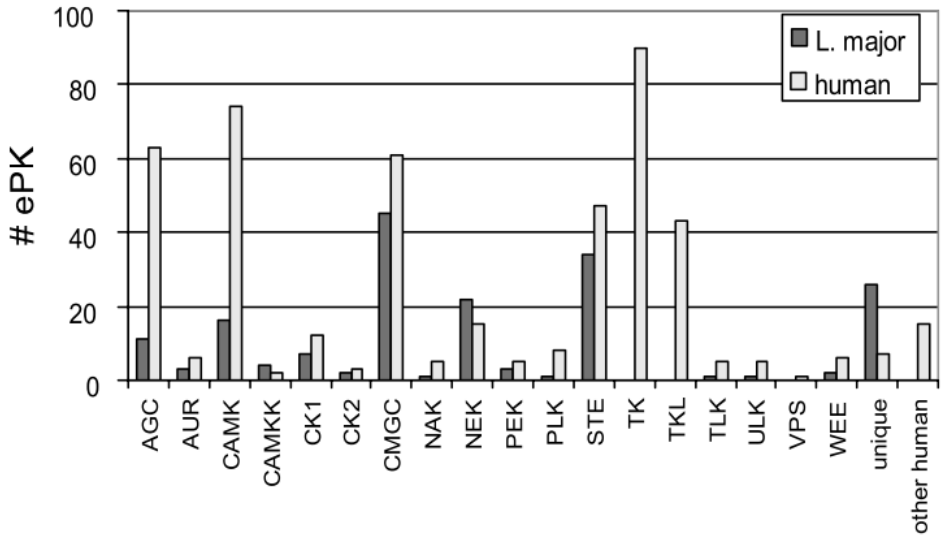


Figure 1:8- Comparison of *L. major* and human ePK classification. Adapted from (Parsons et al. 2005).

Further research into the function of the protein kinome will enable the identification of potentially powerful regulators of the cell cycle and differentiation of *Leishmania*. Our knowledge that protein kinases are 'druggable' targets further establishes the promise of these proteins in therapy, whilst the expansion of unique *Leishmania* protein kinases holds promise for specific inhibition in the absence of human kinase inhibition. Advances in regulation of gene expression and testing of compound screens against these targets will potentiate the discovery of antiparasitic agents.

1.4.3 Mining the kinome: RNAi kinome screens in *T. brucei* as a reference point for *Leishmania* drug target validation

The published *Leishmania* kinome enables the functional analysis of other ePKs by the available genetic methods to establish whether the deletion of the encoded gene confers a suitably pronounced effect on survival to be a target for drug inhibition. Current methods do not exist to conduct genetic analysis in a high-throughput manner for *Leishmania* (as discussed in section 1.3), however the utilisation of a kinome-wide RNAi screen of 190 protein kinases in *T. brucei* has enabled the identification of protein kinases which are essential for proliferation of the mammalian infective bloodstream form (Jones et al. 2014). In addition, two protein kinases termed repressor of differentiation (RDK) 1 and 2 were identified which regulate the differentiation of bloodstream to procyclic

forms. The regulation of differentiation is a key step in maintenance of the parasite's life cycle, therefore these kinases represent very interesting targets for inhibition to induce inappropriate cellular differentiation during infection.

Due to the highly conserved kinases between the kinetoplastids (Parsons et al. 2005), the transfer of information from such a comprehensive screen to form rational hypotheses for functional analysis of *Leishmania* kinases in a more targeted manner is feasible. Chapter 6 of this thesis utilises this strategy to study a potential repressor of differentiation (RDK1) identified by kinome-wide screening of *T. brucei* for application to the *L. mexicana* homologue, with the application of this method to the other protein kinases having important implications in identifying proteins with a key role in regulating many aspects of the *Leishmania* life cycle.

1.5 Project aims

Leishmaniasis remains a considerable public health issue with the development of drug discovery programmes an important step in identifying novel anti-leishmanial targets. *Leishmania* protein kinases are important regulators of cell signalling pathways. The cdc2-related protein kinase, CRK3 represents a partially validated drug target due to its essential function in regulating the cell cycle. The efficacy of protein kinase inhibition by small molecules is further evidence of the potential for small molecule inhibition of CRK3 as a route of therapy, yet our understanding of how this protein functions in the mammalian infective amastigote is currently limited by an absence of an existing method for inducible gene deletion. This prevents the regulation of gene expression during *in vitro* or *in vivo* infection, and is a severe limitation in assessing the protein as useful drug target.

In addition, functional assessment of *T. brucei* kinases by RNAi has identified a repressor of differentiation kinase (RDK1) with a homologue of unknown function in *L. mexicana*. If implicated in regulating differentiation in the amastigote stage, no method exists with which to regulate gene expression during this life cycle stage. To further study proteins implicated as important regulators of amastigotes, an *in vivo* model to assess the onset of a productive immune response to *L. mexicana* infection would enable the study of host-parasite

interactions. By conditional deletion of a target gene, the result on the mammalian immune response to infection could be assessed.

This projects aims to:

1. Develop a method for conditional deletion of essential *L. mexicana* genes using the diCre system.

2. Apply the system to conditionally delete the essential protein kinase gene *CRK3 in vitro* to assess the function of CRK3 in procyclic promastigotes and essentiality for *in vivo* infection of Balb/c mice.

3. Generate a model for the study of immune cell recruitment to infection with bioluminescent *L. mexicana* for application in conjunction with inducible gene deletion.

4. Generate a repressor of differentiation (RDK1) deficient *L. mexicana* cell line to assess a hypothetical function in regulating differentiation.

2 MATERIALS AND METHODS

2.1 Bioinformatics

2.1.1 Genome sequence retrieval

DNA and amino acid sequences for genes of interest were retrieved from TriTrypDB in FASTA format (http://tritrypdb.org/tritrypdb/). TriTrypDB was also used to identify syntenic genes between *Leishmania mexicana* and *Trypanosoma brucei* and to identify the splice and poly-adenylation site locations for each gene of interest.

2.1.2 Sequence manipulation and vector design

In silico manipulation of DNA and amino acid sequences was conducted using CLC Genomics Workbench (CLC Bio). Features of this software allowed the design of oligonucleotide primers, sequence alignment, *in silico* cloning, restriction digest mapping and reverse translation of proteins. In addition, the Gateway cloning process was conducted *in silico* to design primers conferring the desired *att* sites for recombination. This software allows PFAM searches to identify protein domains to be carried out within it (http://pfam.sanger.ac.uk/). Sequences were analysed using the full PFAM library downloaded as an add-on from CLC Genomics plugins (http://www.clcbio.com/products/clc-genomics-workbench/). A number of vector fragements were designed using the CLC software and subsequently synthesised by Dundee Cell Products Ltd (James Lindsay Place, Dundee Technopole, Dundee, DD1 5JJ) or GenScript USA Inc. (860 Centennial Ave, Piscataway, NJ 08854, USA).

2.2 Bacterial strains and culture

2.2.1 *E. coli* strains used

For routine transformations of ligation reactions, DH5α competent cells (Invitrogen) were used. This strain contains the genetic markers *rec*A1, to improve insert stability and prevent unwanted recombination, and *end*A1, to improve yield and quality of plasmid DNA from minipreps. It also contains *lac*ZΔM15 for blue/white colour screening of colonies. MAX efficiency DH5α

competent cells (Invitrogen), a strain with improved transformation efficiency, or OneShot® TOP10 chemically competent *E. coli* contining *hsdR* for efficient transformation of unmethylated DNA from PCR amplifications were used for transformation of Multisite Gateway reactions. For transformation of difficult ligation reactions, XL10-Gold Ultracompetent cells (Stratagene) were used. In addition to *endA, recA* and *lac*ZΔM15, these bacteria exhibit the Hte phenotype, which increases the transformation efficiency of ligated or large supercoiled DNA.

2.2.2 Transformations

Aliquots of 50 µl competent cells were thawed on ice before adding 1 – 5 µl (1 - 10 ng) DNA and incubating on ice for 30 min. Cells were subjected to heat shock for 45 sec in a 42^{o}C water bath then incubated on ice for 2 min. 950 µl of pre-warmed medium were added to each tube, which were then incubated for 1 hour at 37^{o}C with shaking at 225 rpm. ≤200 µl each transformation were plated out onto selective LB agar plates and incubated overnight at 37^{o}C. For blue/white colour screening of ligation reaction transformations, cells were plated out on selective LB agar plates containing IPTG/X-Gal before overnight incubation at 37^{o}C.

2.2.3 Bacterial culture and storage

Individual colonies of transformed bacteria were inoculated into LB broth with suitable antibiotic (Ampicillin 100 µg/ml; Kanamycin 50 µg/ml (Sigma)) and cultured overnight, to select for bacteria expressing plasmid DNA of interest, at 37^{o}C with shaking at 225 rpm. For long term storage of bacteria, 0.5 ml of culture was mixed with an equal volume of 2 % (w/v) peptone and 40 % (v/v) glycerol and stored at -80^{o}C.

2.2.4 Preparation of plasmid DNA from *E. coli*

Plasmid DNA from *E. coli* was purified from a cell pellet harvested from 5 ml of LB culture using the QIAprep Spin Miniprep kit (Qiagen), following the manufacturer's protocol for "QIAprep Spin Miniprep Kit Using a Microcentrifuge".

2.3 Molecular Biology

2.3.1 DNA Sequencing

DNA sequencing was primarily performed by DNA Sequencing & Services (MRCPPU, College of Life Sciences, University of Dundee, Scotland, www.dnaseq.co.uk) using Applied Biosystems Big-Dye Ver 3.1 chemistry on an Applied Biosystems model 3730 automated capillary DNA sequencer. After two years the sequencing provider was changed to Eurofins MWG Operon (i54 Business Park, Valiant Way, Wolverhampton, WV9 5GB) SmartSeq kit sequencing by cycle sequencing technology on Applied Biosystems model 3730XL DNA sequencer.

2.3.2 Polymerase Chain Reaction

Oligonucleotide primers were designed using CLC Genomics Workbench and synthesised by Eurofins MWG Operon (Ebersber, Germany).

Standard PCRs such as colony PCR and PCR amplification of genomic DNA to identify inducible loss of a floxed gene: template DNA (100 ng genomic DNA, 100pg plasmid DNA or bacterial inoculation), 2.5 µl of 10 x PCR mix (1.13 mg ml^{-1} BSA, 450 mM Tris pH 8.8, 110 mM ammonium sulphate, 45 mM $MgCl_2$, 68.3 mM ß-mercaptoethanol, 44 µM EDTA pH 8.0, 10 mM dCTP, 10 mM dATP, 10 mM dGTP, 10 mM dTTP), 5 pmol of each primer, 1 unit of *Taq* polymerase and sterile distilled deionised water to a final volume of 25 µl. Thermocycling was programmed for an initial denaturation step of 96°C for 5 minutes, followed by 30 cycles of: denaturation 96°C for 30 seconds; annealing at [prmer specific Tm] for 30 seconds; extension at 72°C for *Taq* polymerase, 30 seconds per 0.5 kb of sequence. PCR conditions were subject to optimisation for specific primer pairs/templates and primer annealing temperature and elongation time were adjusted as required.

For generating PCR products for Gateway cloning or molecular cloning a proofreading enzyme was used, in this study Phusion (New England Biolabs) was used exclusively due to its robustness and reliability. Phusion reactions were set up as follows; Phusion HF buffer at 1x (stock at 5x), each dNTP at 200 µM,

primers at 0.5 μM each, template DNA and Phusion polymerase at 0.02 U/μl. Reactions were usually set up to 50 μl.

2.3.3 Quantification of DNA concentration and purity

DNA concentration, given in ng ml^{-1}, and purity were quantified on a NanoDrop 1000 Spectrophotometer (NanoDrop), through absorption at 260 nm (A_{260}), and the ratio of A_{260} to A_{280} (nucleic acids to proteins) respectively.

2.3.4 Restriction Enzyme Digests

All restriction endonucleases used in this study were sourced from New England Biolabs (NEB) and used according to the manufacturers instructions and buffers. For double digests, the optimum reaction buffer was checked using the Double Digest Finder tool (https://www.neb.com/tools-and-resources/interactive-tools/double-digest-finder#.T0ODjfVLeU4). For restriction mapping of plasmids or generating fragments/backbones for cloning reactions were allowed to proceed for at least 20 minutes if using a time-saver enzyme prior to band resolution by agarose gel electrophoresis. Plasmids that were being linearized in 10 μg amounts in preparation for transfection were generally digested overnight in 100ul volumes to ensure complete digestion.

2.3.5 Agarose Gel Electrophoresis

DNA sequences in this study were analysed by agarose gel electrophoresis which also serves as a method for purifying DNA fragments by size. UltraPure agarose (Invitrogen) was dissolved in 0.5 x TBE buffer (20mM Tris, 20 mM boric acid, 0.5mM EDTA, pH 7.2) at 1% w/v. For resolving larger fragments this was reduced to 0.8% w/v. After allowing the gel to cool SYBR-safe DNA stain (Invitrogen) was added to allow the visualization of DNA. Gels were then cast in the required size of gel support. DNA was prepared by addition of 6x Loading Buffer (0.25 % (w/v) bromophenol blue, 0.25 % (w/v) xylene cyanol FF, 30 % (v/v) glycerol, in H_2O) and loaded into wells. Gels were run at between 80-120 V for 60 min depending on expected fragment sizes. For Southern blotting, 0.8% w/v gels were run at 50V for over 5 hours to resolve the bands. Gels were imaged using a GelDoc (BioRad) and the associated Quantity One Software (BioRad).

In order to purify DNA fragments from agarose gels the DNA was visualised using a DarkReader blue light transilluminator, the band excised using a sterile scalpel blade and extracted from the agarose gel with a Gel Extraction kit (Qiagen) for general purposes, or a MinElute Kit (Qiagen) for more sensitive applications such as Gateway cloning. This was performed on a Qiacube (Qiagen) according to the manufacturer's instructions.

2.3.6 Ligations

When molecular cloning constructs by the cohesive end technique compatible DNA fragments were ligated together using T4 DNA ligase (NEB) and the 10x Ligase Buffer supplied with it. Insert and plasmid backbone DNA fragments were mixed in equimolar amounts, 1:3 or 1:5 backbone:insert ratios in a 10 µl volume of 1xLigase buffer plus T4 DNA Ligase. These were incubated overnight at 16°C or room temperature for 1 hour. Some inserts were first subcloned into P-GEMT Easy (Promega) or PCR-Script (Stratagene) depending on the size of the insert. PCR-Script was typically used for inserts over 2 Kb due to its greater efficiency for larger inserts.

2.3.7 Site Directed Mutagenesis

Mutations of single bases and deletions of multiple bases were conducted using the Q5 Site-Directed Mutagenesis kit (NEB) according to manufacturers instructions. Mutagenesis primers were designed using the NEBaseChanger software (http://nebasechanger.neb.com). Mutations were carried out to remove and insert restricition sites into existing vectors such as the loxP pDONR221 vector. In addition, mutagenesis of the T-loop residue in *CRK3* was conducted by this method. All mutations were checked by subsequent DNA sequencing (MWG Operon).

2.3.8 MultiSite Gateway® 3-fragment vector construction

Gene replacement vectors were generated using the Multisite Gateway three fragment vector construction kit (Invitrogen), following the manufacturer's guidelines. The aim was to create diCre and loxP expression plasmids for specific integration at a target gene locus by the addition of 500bp of 5' and 3' gene flanking regions. These knockout constructs could then be transfected into

Leishmania to replace the target gene by homologous recombination. A detailed diagram of this approach is represented in section 3. The diCre and loxP vectors were synthesised by GenScript and subcloned into pDONR 221 vectors. *attB* primers were designed to amplify 5' and 3' flanking regions from *L. mexicana* genomic DNA using the primers annotated below. These amplicons were resolved by gel electrophoresis, and purified by Gel extraction (Qiagen). 50fmoles of each amplicon was used in a BP recombination reaction with 150ng of pDONR P4-P1r or P2R-P3, 2ul of BP clonase II enzyme mix to a final volume of 10ul with 1xTE buffer (10mM Tris-HCl, 1mM EDTA, pH8.0). The reaction was performed at 25°C for 1 hour and stopped by the addition of 2ug Proteinase K (2ug/ul in 10mM Tris-HCl pH7.5, 20mM $CaCl_2$, 50% glycerol) and incubation at 37°C for 10 minutes. 2µl of each reaction was used to transform either max-efficiency DH5a or OneShot TOP10 *E. coli* which were spread onto Kanamycin infused plates. Colony PCR was used to check insertion of the amplicon into the vector and integrated plasmids picked for mini-preparations of entry clones (Qiagen).

The final vector was generated by combining the entry clones with either the diCre, loxP or drug resistance cassette pDONR221 vectors (pGL2206, 2207, 2208 and 2209) into a final pDEST expression clone in an LR clonase mediated reaction. 10fmoles of each entry clone and 20fmoles of the pDEST R4-R3 vector II were combined in a 10µl reaction with TE buffer and 2µl of LR Clonase II Plus enzyme mix. The reaction was performed at 25°C overnight and stopped by the addition of 2ug Proteinase K and incubation at 37C for 10 minutes. 2µl of each reaction was used to transform either max-efficiency DH5α or OneShot TOP10 *E. coli* which were spread onto Ampicillin infused plates. Colonies were picked and plasmid prepared by mini-preparation (Qiagen). Restriction enzyme digest and sequencing was utilised to identify appropriate recombination and generation of gene replacement vectors.

2.3.9 Ethanol precipitation

To purify and concentrate DNA for transfection into *Leishmania spp.*, a 10% volume of 3 M sodium acetate pH 5.2 and one volume of 100% isopropanol were added to the sample. The sample was centrifuged at 13 000 x *g* for 30 min at 4 °C. The DNA pellet was washed in 700 µl 70% ethanol and centrifuged at 13 000 x *g* for 30 min at 4 °C. The supernatant was removed and the DNA pellet was air-

dried in a fume hood. The DNA pellet was resuspended in 30 µl molecular grade water for *Leishmania* transfection.

2.3.10 Southern blotting

For analysis of *L. mexicana ΔRDK1* mutants, 5 µg DNA from resistant clones were digested with *Sac*I and resolved overnight on a 0.7 % agarose gel in TBE buffer at 30 volts. The DNA in the gel was depurinated by soaking for 10 min in 0.25 M HCl, denatured for 15 – 30 min in denaturation solution (1.5 M NaCl, 0.5 M NaOH), then neutralised by soaking for 15 – 30 min in neutralisation solution (3 M NaCl, 0.5 M Tris-HCl, pH 7), washing between these steps with distilled water. The DNA was transferred onto a Hybond-N^+ nylon membrane (Amersham, GE Heatlhcare) in 20 x SSC (3 M NaCl, 0.3 M sodium citrate, pH 7). The membrane was layered on top of the gel, with two sheets of blotting paper and excess weighted paper towels added to draw the 20 X SCC buffer up the wick by capillary transfer overnight. Transferred DNA fragments were covalently cross-linked to the membrane by UV cross-linking in a UV Stratalinker 2400 (Stratagene) at 1200 mJoules. The probe (5' UTR sequence of *L. mexicana RDK1*) was prepared at 10 ng/µl, labelled with an AlkPhos Direct labelling kit (Amersham, GE Healthcare) following manufacturer's instructions, and incubated with the membrane overnight at 60^{o}C in hybridisation buffer (GE Healthcare). After washing, signal was detected using CDP-Star detection reagent (Amersham, GE Healthcare) and exposed using Kodak photographic film.

For detection of floxed *GFP* loss, the process was repeated but with the following amendments; 2.7µg of genomic DNA was digested per sample with *Sac*I and *Spe*I double digests and the probe was prepared from a *GFP* CDS amplicon.

For detection of floxed *CRK3* loss; the *Nru*I restriction enzyme was used to digest 3ug of genomic DNA per sample and the probe was prepared from a *CRK3* CDS amplicon.

2.3.11 RNA extraction

For each sample, RNA was isolated from 1 x 10^7 promastigotes using the RNAeasy kit (Qiagen) processed in the automated QIAcube (Qiagen) according to

manufacturer's instructions. An additional DNAse step was included once purified RNA was derived. cDNA was produced from 1 µg of isolated RNA using random hexamer primers (Invitrogen) and SuperScript Reverse Transcriptase III (Invitrogen) according to manufacturer's instructions. To monitor genomic DNA contamination the procedure was performed without reverse transcriptase (-RT).

Table 2-1- List of primers used for real-time PCR in this study

Oligo No.	Gene ID	Gene	Sequence
OL4593	LmxM.36.0550	*LmxCRK3*	GATCGCACTGAGGAGGGTAT
OL4594			GTGGTCGAACTCTTGCAGAA
OL4595	LmxM.18.0360	*LmxGPI8*	GGCTGTCATTGTCTCCTCCT
OL4596			GTACATGGTAAGCGCATTGG
OL4691	N/A	*Cre59*	GGCTCCACCTCTGATGAAGT
OL4692			CAGGTGTGTTCAGAGAAGGC
OL4693	N/A	*Cre60*	ACCTGAGGATGTGAGGGACT
OL4694			AGGTGCTGTTGGATGGTCTT

2.3.12 Quantitative real time PCR

qPCR reactions were set up using Applied Biosystems SYBR Green PCR master mix, each reaction was performed in triplicate and contained 12.5 µl Mastermix, 2.5 µl of each primer (3 µM), 5.5 µl H_20 and 2 µl of the template cDNA. Reactions were set up in MicroAmp® Optical 96-Well Reaction Plate (Applied Biosystems) and sealed with MicroAmp® Optical Adhesive Film (Applied Biosystems). Samples were analysed in an Applied Biosystems Prism 7500 using the default thermocycling settings with the reaction volume set to 25 µl. A denaturation step was added to check that only a single product was being formed by each primer pair.

Table 2-2-List of primers used for generation of Gateway entry clones in this study

Oligo No.		Flank	Description	Gene ID	Sequence	Annotations
OL3966	F	5'	Amplification of *RDK1* flanking regions for Gateway entry clone generation	LmxM.31.0810	GGGGACAACTTTGTATAGAAAAGTTG**AAGCTT**GCACAGCAATGTTTAAGGGC	attB4, ***Hind*III**
OL3967	R				GGGGACTGCTTTTTTGTACAAACTTGGGTCGAGGCCGTGCCAGCCGTT	attB1r
OL3968	F	3'			GGGGACAGCTTTCTTGTACAAAGTGGGAACCCTGCGTCCCCGTACC	attB2r
OL3969	R				GGGGACAACTTTGTATAATAAAGTTG**GAGCTC**AAGACAACGTCGCTCCATGAC	attB3, ***Sac*I**
OL4249	F	5'	Amplification of *CRK3* flanking regions for Gateway entry clone generation	LmxM.36.0550	GGGGACAACTTTGTATAGAAAAGTTGCCC**TTAATTAA**AAAGGTAGAGGATGCCGTTTT	attB4, ***Pac*I**
OL4250	R				GGGGACTGCTTTTTTGTACAAACTTGCTTGAAATGTTGCAGGGAGAAA	attB1r
OL4251	F	3'			GGGGACAGCTTTCTTGTACAAAGTGGGGAGTGGAAAAGGCATGACTGAA	attB2r
OL4252	R				GGGGACAACTTTGTATAATAAAGTTGC**GTTTAAAC**TTTCCTCCCCAGCACGCACAC	attB3, ***Pme*I**
OL4397	F	5'	Amplification of ribosomal 18S flanking regions from pGL631 for Gateway entry clone generation	LmxM.27.rRNA	GGGGACAACTTTGTATAGAAAAGTTGCC**TTAATTAA**TGACGAACAACTG	attB4, ***Pac*I**
OL4398	R				GGGGACTGCTTTTTTGTACAAACTTGCGTTACTCGATATTGGATGGGT	attB1r
OL4399	F	3'			GGGGACAGCTTTCTTGTACAAAGTGGGGCGACTAGACCGTAACGCCTTT	attB2r
OL4400	R				GGGGACAACTTTGTATAATAAAGTTGG**GTTTAAAC**ATGAGCTGCGCCT	attB3, ***Pme*I**
OL4512	F	5'	Amplification of *NMT* flanking regions for Gateway entry clone generation	LmxM.31.0080	GGGGACAACTTTGTATAGAAAAGTTG**TTAATTAA**GTGGAAGGGAGAGCCTTGCTG	attB4, ***Pac*I**
OL4513	R				GGGGACTGCTTTTTTGTACAAACTTGTCGTGTGCGTGTGCGTACAGC	attB1r
OL4405	F	3'			GGGGACAGCTTTCTTGTACAAAGTGGTCTACGCACTGCCCCACC	attB2r
OL4406	R				GGGGACAACTTTGTATAATAAAGTTG**GTTTAAAC**GGGAACAAGGCGACAAAC	attB3, ***Pme*I**
OL4415	F	5'	Amplification of *MIF* 1 and 2 flanking regions for Gateway entry clone generation	LmxM.32.1740 LmxM.32.1750	GGGGACAACTTTGTATAGAAAAGTTGCC**TTAATTAA**GTGGAGCTATGTAGGCTGATT	attB4, ***Pac*I**
OL4416	R				GGGGACTGCTTTTTTGTACAAACTTGCTTTCGCTAAGGGGGAGGGGGG	attB1r
OL4417	F	3'			GGGGACAGCTTTCTTGTACAAAGTGGCTAGCGCCGCGGCCATCACGAAGGA	attB2r, STOP
OL4418	R				GGGGACAACTTTGTATAATAAAGTTGC**GTTTAAAC**CCTTCGTTTTGGCGCCTCGGA	attB3, ***Pme*I**
OL4705	F	5'	Amplification of ß-Tubulin flanking regions and the *CRK3* CDS with G418r cassette from pGL2374 for pDONR221 insertion and Gateway entry clone generation	LmxM.32.0792 pGL2374	GGGGACAACTTTGTATAGAAAAGTTG**TTAATTAA**AGAAACACAAACATTTTCGCA	attB4, ***Pac*I**
OL4706	R				GGGGACTGCTTTTTTGTACAAACTTGGATGGCAGAGTGGCGAAAGGC	attB1r
OL4715	F	CDS			GGGGACAAGTTTGTACAAAAAAGCAGGCTCTCGAGATGTCTTCGTTTGGC	attB1
OL4708	R				GGGGACCACTTTGTACAAGAAAGCTGGGTCGGACGCGGGCAGCGAGGGGA	attB2
OL4709	F	3'			GGGGACAGCTTTCTTGTACAAAGTGGACGGTGTGTGGGTGAGGTGCG	attB2r
OL4710	R				GGGGACAACTTTGTATAATAAAGTTG**GTTTAAAC**CTGGTCCGTCGTGCAGAGACA	attB3, ***Pme*I**

Table 2-3- Primers used for cloning of a loxP vector for insertion of a target gene flanked by loxP sites

Oligo No.		Description	Sequence	Annotations
OL4065	F	Amplification of puromycin resistance cassette from pGL631	GATC**CTGCAG**CGCGTGGATGTCGCGCAG	***Pst*I**
OL4066	R		GATC**GCTAGC**CTAGGCACCGGGCTTGCG	***Nhe*I**
OL4293	F	Amplification of SAS-HASPB-*mCherry* from pGL1893 to integrate at reporter site	GATC**CTCGAG**AATTGCCCGCTTTCCAT	***Xho*I**
OL4294	R		GATC**GCGGCCGC**GGGATCCTCAATGATGA	***Not*I**
OL4316	F	Amplification of GFP from pGL1773 for integration as N-terminal tag	GATC**CATATG**ATGGTGAGCAAGGGCGAG	***Nde*I**
OL4317	R		GATC**GGTACC**CTTGTACAGCTCGTCCAT	***Kpn*I**
OL4318	F	Amplification of 6xHA integration as N-terminal tag	GATC**CATATG**TACCCTTACGATGTGCCT	***Nde*I**
OL4319	R		GATC**GGTACC**TGCGTAATCGGGCACATC	***Kpn*I**
OL4320	F	Amplification of GFP from pGL1773 for integration as C-terminal tag	GATC**ACTAGT**ATGGTGAGCAAGGGCGAG	***Spe*I**
OL4321	R		GATC**TCTAGA**TCACTTGTACAGCTCGTCCAT	***Xba*I**, STOP
OL4541	F	Amplification of SAS-HASPB-mCherry for insertion via *Hind*III: enables the replacement of HASPB-mCherry by *Xho*I and *Not*I	GATC**AAGCTT**AATTGCCCGCTTTCCATTTCG	***Hind*III**
OL4542	R		GATC**GCGGCCGC**GGGATCCTCAATGATGATGAT	***Not*I**
OL4067	F	Amplification of the *CRK3* CDS for insertion into the loxP MCS: no Stop codon amplified due to C-terminal *GFP* fusion	GATC**CATATG**TCTTCGTTTGGCCGTGTG	***Nde*I**
OL4103	R		GATC**ATCGAT**CCAACGAAGGTCGCTGAA	***Cla*I**
OL4335	F	Amplification of the *RDK1* CDS for insertion into the loxP MCS: no Stop codon amplified due to C-terminal *GFP* fusion	GACC**CATATG**ATCCGGAACGCCACGATC	***Nde*I**
OL4336	R		GCGC**ACTAGT**AATCAGGAAGGCGAAGGA	***Spe*I**
OL4380	F	Amplification of the *RDK1* CDS for insertion into the loxP MCS: Stop codon amplified due to N-terminal *GFP* fusion	GATC**ACTAGT**ATCCGGAACGCCACGATCCCG	***Spe*I**
OL4381	R		GATC**TCTAGA**CTAAATCAGGAAGGCGAAGGAAAG	***Xba*I**, STOP
OL4388	F	Amplification of the *CRK3* CDS for insertion into the loxP MCS: Stop codon amplified due to N-terminal *GFP* fusion	GATC**ACTAGT**TCTTCGTTTGGCCGTGTGACC	***Spe*I**
OL4389	R		GATC**TCTAGA**CTACCAACGAAGGTCGCTGAA	***Xba*I**, STOP
OL4407	F	Amplification of the *NMT* CDS for insertion into the loxP MCS: Stop codon amplified due to N-terminal *GFP* fusion	GATC**ACTAGT**TCTCGCAATTCATCGAACTCT	***Spe*I**
OL4408	R		GATC**TCTAGA**CTACAGCATCACCAAGGCAAC	***Xba*I**, STOP
OL4419	F	Amplification of the *MIF 1/2* CDS for insertion into the loxP MCS: Stop codon amplified due to N-terminal *GFP*	GATC**GAATTC**ATGCCGGTCATTCAAACGTTT	***Eco*RI**
OL4420	R		GATC**TCTAGA**CTAAAGTTAGTGCCGTTCCA	***Xba*I**, STOP

		fusion		
OL4591	F	Amplification of *CRK3-his* for insertion into pGL2277: generate an 18S RNA integration vector for complementation of the floxed *CRK3* inducible deletion line	**CTCGAG**ATGTCTTCGTTTGGCCGT	***Xho*I**
OL4592	R		**GCGGCCGC**CTAATGATGATGATGATGATGCCAAC GAAGGTCGCTGAA	***Not*I**, STOP, 6xhis

Table 2-4- Primers used for analysis of inducible floxed gene loss and for integration confirmation

Oligo No.		Binding Region	Description	Sequence
OL4097	F	Upstream	Detection of *RDK1* loss by amplification of a 5' fragment	TTGCGAAGCATCTCCAGC
OL4098	R	*RDk1*		CGAGTGACAGCAAAAGGC
OL4099	F	*RDK1*	Detection of *RDK1* loss by amplification of a 3' fragment	ATTGCTGTCGACATTCCC
OL4100	R	Downstream		GTAGTCCTCATCATCAGC
OL4101	F	*BLAr*	Internal forward and reverse primers to detect diCre integration into the genome	CTGGTTATGTGTGGGAGG
OL4102	R	*FKBP12*		GATGGTTTCCACCTGCAC
OL4287	F	Upstream	Amplification of floxed a *GFP* fragment to detect gene loss by diCre induction	GCTCGCGTGTGTTGAGCC
OL4288	R	Downstream		CATTCGTGGGCTCCAGCT
OL4296	F	Upstream	Primers binding out-with the *CRK3* integration site	GATCGTGGGAAGGGGAAG
OL4297	R	Downstream		GGAAGTCCAAGTAGCGCG
OL4298	R	*CRK3*	Primers binding the *CRK3* gene	GGTCACACGGCCAAACGA
OL4299	F	*CRK3*		GCCAAGGAGGCCCTACAG
OL4300	R	loxP vector SAS	Primers binding the loxP vector at the 5' splice acceptor site (SAS) and 3' poly-adenylation site (PAS)	GGTGGACGGCTCAACACA
OL4301	F	loxP vector PAS		GTGTGCTGTGCGTTCAGC
OL4571	F	Upstream	*MIF* integration primers which bind upstream or downstream of the integration site	CATGGCAGTGCTCTTCAG
OL4572	R	Downstream		CAATACGCGGTGAGCTAC
OL4573	F	Upstream	*NMT* integration primers which bind upstream or downstream of the integration site	GAGTGGGCGCATTGCTGC
OL4574	R	Downstream		CTGCCCCATCAATGACAG
OL4781	F	Upstream	Amplification of a floxed *CRK3-GFP* fragment to detect gene loss by diCre induction	AACTGGCAGCAGCGATTTGGCAGGGG
OL4782	R	*PACr*		GCACCGTGGGCTTGTACTCGGTCATG
OL4748	F	Upstream	Primers to check for integration of *RE9H* construct (pGL2398) into the ribosomal locus; primers 4749 and 4750 can be used to check for episomal retention	TCGTGAGACGCCCAGCGAATG
OL4749	F	*RE9H* (middle)		GAAGGCGATGGTGCCTGGCAC
OL4750	R	*RE9H* (middle)		ACCGACGCCCACATCGAGGTG
OL4751	R	*RE9H* (3' end)		GCCACGTAGTCCACGATCTCC

Table 2-5- Sequencing primers used in this study

Oligo No.		Binding Region	Description	Sequence
OL4349	F	Backbone	Sequencing primers to check for loss of *Nde*I codon from mutated pGL631	GCGCGTTTCGGTGATGACGG
OL4350	R	5'SSU flank		TTGTTACTCGATATTGGATG
OL4351	F	Backbone	Sequencing primers for final loxP vector (pGL2314): these primers give full coverage of the plasmid	ATAATGCCAACTTTGTACAAA
OL4352	R	*6xHA*		TACCCTTACGATGTGCCTGA
OL4353	F	*6xHA*		GGCACGTCGTATGGGTACGC
OL4354	R	*PACr*		GCGGGGTAGTCGGCGAACGC
OL4355	F	*PACr*		CCCCGGGCCGTACGCACCCT
OL4356	R	*PAS*		TCGCGGGGCACCACGACTTG
OL4357	F	*PAS*		GAGCAGGCGCGCTGTGAATC
OL4358	F	5' *CRK3*	Primers to sequence the whole *CRK3* gene	ATGTCTTCGTTTGGCCGTGT
OL4359	R	3' *CRK3*		CTACCAACGAAGGTCGCTGA
OL4360	R	*RDK1*	Primers to sequence the whole *RDK1* gene (4.095 Kbp)	TGCGGGTCTGATTCGCATCG
OL4361	R			GCATCTCTCGCAAACTCCGC
OL4362	F			TTCGCGGCATTCACGAGGCC
OL4363	F			GTGCTGCGATGTACGACTGC
OL4364	F			CATGCCAGGGGGCTCGCTGC

Table 2-6-Mutagenesis primers used in this study

Oligo No.		Gene/Mutation	Description	Sequence	Annotations
OL4253	F	diCre/ Deletion	Deletion of extra ATG start codon from 5' actin region within diCre expression construct	CTTGCCTTTCAGTGCGGG	-
OL4254	R			GGTATTCGACCTACACCG	-
OL4265	F	diCre/ Insertion	Insertion of an *Xho*I *Not*I site to enable integration of an extended 3' actin flank into diCre vector	ATCGTTTTGGCGGCCGCTTTCTTCCATCTGCACTCG	***Not*I**
OL4266	R			GATTGAAAGTAGGATCCGAGCAACGCACACTCTAAC	***Xho*I**
OL4267	F	Actin	Amplification of a 655bp 3' acting flank containing a poly adenylation site for diCre vector insertion	GATC**GGATCC**CTGCCTGGTTCTCGTGCTTA	***Xho*I**
OL4268	R			GATC**GCGGCCGC**GCGTGCACTTGATGCCAAAT	***Not*I**
OL4289	F	Deletion	Removal of E1α antigen tag from pGL1893 for mCherry amplification (with OL4293/4)	AGGATCCAATGGTTAGTAAAG	-
OL4290	R			CCATAGATCCATCCGCAC	-
OL4291	F	Deletion	Removal of *Xho*I site from pGL1893 to ready vector for mCherry amplification (with OL4293/4)	TGATCCTATCTATCTCCCCCG	-
OL4292	R			GCTACGGTGGACGGCTCA	-
OL4339	F	Deletion	Removal of extra *Nde*I restriction site upstream of 5'SSU flank in pGL631 vector	CGGTGTGAAATACCGCAC	-
OL4340	R			TATGGTGCACTCTCAGTAC	-
OL4553	F	loxP vector/ Substitution	Replacement of *Xho*I restriction site in loxP vector with *Hind*III to insert to enable replacement of reporter cassette by *Xho*I and *Not*I digest	GGCTAGCGCT**AAGCTT**ATGGTGAGCAAGGGCGAGGAG	***Hind*III**
OL4544	R			TAGGCACCGGGCTTGCGG	-
OL4601	F	*CRK3* mutagenesis	Mutation of T178 residue to glutamic acid to create $CRK3^{T178E}$	GCACACCTACgaGCACGAGGTGG	mutated site
OL4602	R			ATGGGCACTTGAAACGCAC	-
OL4689	F		Mutation of ATP binding pocket at 33-34 from TY to AF to create $CRK3^{AF}$	GGGAGAGGGAgcgttCGGCGTTGTG	mutated site
OL4690	R			AAAACATCCAAGCGATTGTACCGG	-

pGL No.	Gene ID	Gene Name	Backbone	Description	Drug Resistance
			Reporter Plasmids		
2217	N/A	*LUC*	pGL631	Luciferase bioluminescent protein in pRib construct	AMPr/ PACr
2233	N/A	*tdTomato*	pGL631	tdTomato red-fluorescent protein in pRib construct	AMPr/ PACr
2234	N/A	*RE9H*	pGL631	Red-shifted luciferase bioluminescent protein in pRib	AMPr/ PACr
2275	N/A	*CFP*	pGL631	Cerulean blue-fluorescent protein in pRib construct	AMPr/ PACr
2398	N/A	*RE9H*	pGL631	Red-shifted luciferase bioluminescent protein in pRib	AMPr/ G418r
2461	N/A	*GFP* ^{flox}	pGL631	Floxed GFP in pRib: for functional analysis of diCre	AMPr/PACr
			Gateway entry plasmids		
2313	N/A	*diCre*	pDONR221	DiCre expression cassette entry vector	KANr
2314	N/A	*loxP- C-6xHA*	pDONR221	LoxP (empty) expression cassette: c-terminal 6xHA tag	KANr
2315	N/A	*loxP-C-GFP*	pDONR221	LoxP (empty) expression cassette: c-terminal GFP tag	KANr
2316	N/A	*loxP-N-GFP*	pDONR221	LoxP (empty) expression cassette: n-terminal GFP tag	KANr
2445	LmxM.36.0550	*5' CRK3 flank*	pDONR P41-Pr	5' Flank (500bp) ready for Gateway recombination	KANr
2446	LmxM.36.0550	*3' CRK3 flank*	PDONR P2r-P3	3' Flank (500bp) ready for Gateway recombination	KANr
2447	LmxM.31.0810	*5' RDK1 flank*	pDONR P41-Pr	5' Flank (500bp) ready for Gateway recombination	KANr
2448	LmxM.31.0810	*5' RDK1 flank*	PDONR P2r-P3	3' Flank (500bp) ready for Gateway recombination	KANr
2449	LmxM.31.0080	*5' NMT flank*	pDONR P41-Pr	5' Flank (500bp) ready for Gateway recombination	KANr
2450	LmxM.31.0080	*3' NMT flank*	PDONR P2r-P3	3' Flank (500bp) ready for Gateway recombination	KANr
2451	LmxM.32.1740/	*5' MIF flank*	pDONR P41-Pr	5' Flank (600bp) ready for Gateway recombination	KANr
2452	LmxM.32.1750	*3' MIF flank*	PDONR P2r-P3	3' Flank (300bp) ready for Gateway recombination	KANr
2453	LmxM.27.rRNA	*5' 18S SSU*	pDONR P41-Pr	5' Flank (254bp) ready for Gateway recombination	KANr

2454	LmxM.27.rRNA	*3' 18S SSU*	PDONR P2r-P3	3' Flank (960bp) ready for Gateway recombination	KANr
			Gateway expression plasmids		
2399	LmxM.27.rRNA	*18S*	pDEST R4-R3	DiCre cassette flanked with 18S *SSU* homologous arms	AMPr/ BLAr
2455	LmxM.36.0550	*CRK3*	pDEST R4-R3	DiCre cassette flanked with *CRK3* homologous arms	AMPr/ BLAr
2456	LmxM.36.0550	*CRK3*	pDEST R4-R3	*CRK3-GFP*^{flox} cassette flanked with *CRK3* homology	AMPr/ PACr
2457	LmxM.31.0080	*RDK1*	pDEST R4-R3	DiCre cassette flanked with *RDK1* homologous arms	AMPr/ BLAr
2458	LmxM.32.1740/50	*MIF1/2*	pDEST R4-R3	DiCre cassette flanked with *MIF* locus homologous arms	AMPr/ BLAr
2459	LmxM.31.0810	*RDK1*	pDEST R4-R3	Blasticidin resistance cassette with 500bp *RDK1* flanks	AMPr/ BLAr
2460			pDEST R4-R3	Puromcyin resistance cassette with 500bp *RDK1* flanks	AMPr/ PACr
2235			pDEST R4-R3	Streptothrcin resistance cassette with 500bp *RDK1* flanks	AMPr/ SATr
2236			pDEST R4-R3	Hygromycin resistance cassette with 500bp *RDK1* flanks	AMPr/ HYGr

Table 2-7- List of plasmids (pGLs) generated in this study by restriction enzyme and Gateway mediated cloning

2.4 *Leishmania* culture methods

2.4.1 Culture of *Leishmania* promastigotes

Leishmania mexicana (MNYC/BZ/62/M379) were grown in modified Eagle's medium (designated HOMEM) supplemented with 10 % (v/v) heat-inactivated foetal calf serum (HIFCS) and 1 % (v/v) penicillin streptomycin solution (Sigma) at 25^{o}C. When referring to the stage of growth of cultures, mid-log phase corresponds to ~5 x 10^6 parasites/ml and stationary phase to ~2 x 10^7 parasites/ml. Transgenic parasites were maintained in appropriate antibiotics: G418 (Neomycin) at 50 µg/ml; Hygromycin at 50 µg/ml; Blasticidin S at 10 µg/ml; Puromycin at 10 µg/ml (InvivoGen).

2.4.2 Determination of cell density

Culture flasks were homogenised by gentle swirling and pipetting. 50 µl parasites were mixed with 50 µl 2 % formaldehyde and mixed by pipetting. 10 µl fixed cells were placed in a Neubauer haemocytometer (Weber Scientific) and cells counted under a light microscope. To set up a growth curve, a culture was started bewteen 1 x 10^5 to 1x10^6 cells/ml and cells counted every day for up to 7 days.

2.4.3 Creating *Leishmania* stabilates

For long term storage of *Leishmania* cell lines, stabilates were prepared by mixing 500 µl cells with 500 µl HOMEM + 20 % HIFCS + 10 % DMSO in a 1.5 ml cryotube. These were stored overnight at -80^{o}C before transferring to liquid nitrogen.

2.4.4 Transfection and selection of clones

Transfections of *Leishmania* were performed using an Amaxa human T cell nucleofector kit (Lonza) following the manufacturer's instructions. For each transfection, 5 x 10^7 cells in mid-log phase of growth were harvested by centrifugation at 1000 *g* for 10 min at 4^{o}C, and then resuspended in 100 µl T cell

nucleofector solution and transferred to a cuvette. Approximately 10 µg DNA in 30 µl sterile dH_2O were added to the cells and tapped gently to mix. Cells were electroporated using the U-033 program before being transferred to 10 ml fresh HOMEM + 20 % HIFCS. The culture was split between two flasks to select for independent transfection events and incubated overnight at 25°C to allow recovery. For each transfection a negative control was performed where 30 µl dH_20 was added instead of DNA. The following day appropriate antibiotics were added to select for transfectants. Cells transfected with integrative DNA were prepared in serial dilutions of 1 in 5, 1 in 50 and 1 in 500 in HOMEM + 20 % HIFCS + antibiotics and plated out onto 96 well microplates. Plates and flasks were maintained at 25°C for 3-5 weeks until transfectants appeared.

2.4.5 Induction of diCre mediated gene deletion

Inducible gene deletion in cell lines containing both diCre and loxP flanked genes of interest was induced by addition of between 1nM-1µM rapamycin (Abcam) from a 100µM stock solution to the culture medium. Promastigotes were induced at day 0 when preparing growth curves or for 24 hours for stationary phase deletion induction.

2.4.6 Preparation of protein extracts

A 10 ml culture of *L. mexicana* in the desired life cycle stage was pelleted by centrifugation at 1,200 *g* for 10 min, washed in PBS and then lysed by resuspending in 1 x SDS-PAGE loading buffer to a cell density of 1 x 10^7 parasites/10 µl. Samples were then boiled for 10 min at 100°C on a heat block before loading directly into SDS-PAGE gels or storing at -20°C.

2.4.7 Purification of *Leishmania major* metacyclic promastigotes

Metacyclic promastigotes were isolated from late stationary phase cultures using peanut lectin agglutination (Sacks et al., 1985). Parasites were centrifuged at 1000 *g* for 10 min, washed and resuspended in PBS to a density of 1 x 10^8/ml. Peanut lectin (Sigma) was added to a final concentration of 50 µg/ml and incubated for 10 min at room temperature. Metacyclic promastigotes were recovered from the supernatant after separation of agglutinated procyclic promastigotes by centrifugation at 100 *g* for 5 min.

2.4.8 Extraction of murine bone-marrow for bone marrow macrophage differentiation

Non-differentiated monocytes were extracted from the femurs and tibia of BALB/c mice by dissection to remove the bones. RPMI 1640 medium was used to was the bone marrow out of the intact bones by syringing with a 25G needle. Extracted cells were quantified by dilution in Trypan blue (1:1) and counting with a haemocytometer. Monocytes were seeded at $5x10^5$ cells/ml in MΦ Medium (DMEM + L-Glut + 20%FCS + 1% P/S + 30% L-Cell M) in 8ml volumes in Petri dishes and incubated at 37C with 5% CO2 for 3 days to induce differentiation to monocyte-derived macrophage. After this period the medium was replaced and by day 5 the cells were removed from the dishes using a cell scraper with ice-cold RPMI 1640. Purified macrophage were then counted once more and used for subsequent assays.

2.4.9 Macrophage infection

Bone marrow derived macrophage were adhered overnight in DMEM medium (PAA) with 10 % HIFCS onto 8-chamber tissue culture slides (LAB-TEK) at 37°C in 5 % CO_2. Macrophages were then infected with lesion derived *L. mexicana* amastigotes at a ratio of between 2-5 parasites per macrophage and imaged between 24 and 120 hours after infection in the DeltaVision Core environmental chamber at 37°C and 5 % CO_2, after DAPI staining.

2.4.10 DNA content analysis

To determine DNA content of cells by flow cytometry cells were fixed in 70% methanol 30% PBS overnight at 4°C. Cells were washed in PBS then resuspended in PBS + 10 μg/ml propidium iodide, 10 μg/ml RNase A at 37°C for 45 min. Cell fluorescence was measured on a MACSQuant Analyzer (Miltenyi Biotec) using the B3 channel (655-750nm) for PI detection. Data analysis was performed using FlowJo software (Tree Star Inc.).

2.5 Fluorescent microscopy

2.5.1 DeltaVision systems

Fluorescent microscopy was performed on an Applied Precision DeltaVision Core deconvolution microscope equipped with a Photometrics CoolSNAP HQ^2 camera and DAPI (381 - 399 nm), CFP (426 - 450 nm), GFP (461 – 489 nm), FITC (461 – 489 nm), YFP (505 – 515 nm), mCherry (563 – 588 nm), and Alexa594 (621 – 643 nm) filters. Earlier imaging experiments were carried out using an Applied Precision DeltaVision RT deconvolution microscope with a Photometrics CoolSNAP HQ camera and DAPI (381 - 399 nm), FITC (461 - 489 nm) and mCherry (529 - 556 nm) filters. Both microscopes are fitted with environmental chambers with temperature and CO_2 concentration regulated by a Weather Station temperature controller (PrecisionControl). DIC images were obtained under polarised light. Imaging of parasites was performed at 100 x magnification, and infected macrophages at 60 x, using immersion oil with the appropriate refractive index: live parasites in PBS, 1.516; fixed parasites in DAPI Fluoromount-G® (SouthernBiotech), 1.520; infected macrophages at 37^{o}C, 1.518.

2.5.2 Live cell imaging

~1x10^6 cells were used for microscopic analysis. Promastigotes or amastigotes were centrifuged at 1,200g or 2,000g respectively for 10 minutes and the cell pellet washed once in 1xPBS by repeat centrifugation. Parasites were then resuspended in PBS to the appropriate cell density and a small volume was spread thinly on a slide, i.e. 10 µl cells under a 22 x 40 mm coverslip, sealing the coverslip with nail varnish. Mounted parasites were observed on the microscope immediately, and imaged for up to 1 hour to reduce cellular damage occurring due to prolonged exposure to UV light.

2.5.3 DAPI staining

DAPI (4', 6-diamidino-2-phenylindole) was used to stain the DNA of parasites and host macrophages. DAPI was prepared as a stock solution of 10 mg/ml in sterile water and added to cells at a final concentration of 1 µg/ml, incubated at room

temperature for 5 – 10 min then washed in PBS and cells prepared for microscopy as above.

2.6 Biochemical methods

2.6.1 SDS-PAGE

Protein extracts from *Leishmania* were loaded into sodium dodecyl sulphate polyacrylamide gels for separation and visualisation of proteins. 12 % (w/v) polyacrylamide gels were cast in plastic casting cassettes (Invitrogen), and then a 5 % stacking gel was cast over this to allow focussing of the proteins before their separation on the 12 % resolving gel. In some cases acrylamide gels containing 6 M urea were prepared, by dissolving urea in the tris and acrylamide with gentle heating before addition of the other ingredients. Electrophoresis was performed in an XCell SureLock Mini Cell chamber (Invitrogen) with 1 x SDS-PAGE running buffer (10 x running buffer: 25 mM Tris, 192 mM glycine and 0.1 % (w/v) SDS) at 180 volts. A broad range protein marker (New England Biolabs) was loaded alongside protein samples at a concentration of 1 – 2 µg per lane to determine sizes of protein bands on the gel.

2.6.2 Western blotting

For Western blotting, proteins were transferred from a polyacrylamide gel, following electrophoresis, to a Hybond-C nitrocellulose membrane (Amersham, GE Healthcare). Transfer was carried out by semi-dry blotting using a BioRad Trans-Blot SD Semi-Dry Transfer Cell at 20 volts for 30 min, with the membrane and filter paper soaked in transfer buffer (20 mM Tris-HCl, 15 mM glycine, 20 % (v/v) methanol, in distilled water). The membrane was subsequently incubated in a blocking solution of 5 % (w/v) milk powder in TBST buffer (25 mM Tris-HCl pH 8, 125 mM NaCl, and 0.1 % Tween) for 1 hour at room temperature or overnight at 4^{o}C, with agitation. This blocking step prevents non-specific binding of antibodies to the membrane. After blocking, the membrane was incubated with primary antibody diluted to an appropriate concentration in fresh TBST with 5 % milk for 1 hour at room temperature. Relevant concentrations of antibody are described in figure legends. The membrane was washed three times in TBST, incubating for 10 min each time, before incubation with horse radish

peroxidase (HRP)-conjugated secondary antibodies at 1 in 5000 dilution for 1 hour at room temperature. After washing three times in TBST, the membrane was treated with an ECL (enhanced chemiluminescence) kit (SuperSignal West Pico Chemoluminescent Substrate, Pierce) according to manufacturer's instructions and then exposed on Kodak photographic film.

2.7 Immune cell flow cytometry

2.7.1 Extraction of immune cells from ear tissue and lymph nodes

Ears were deposited in PBS and cut repeatedly with surgical scissors, 4 mg ml^{-1} collagenase D (Roche) and 100 U ml^{-1} DNase I were added, then the tissue was incubated in a Thermomixer Comfort Eppendorf shaking incubator (Eppendorf) at 37 °C shaking at 1000 rpm for 45 min. Digested tissue was transferred to a gentleMACS C tube (Miltenyi Biotec) containing RPMI 1640 and processed in the gentleMACS dissociator (Miltenyi Biotec). Tissue homogenates were filtered through a 40 µm cell strainer (BD Biosciences).

Draining retromaxillary lymph nodes were collected and mechanically dissociated by tearing apart with 26G needles in RPMI 1640. Cells were then passed through a 40µm cell strainer (BD Biosciences).

Homogenised tissue samples were centrifuged at 380 x *g* for 10 min at 4 °C, washed in PBS, and resuspended in 1 ml PBS. Cell number was then determined by taking 10 µl of cells with a 1:1 ratio of cells to trypan blue (Sigma), and counting on a Neubauer chamber.

2.7.2 Staining cell surface antigens

All steps were performed at 4 °C and all centrifugation steps were performed at 380 x *g* for 5 min. Single-cell suspensions from mouse ear or lymph nodes were incubated with an anti-Fc-γ III/II (CD16/32) receptor antibody (2.4G2, BD Biosciences) for 30 min, washed twice with PBS, then stained in the dark with the fluorochrome-conjugated antibodies for 30 min. The appropriate rat IgG2a, rat IgG2b, rat IgG2c, or Armenian hamster IgG isotype controls were used. After staining of surface markers, the cells were washed twice with PBS, and stained with Fixable Viability Dye eFluor 506 or eFluor 660 (eBioscience) for 30 min,

following the manufacturer's protocol. The cells were washed twice in FACS buffer (1% dialysed FCS, 0.05% sodium azide, 2 mM EDTA, in PBS) then fixed with methanol-free formaldehyde (Thermo Scientific) for 5 min. Cells were washed twice in FACS buffer, then resuspended in FACS buffer and passed through a Nitex mesh with a pore size of 50 µm (Cadisch).

2.7.3 Data acquisition and analysis

The data were collected using either a MACSQuant Analyzer (Miltenyi Biotec), and analysed using FlowJo. Compensation settings were optimised using lymph node cells single-stained with anti-mouse CD4 antibodies conjugated to the corresponding fluorophores used in Table 2-5, (RM4-5, eBioscience and GK1.5, BD Biosciences). Live (based on Fixable Viability Dye staining) innate immune cells from the ear and draining lymph node were identified based on size (forward scatter) and granularity (side scatter), as well as by surface phenotype as indicated in the text and figure legends.

2.8 Bioluminescence imaging (BLI)

2.8.1 Preparation of luciferin

A stock solution of D-luciferin potassium salt (Promega) was prepared at 15 mg/ml in PBS. The solution was filtered through a 0.2 µm syringe filter. Luciferin was administered at 150 mg per kg body weight, 10 µl per g body weight, intraperitoneally.

2.8.2 Preparation of luminol sodium salt

A stock solution of luminol sodium salt (Sigma) was prepared at 50 mg/ml in PBS. The solution was filtered through a 0.2 µm syringe filter. Luminol was administered at 200 mg per kg body weight, 4 µl per g body weight, intraperitoneally or by cutaneous injection in the scruff.

2.8.3 BLI image analysis

Mice were anaesthetised at 4% isoflurane/1.5 L O_2 per min then bioluminescent light emission was imaged at 10 to 15 min after luciferin or luminol injection,

using the IVIS Spectrum bioluminescence imaging system (Caliper Life Sciences). Mice were maintained under anaesthesia at 1.5% isoflurane/1.5 L O_2 per min whilst in the IVIS. Imaging was performed with an open emission filter, for 1 min exposures, large binning, and 1 f/stop, and captured with a charge-coupled device (CCD) camera. Analysis was performed using Living Image software (Caliper Life Sciences). The absolute unit of photon emission was given as radiance (photons/second/cm^2/steradian). Regions of interest (ROIs) were manually selected over the entire ear to quantify the amount of photon emission as total photon flux in photons per second (photons/sec).

2.8.4 Statistical analysis

Statistical analysis was performed using GraphPad Prism 5. The analysis of significance of the data was performed by an unpaired t-test and 2-way ANOVA when comparing data from induced (+Rap) and uninduced (-Rap) Δ*crk3*::*DICRE*/Δ*crk3*::*CRK3*^{FLOX} [*SSU RE9H*] infections.

3 Developing Inducible Gene Deletion in *Leishmania mexicana*

3.1 Introduction

3.1.1 The importance of conditional deletion in Leishmania

Functional genomics of kinetoplastids are limited by the available techniques to manipulate target genes, test their function and thereby identify appropriate therapeutic targets. A number of genes encoding proteins essential for *Leishmania* survival have been discovered which represent potential targets for drug inhibition (M P Barrett et al. 1999), however the chief obstacle preventing further functional analysis of such genes is an absence of an effective conditional gene deletion system in *Leishmania*. There are a number of useful methodologies for functional analysis of *Leishmania* genes and proteins, such as destabilising domain tagging of protein (Madeira da Silva et al. 2009), the potential for small RNA inhibition of target gene transcript (Lye et al. 2010) and transposition systems (Damasceno et al. 2010), however no analysis of essential targets by these methods have been published to date. Homologous recombination of drug selectable markers is a long established methodology to replace endogenous *Leishmania* genes for phenotypic analysis of null mutants (Cruz et al. 1991). By this method, *null* mutant promastigotes can been generated to identify the function of the encoded protein as a virulence factor (Morrison et al. 2012) for example. However, this method cannot be used to generate *null* mutant lines deficient in an essential gene as a result of the intrinsic cytoxicity following gene removal (Ilgoutz et al. 1999; Hassan et al. 2001; Ambit et al. 2008). The strong selective pressure for retention of an essential gene results in the generation of drug resistant lines retaining an extra copy of the gene through altered ploidy, and necessitates the complementation of an exogenous gene copy for removal of the endogenous gene and an absence of altered ploidy. ‘Plasmid shuffle’ develops this further to more definitively demonstrate essentiality through retention of the ectopic gene copy following negative selection by ganciclovir treatment (Murta et al. 2009), and recent studies have further utilised the technique to generate partial essential gene *null* mutants for functional analysis of protein domains (M. a Morales et al. 2010; Dacher et al. 2014) and for *in vivo* confirmation of essentiality (Paape,

manuscript in production). Finally, overexpression of a gene can also be used as a tool for functional analysis, particularly when the encoded protein is essential for survival by mediating drug resistance (Drummelsmith et al. 2004).

Despite this range of methods for manipulation of gene and protein expression, an absence of a strategy to conditionally regulate essential gene expression and carry out direct phenotypic analysis remains a barrier to researching crucial aspects of *Leishmania* biology. The ability to modulate expression of proteins, such as by inducible RNAi in *T. brucei* has allowed many key enzymes involved in an array of parasitic processes such as immune evasion, DNA repair, differentiation and mechanisms of drug resistance to be identified by phenotypic analysis of 'knock-down' cells. When the targeted protein is essential for such biological functions, such studies would have been unfeasible in the absence of a conditionally regulatable system. As cited above, the current molecular techniques available for studying such a diversity of biologically essential cellular and pathological processes in *Leishmania* hinders our current understanding of the parasite, therefore the application of such a tool would facilitate a greater understanding of these processes and aid studies into novel drug targets.

3.1.2 Cre recombinase

The system of Cre-lox recombination was first identified from the linear genome of the *Escherichia coli* bacteriophage P1 by Sternberg and Hamilton (1981). This seminal work demonstrated that two elements are essential for the recombination of this product; a *cre* sequence encoding an enzyme necessary to catalyse recombination and the presence of two 'locus of crossover of P1' (loxP) sites where recombination occurs. Also established was the necessity of Cre-lox for integration of the linear bacteriophage genetic sequence into the genome of its *E. coli* host via Cre mediated loxP:loxB recombination (Sternberg et al. 1981). Further investigation revealed that these loxP sites were smaller than 60bp (Abremski et al. 1983) and electron microscopy of liner DNA containing two loxP sites in the presence of Cre allowed the formation of a Cre-lox synapse, recombination and subsequent circularisation of the DNA fragment to be imaged (Hamilton & Abremski 1984). Further dissection of the loxP site revealed a 34bp region containing two homologous, 13bp inverted repeats flanking an 8bp spacer

where cleavage and recombination occurs (Hoess et al. 1986), and mutagenesis of the *cre* coding sequence identified regions crucial to its function as a recombinase (Wierzbicki et al. 1987). Such fundamental analysis of the mechanisms of Cre-lox recombination formed the basis for the development of the recombinase system as a transgenic tool for genome engineering. Cre-lox recombination has been used as a method to conduct genome engineering for thirty years. Indeed, a PubMed search for 'cre loxP' resulting in over three thousand hits is indicative of the wealth of knowledge derived from the use of this technique. Following its discovery, the system was rapidly adapted for use in a variety of eukaryotic organisms such as yeast (Sauer 1987), mammalian cells (Sauer & Henderson 1988) and plants (Russell et al. 1992) to generate knock-out of reporter transgenes flanked by loxP sites ('floxed' genes). Since its implementation (Orban et al. 1992, Lakso et al. 1992), the use of Cre-lox in the generation of mouse deletion models by crossing mice expressing Cre controlled by a specific promoter and with a partner containing a floxed gene has become an established and significant method for generating mice deficient in a number of proteins regulating immunological, cancer or other disease pathways, reviewed by Nagy (2000).

3.1.3 Inducible Cre recombinase

A major benefit of Cre-mediated recombination is the very efficient levels of recombination and therefore gene loss, but constant expression of the enzyme means a lack of temporal control over knock-out and can result in toxic side-effects. To address this lack of conditional regulation, a number of systems for conditional regulation of Cre expression have been developed.

Fusion of Cre to the ligand-binding domain (LBD) of the human estrogen receptor (ER) allows induction of CreER activity by administration of the prodrug tamoxifen, metabolism to 4-hydroxytamoxifen (OHT) and subsequent binding to the CreER complex to stabilise the complex and induce activity (Metzger et al. 1995). An optimised $CreER^{T2}$ version of this has been generated through triple mutagenesis of the LBD and is currently the recommended tool for inducible mouse genome engineering (Parkitna et al. 2009), however such a system would not function in *Leishmania* due to necessary metabolism of tamoxifen to OHT and the toxicity of tamoxifen to *Leishmania* (Miguel et al. 2008). A more

recently developed methodology involving destabilisation domain tagged Cre (DD-Cre) represents an alternative to the CreER methodology, as inducible stabilisation of the protein by trimethroprim (TMP) treatment results in recombinase activity in the brain of DD-Cre transgenic mice (Sando et al. 2013). Such a method may represent a useful mechanism to regulate Cre activity in *Leishmania*, however the authors observe some background recombination in the absence of TMP, whilst TMP itself is an inhibitor of the essential fusion protein dihydrogofolate reductase-thymidilate synthase (DHFR-TS) activity in *L. braziliensis* (Osorio et al. 2013). As such, an alternative strategy for conditional regulation of Cre in *Leishmania* is required.

The integration of *cre* downstream of a tetracycline inducible promoter represents a potential method for inducible control of Cre expression. By conjugating an *E. coli* tetracycline repressor (TetR) with the activation domain VP16 from Herpes Simplex Virus, Bujard and Gossen (1992) were able to engineer the tetracycline transactivator protein (tTA) in HeLa cells. Expression of a luciferase gene downstream of a promoter region containing *tet* operator (*tet*O) sequences was ablated by tetracycline treatment, as tetracycline binding to the tTA complex prevents *tet*O binding and gene expression. The adaptation of this system in *Trypanosoma brucei* represents an important progression in kinetoplastid manipulation (Wirtz & Clayton 1995). Integration of the *tetR* sequence into the tubulin locus conferred expression of the repressor machinery, coupled with the insertion of *tet*O sequences into the characterised procyclic acidic repetitive protein (PARP) promoter region (later renamed *GPEET*/*PAG3*) with a downstream *luc* cassette generated clones whereby PolI mediated luciferase expression could be induced upon tetracycline treatment. When applied to regulating the expression of *cre* however, there was 'leaky' expression of the protein in the absence of tetracycline despite the presence of two upstream *tet*O elements (Barrett et al. 2004). In conjunction, induction of high-level Cre expression through tetracycline treatment induced death in the cells, and the authors speculate that this could be a result of recombination of cryptic lox sites in the genome. Despite the cytoxicity, the rates of cre-loxP recombination were highly efficient and could still be utilised with the appropriate modifications such as more stringent control of Cre expression. More recent work has attempted to address these issues by modifying the splice-

acceptor site and incorporating a temperature sensitive motif in the 3'untranslated region (UTR), with both steps reducing expression to significantly lower levels (Scahill et al. 2008). In conjunction, their use of bloodstream form *Trypanosoma brucei* results in overall reduced expression due to the GPEET promoter being required for procyclin expression during the procyclic life cycle stage. The authors also applied transient transfection of Cre to circumvent the toxic phenotype resulting from prolonged Cre expression, demonstrating this as a useful method. The aim of this work is to recycle floxed drug resistance cassettes following gene deletion, or to integrate floxed constructs containing an add-back gene in array with a drug resistance marker to facilitate excision of the gene and markers following induced Cre expression (Kim et al. 2013).

A limitation to this approach is the absence of a tet-inducible system in *Leishmania* to induce expression of the Cre enzyme. This was despite a previous effort to regulate expression in *L. donovani* (Yan et al. 2001), where background activity was high and expression varied depending on the RNA polymerase mediated gene expression. A recent study by Kraeva *et al* (2014) generated a *Leishmania mexicana* cell line expressing the TetR and T7 polymerases from the ribosomal locus which may resolve this issue, but was unavailable at the start of this project. The integration of a cassette into the tubulin locus containing a catalase enzyme, upstream T7 promoter region and *tet*O facilitates expression of the enzyme in the presence of tetracycline. The reverse orientation of this cassette aims to reduce background expression of a catalase enzyme marker from *L. pyrrhocoris* in the absence of tetracycline, and based on immunoblotting in the absence of tetracycline regulation of the protein is stringent. Such a system represents a useful method to induce Cre recombinase activity in *L. mexicana*, however as demonstrated previously in *T. brucei* there can be deleterious side-effects of uncontrolled and leaky Cre expression which may arise when utilising this system. In addition, this cell line has undergone three separate transfections to select for integration and expression of the inducible machinery and the regulatable enzyme, leaving only *blasticidin deaminase* (*BLA*), the *phleomycin resistance gene* (*PHLEO*) and *puromycin acetyltransferase* (*PAC*) selectable markers for integration of floxed gene and reporter gene cassettes. In the absence of any toxic side-effects of Cre expression, this methodology would represent a useful method for Cre mediated gene excision.

As an alternative to regulating *cre* expression in *Leishmania*, we looked to a conditional version of Cre developed in 2003 (Jullien et al). In this work the authors separated the Cre enzyme into two inactive fragments fused to FK506-binding protein (FKBP12) and the binding domain of the FKBP12-rapamycin associated protein (FRB). Treatment with rapamycin therefore dimerises the Cre (diCre) subunits to reconstitute recombinase activity, and the expression of Cre as non-functional subunits is an elegant method to alleviate any side-effects of overexpression of active, cytotoxic Cre. Induction of diCre activity through rapamycin treatment of rat fibroblasts was detectable *in vitro* through loss of a floxed spacer region, resulting in promoter transfer to the 5' end of a beta-galactosidase cassette and highly efficient induction of X-gal positive cells. In addition, the low background activity of this assay demonstrates that diCre induction is very tightly regulated. Importantly, the functionality of diCre *in vivo* (Jullien et al. 2007) has implications for the utilisation of this system for deletion of genes during infection, a desirable property for researchers interested in regulating genes involved in pathogenesis. To this end, the system represents a desirable tool for use in molecular parasitology, where there is a paucity of systems to conditionally regulate essential genes in a disease context. Our collaborator Markus Meissner recognised the potential for this system and utilised it to great effect in *Toxoplasma gondii* to investigate the role of proteins thought to be necessary for cellular invasion (Andenmatten, Egarter & Jackson 2012). Since this study, the rapid nature of diCre mediated gene excision has been utilised to efficiently excise drug selectable markers during the course of the forty eight hour *Plasmodium falciparum* erythrocytic life cycle (Collins et al. 2013). This study utilised a strategy to excise the floxed 3'UTR of a target gene in order to ablate the poly-adyenylation site (PAS), yet down regulate gene expression was unsuccessful due to an alternative PAS. A more successful strategy involved integrating a floxed version of the apical membrane antigen 1 (PfAMA1) coding sequence which was successfully excised following diCre activation, thereby preventing invasion of erythrocytes by the merozoites (Yap et al. 2014). This complementation method of a floxed target gene represents the most efficient method for gene deletion. The high levels of diCre mediated gene excision and protein loss presented in these studies demonstrates the efficiency of the system in the organisms, indicative of the potential for efficient activity in *Leishmania*. In terms of kinetoplastids, only one study to

date has been published using diCre for the removal of drug selectable markers in *T. cruzi* (Kangussu-Marcolino et al. 2014), however the absence of an appropriately designed and conducted method to ablate endogenous genes in this study does not address the lack of useful techniques to manipulate gene expression in this organism.

3.1.4 Research aims

We aimed to design a robust, targeted and flexible approach to induce deletion of endogenous genes in *Leishmania mexicana* using the diCre system. In particular, we were concerned with adapting this system to induce deletion of genes essential to parasite survival due to the lack of a conditional gene deletion system for such genes in *Leishmania*. Therefore we will:

1. Design a Gateway based cloning strategy to generate diCre and floxed gene vectors to replace endogenous target genes

2. Apply this strategy to generate an *L. mexicana* line expressing diCre from the CRK3 locus and a floxed green fluorescent protein transgene at the ribosomal locus

3. Test the efficiency of diCre mediated recombination by loss of a GFP expression in promastigotes and amastigotes

3.2 Results

3.2.1 Design of a DiCre expression construct for *L. mexicana*

Two elements are necessary in order to carry out diCre mediated site-specific recombination; sufficient expression of the *dicre* coding sequence and a loxP flanked copy of the target gene of interest. Previous studies have conferred diCre expression through transient plasmid transfection (Yap et al. 2014), by integrated expression to generate a diCre 'parental' line at multiple loci (Andenmatten, Egarter & Jackson 2012) or in array with the floxed gene (Collins et al. 2013). These studies were carried out in haploid, apicomplexan parasites, therefore in the context of the diploid *Leishmania* expression of the diCre by

integration and subsequent replacement of a target gene was deemed a practical strategy.

We obtained the in silico *dicre* sequence from our collaborators in order to design and synthesise an appropriate vector for diCre expression. The *dicre* construct encodes each subunit separately, with each open reading frame encoding a 5'MAPKKKRKVV eukaryotic nuclear localisation signal (NLS) peptide (Kalderon et al. 1984). The N-terminal domain residues 19-59 of Cre (Cre59) are coupled to the FK506-binding protein (FKBP12) by a 15 amino acid linker, and the C-terminal domain residues 60-343 of Cre (Cre60) are linked to the binding domain of the FKBP12-rapamycin-associated protein (FRB) via a 12 amino acid linker. Due to the poly-cistronic expression of genes by PolII in *Leishmania*, there is no requirement to include promoter regions into an expression vector. However, appropriate gene flanks must be introduced to a transgene cassette to confer the necessary splice acceptor site (SAS) and poly adenylation sites (PAS) for trans-splicing and polyadenylation to generate mature messenger RNA. To confer stable expression of diCre in both promastigotes and amastigote forms, each encoded polypeptide was flanked with 198bp 5' and 3'actin (LmjF.04.1230) or beta-tubulin (LmjF.08.1230). Annotated mapping of the splice and poly-adenylation sites are only available from TriTrypDB for *Leishmania major*, showing the appropriate splice sites 73bp and 75bp upstream of the coding sequence (CDS) respectively. The synteny between different species of *Leishmania* allows efficient expression of transgenes from vectors containing regulatory flanks derived from *L. major* (Misslitz et al. 2000). In order to select drug resistant clones following transfection and integration of the construct, a blasticidine resistance cassette was incorporated downstream of *dicre*. To confer stable expression of the marker, the cassette was flanked by 300bp regions derived from the 5' and 3' arms of the dihydrofolate reductase-thymidylate synthetase (*DHFR-TS)* gene from *L. major* (Goyard & Beverley 2000), and this region was flanked by multiple cloning sites to exchange the drug resistance cassette if required in the future. Following this *in silico* design process, the construct was synthesised (**Figure** 3:1a.).

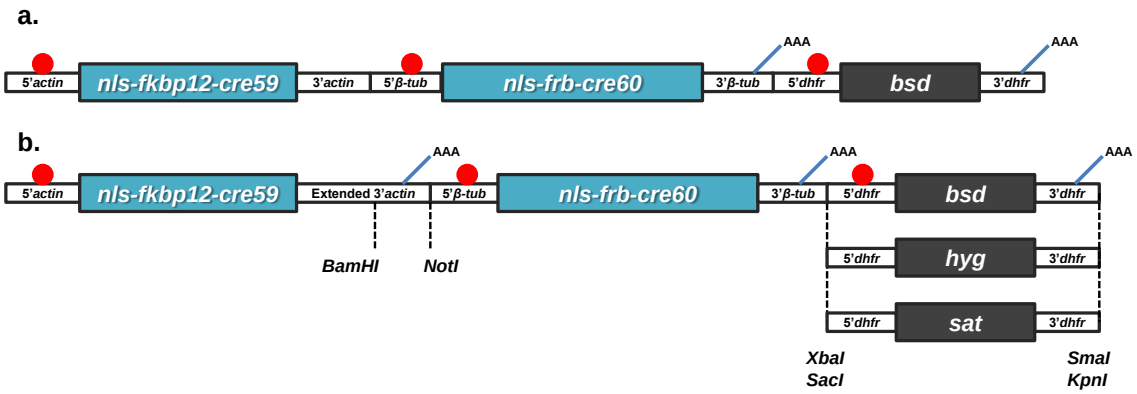


Figure 3:1- Schematic depicting the design of the L. mexicana diCre expression construct. Panel a. represents the original, synthesised construct encoding each dicre subunit and the blasticidine resistance marker (bsd) flanked with regulatory regions to confer expression by transplicing (red circle) and polyadenylation (AAA). Panel b. represents the optimised diCre vector with extended 3' actin flank to confer the appropriate polyadenylation signal and enhance expression of the FKBP12-Cre59 subunit. Also shown is the strategy for restriction enzyme mediated replacement of the resistance cassette.

The synthesised construct was flanked with 5' and 3' arms of homology from the flanking regions of the *crk3* gene locus (detailed in section 3.2.3), allowing transfection and homologous recombination to replace the first *crk3* allele. Integration and replacement was confirmed by PCR, however following poor detection of the FKBP12-Cre59 subunit by immunoblotting, analysis of the available gene expression data from TriTrypDB revealed the primary site of regulation to be 585bp downstream of the *actin* stop codon. This is not present in the synthesised vector, so in order to create an 'optimised' expression vector additional 650bp fragment was inserted through mutagenesis of the original vector to insert *BamHI* and *NotI* sites, PCR amplification of the extended fragment containing these sites and subsequent ligation (**Figure** 3:1b.). This optimised construct was used for all future experiments.

3.2.2 Design of a loxP construct for *L. mexicana*

In order to regulate the expression of a gene using diCre site-specific recombination, the target gene must be flanked by loxP sites. The design rationale is the integration of a gene of interest (GOI) into a floxed multiple cloning site (MCS) by restriction enzyme cloning, a practical strategy which has been utilised by others to effectively excise a number of genes through diCre mediated recombination (Andenmatten et al. 2012, Yap et al. 2014). In contrast to the diCre vector, *in silico* design and synthesis of a small (1.1kbp) loxP vector

backbone was constructed, allowing other elements to be incorporated through restriction digest cloning (Figure 3:2a). This backbone consisted of a SAS from the established pGL631 transgene expression construct (Misslitz et al. 2000) upstream of a MCS. The addition of 6x hemagglutinin (HA) downstream of the MCS to confer a C-terminal epitope tag to the expressed gene of interest (GOI), thereby facilitating subsequent expression monitoring through western blotting or immune fluorescence. The inclusion of unique restriction sites flanking this tag allows 6xHA replacement by other epitope tags, such as the *GFP* coding sequence at the N or C-terminal of the GOI. A 3' flank conferring a PAS from the vector pGL631 was included downstream of the GOI, and two locations flanked by restriction sites formed the template for which a puromycin resistance cassette (Figure 3:2b) and an mCherry cassette (Figure 3:2c) could be cloned in by restriction digest.

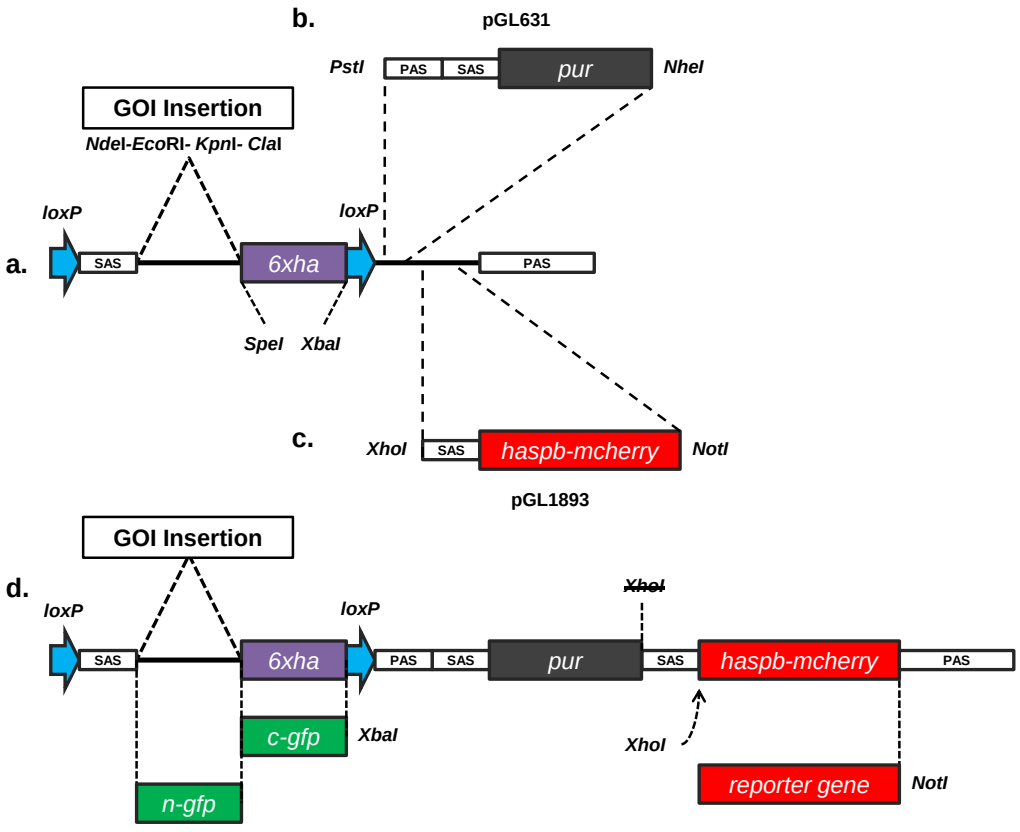


Figure 3:2- Schematic depicting the construction of a loxP vector. a. The in silico designed and synthesised vector backbone ready for restriction digest cloning of; b. the puromycin resistance cassette from pGL631, and c. the HASPB18-mCherry expression cassette from pGL1893. d. The final vector ready for gene of interest (GOI) insertion by restriction digest mediated cloning for loxP site flanking (blue arrows). The 6xHA can be readily exchanged with other epitope tags such as GFP. Mutagenesis of the XhoI site to the 5'CDS of mCherry facilitates the replacement of HASPB18-mCherry with other reporter cassettes.

Following synthesis of a loxP backbone, drug resistance and fluorescent gene cassettes were PCR amplified from pGL631 and pGL1893 respectively and inserted through restriction digest cloning to generate the full-length loxP vector (Figure 3:2d). The loxP vector contains a loxP flanked GOI site with a C-terminal 6xHA sequence, a puromycin resistance cassette and an mCherry reporter with an N-terminal hydrophilic acylated surface protein B (HASPB18) domain for membrane trafficking (Denny et al. 2000). Mutagenesis was also performed to exchange the *XhoI* restricition site from upstream to downstream of the SAS regulating mCherry expression in order to replace this cassette with the open reading frame of an alternative reporter. The result is a construct that once transfected into *Leishmania mexicana* permits puromycin selection of clones with expression of a conditionally regulatable target gene. Induction of diCre mediated site specific recombination will excise the gene, resulting in detectable protein loss through immunoblotting with an antibody raised against the protein tag. An mCherry cassette was included to assist phenotypic analysis by fluorescent microscopic imaging or flow cytometry analysis.

3.2.3 A flexible method for homologous flank addition

In contrast to previous studies utilising diCre in haploid organisms, the implementation of a flexible system for gene replacement of the diploid (and sometimes polyploid) *Leishmania* poses a challenge. An advantage of genetic manipulating of *Leishmania* is the well established protocol for transfection and replacement of a target gene through recombination of homologous flanks (Cruz et al. 1991), therefore the replacement of one allele with the diCre cassette and the second with a floxed gene of interest represents a feasible strategy. However, the addition of gene homologous flanks to these constructs by restriction enzyme cloning represents a laborious cloning method, one which would impede the utilisation of diCre for targeting multiple genes. An alternative cloning methodology is Gateway recombination, whereby the addition of *att* sites on different constructs enables enzyme mediated recombination and linkage in a manner more conducive to high-throughput cloning. As such, we utilised the Multisite Gateway three-fragment vector construction kit for the addition of gene homologous flanks the diCre and loxP vectors by sub-cloning each construct into a pDONR221 vector. PCR amplification of between 0.5-1kbp of 5’ and 3’ flanks surrounding a target gene from gDNA

with oligos containing the appropriate *att* sites generates amplicons which can be recombined into pDONR vectors in a reaction catalysed by BP clonase (**Figure 3:3**). The incorporation of unique restriction sites (such as *PacI* and *PmeI*) into these oligos enables the transfection fragment to be digested following flank addition. Finally, these individual elements can be cloned into a single vector by an LR clonase mediated reaction, thereby flanking the diCre and loxP vectors with the appropriate homologous DNA sequences to allow gene replacement by *PacI* and *PmeI* digest and subsequent transfection.

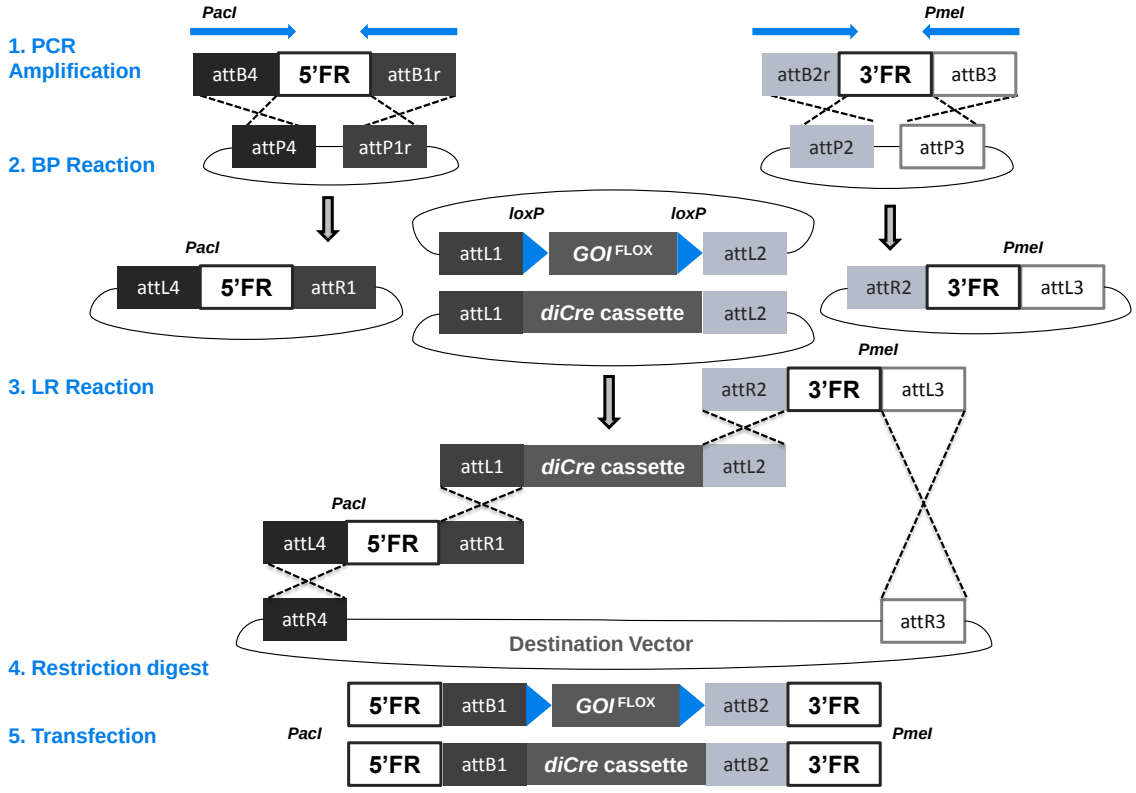


Figure 3:3- Pipeline of Gateway mediated addition of target gene homologous flanks to diCre and loxP vectors. 1. Primers (blue arrows) containing appropriate att sites and 5' *PacI* or 3'*PmeI* unique restriction sites amplify a 0.5-1kbp region up and downstream of the gene. 2. BP clonase catalysis the insertion of these flanks into their appropriate vectors. 3. The resulting 5',3' and diCre or loxP vectors are recombined into a pDEST vector by LR clonase 4. The final vector is linearised by *PacI* and *PmeI* digest for 5. transfection into *L. mexicana*. This method enables flanking of both the floxed gene of interest (GOI) expression cassette and diCre expression cassette.

3.2.4 Functional analysis of diCre activity

By utilising the Gateway system, the 'non-optimised' diCre construct was flanked by 500bp regions of homology from the gene encoding the L. mexicana cdc-2 like protein kinase CRK3 (A detailed description of the process and rationale of regulating CRK3 expression is the topic of Chapter 4). Integrative

replacement of *CRK3* by diCre at this locus was conducted to establish whether expression of diCre from an endogenous gene locus as opposed to the ribosomal locus would be sufficient to induce efficient gene loss. Blasticidin selection yielded a number of surviving clones, with protein extracts prepared from six of these to detect expression of the diCre subunits. Immunoblotting with polyclonal antibodies against residues in the FRB and FKBP12 domains resulted in good detection of expression of the 46kDa FRB-Cre60 subunit, but no detection of the smaller FKBP12-Cre59 (**Figure** 3:4b). This may be as a result of its size or more rapid degradation; however optimisation to confer stability to this subunit by rapamycin treatment to induce diCre complex formation did not improve detection. By in silico expression analysis of the actin gene, it was discovered that the lack of a poly-A site within the 3'flank of Cre59 may result in reduced expression of the protein. Therefore the optimisation shown in Figure 3:1 was carried out to extend the flank and confer this site. In order to circumvent the problematic Western blotting process, a cell line was generated by integration of a loxP flanked GFP open reading frame from the ribosomal locus: [*SSU GFP* ^{Flox}]. This line was strongly fluorescent as confirmed through fluorescence microscopy (Figure 3:4a) and was used as negative control to investigate growth and fluorescence following rapamycin treatment. Transfection of this line with the diCre-CRK3 construct resulted in the heterozygous experimental line (Δ*crk3*::*DICRE*/*CRK3* [*SSU GFP* ^{Flox}]) retaining high levels of GFP expression and replacement of *CRK3* as confirmed by PCR analysis.

3.2.5 diCre conditional deletion of *GFP* in promastigotes

Experimental (Δ*crk3*::*DICRE*/*CRK3* [*SSU GFP* ^{Flox}]) and control ([*SSU GFP* ^{Flox}]) cultures were seeded at 1x10^6 promastigotes/ml with increasing rapamycin doses to identify any cytotoxic effects of rapamycin treatment and establish the optimal dose for diCre activity. There was no effect on the growth of the control or experimental parasites over 5 days even at the highest dose of 250nM (Red plotted lines in **Figure** 3:4c), a dosage of around twenty times less than the *L. major* inhibitory IC_{50} of 4.9±0.5μM (Madeira da Silva et al. 2009). In conjunction, Wt *L. mexicana* seeded at 5x10^5 cells/ml were treated with zero, 100nM, 500nM and 1μM doses of rapamycin to investigate any effect on growth or the cell cycle

(**Figure** 3:4d.), resulting in a minor growth rate reduction at 1μM treatment but no detectable effect on the cell cycle.

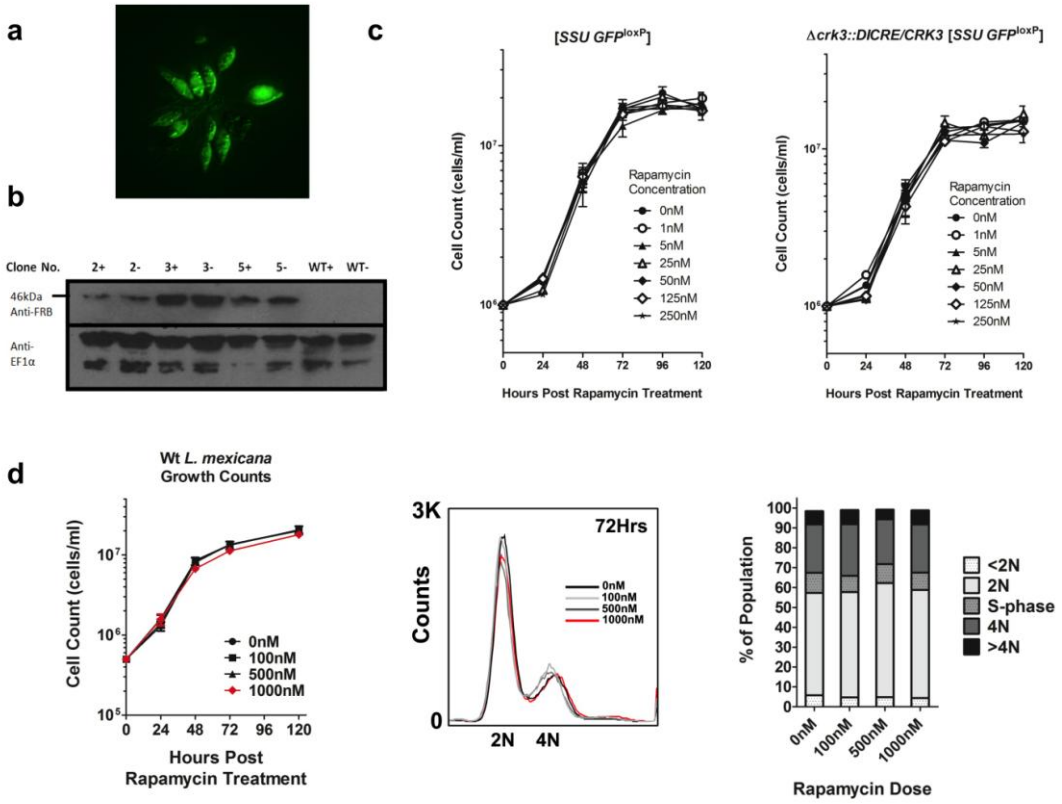


Figure 3:4- Growth rate of L. mexicana promastigotes following rapamycin treatment. Panel **a**. is a representive image of green fluorescent protein expression by the Δ*crk3::DICRE/CRK3* [*SSU GFP* ^{Flox}] experimental line. **b**. Analysis of diCre expression from the *CRK3* locus by western blotting of protein extracted from 3 clones grown in the presence (+) or absence (-) of 50nM rapamycin using an anti-FRB antibody and EF1a loading control. **c.** Experimental Δ*crk3::DICRE/CRK3* [*SSU GFP* ^{Flox}] or control [*SSU GFP* ^{Flox}] *L. mexicana* promastigotes were seeded at 1x10^{6} cells/ml and incubated in the presence or absence of between 1 to 250nM rapamycin. Cell density was determined at 24 hour intervals and the mean result of between 1-3 counts per sample shown (Error shown as SD). **d**. Wild-type *L. mexicana* were seeded at 5x10^{5} cells/ml and incubated in the presence or absence of between 0-1000nM rapamycin. Cell density was determined every 24 hours and the mean triplicate value is plotted (Error shown as SD). At 72 hours DNA content analysis was conducted by methanol fixation and PI staining. Flow cytometry analysis of 100,000 cells per group and graphical representation of the DNA content of each population is shown.

Gene excision efficiency was investigated by PCR amplification of the floxed *GFP* locus following 120 hours in culture. Analysis shows amplification of a smaller 0.6kbp amplicon following treatment and therefore *GFP* excision at all but the lowest dilutions, with no background diCre activity in the untreated experimental sample (**Figure** 3:5a.). Amplification of the excised fragment is not a quantitative measure of loss as small amplicons are preferentially amplified, consequently Southern blotting of these genomic DNA was conducted, demonstrating highly efficient loss of floxed *GFP* above 50nM treatment (**Figure**

3:5b.). Flow cytometry at day 5 demonstrates an equal loss of fluorescence in all samples treated with greater than 50nM of rapamycin, however this reduction in protein expression is not as pronounced as the relative DNA loss (**Figure** 3:5c). Western blotting confirms efficient protein loss by immunoblotting of protein extracts 5 days following treatment with 100nM rapamycin using a monoclonal anti-GFP antibody (**Figure** 3:5d.). This slower loss of protein is likely due to the stability of GFP, in conjunction with the high levels of expression from the ribosomal locus as a reservoir of protein to be degraded and mature mRNA transcript to be expressed in the absence of the gene coding sequence. These data demonstrate that expression of diCre from the CRK3 locus is sufficient to efficiently excise the *GFP* transgene at rapamycin concentrations above 25nM, and importantly that no background diCre activity can be detected in the absence of ligand. 100nM rapamycin was selected as sufficient dose to induce efficient diCre activity and gene loss in promastigotes whilst having no effect on *in vitro* growth or cell cycle progression.

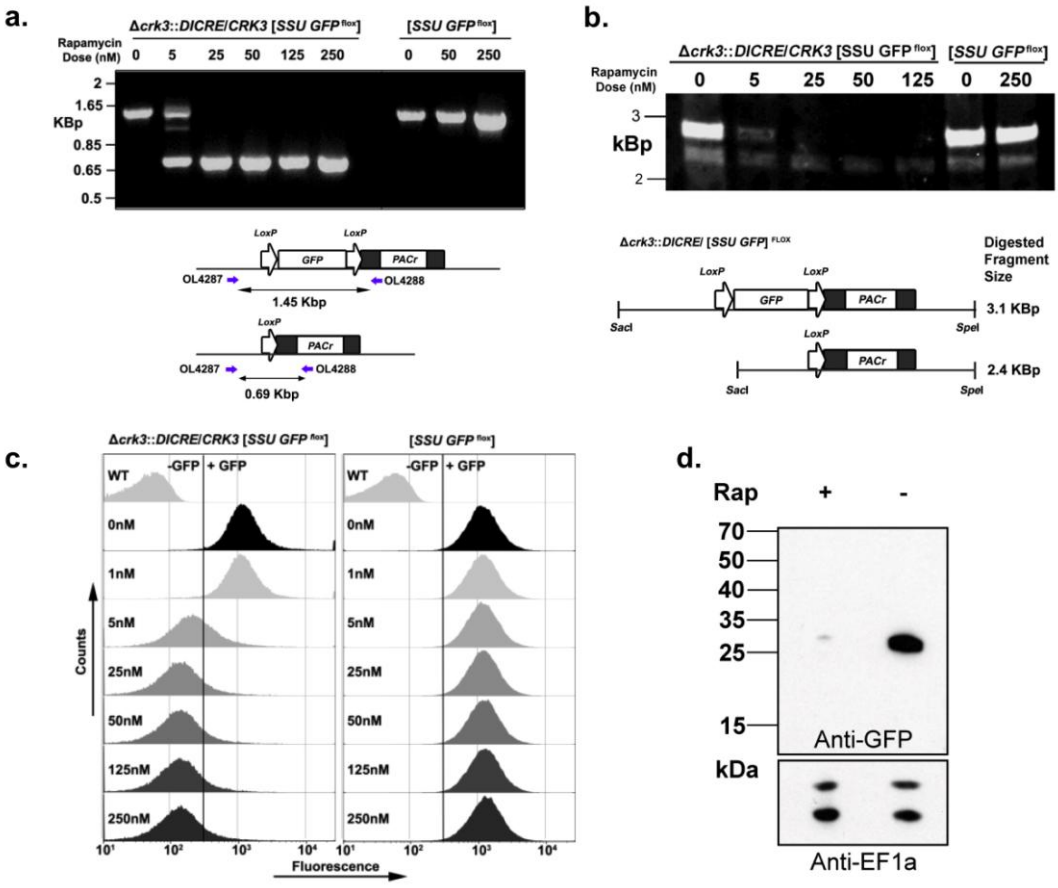


Figure 3:5- DiCre inducible loss of GFP expression in promastigotes. a. Experimental Δ*crk3::DICRE/CRK3* [*SSU GFP* ^{Flox}] or control [*SSU GFP* ^{Flox}] *L. mexicana* promastigotes were seeded at 1x10^{6} cells/ml and incubated in the presence or absence of between 1 to 250nM rapamycin. Genomic DNA was extracted at day 5 post rapamycin treatment from each experimental and control treatment group. Loss of floxed *GFP* was detected by PCR amplification

of the floxed *GFP* region which reduces in size from a 1.45KBp to a 0.69KBp amplicon using the primers shown in the lower schematic. **b**. 3µg gDNA was digested overnight with SacI and SpeI restriction enzymes and the digested DNA resolved on a 0.7% agarose gel followed by overnight transfer of resolved DNA to a Hybond-N membrane. Floxed *GFP* loss by Southern blotting was determined using a *GFP* CDS probe to detect a 3.1kBp digested genomic fragment as depected on the lower schematic. **c.** Flow cytometry analysis of promastigotes at 5 days post treatment. **d**. GFP loss by immunoblotting of protein extracted from cells 120hr post 100nM treatment with anti-GFP Ab with EF1-alpha loading control.

3.2.6 diCre conditional deletion of *GFP* in amastigotes

In order to test if diCre activity could be used in amastigotes, the experimental line (Δ*crk3*::*DICRE*/*CRK3* [*SSU GFP* ^{Flox}]) was inoculated into Balb/c footpads and amastigotes extracted following 2 months infection. Lesion derived amastigotes retained high levels of green fluorescence (**Figure** 3:6c) and were subjected to 0nM, 250nM, 500nM or 1µM rapamycin treatment in Schneider's medium. Cells were then washed and used for 1:2 *in vitro* infection of bone-marrow derived macrophage extracted from Balb/c mice. The rationale for this 'pre-treatment' method was to determine whether diCre activity in amastigotes is efficient after 24 hours to allow infection of macrophages in the absence of the immuno-modulatory rapamycin.

By gDNA extraction at day 5 post infection and PCR amplification using primers amplifying a 1.44kBp floxed *GFP* retained fragment, the amplification of 0.65Kbp fragments are indicative of efficient floxed *GFP* excision at all rapamycin treatment concentrations (**Figure** 3:6a). In contrast, the loss of GFP intensity is not as pronounced as in the promastigote study (**Figure** 3:5c) where rapamycin treatment results in ~90% loss of fluorescence intensity. In addition, it was more difficult to quantify by flow cytometry because of the presence of macrophage cellular debris in the amastigote pre-gate (**Figure** 3:6b). Microscopic analysis of live amastigotes during macrophage infection reveals a very variable rate of GFP loss between individual cells (**Figure** 3:6c), some of which have undergone complete loss (white arrows) and some retaining strong fluorescence. This is independent of the rapamycin treatment, leading again to the conclusion that the integration of floxed *GFP* and subsequent high level of GFP expression from the ribosomal locus requires a long incubation period for the cells to undergo GFP degradation and transcript loss, coupled with the transfer of cytosolic protein between cells during division. This may be particularly true of lesion-

derived amastigotes which undergo replication and protein turnover at a reduced rate compared with axenic or macrophage-derived amastigotes (Kloehn et al. 2015). Despite the reduced rate of protein loss, these data demonstrate the functionality of conditional deletion in amastigotes and their retention of diCre expression and activity following *in vivo* infection and recovery.

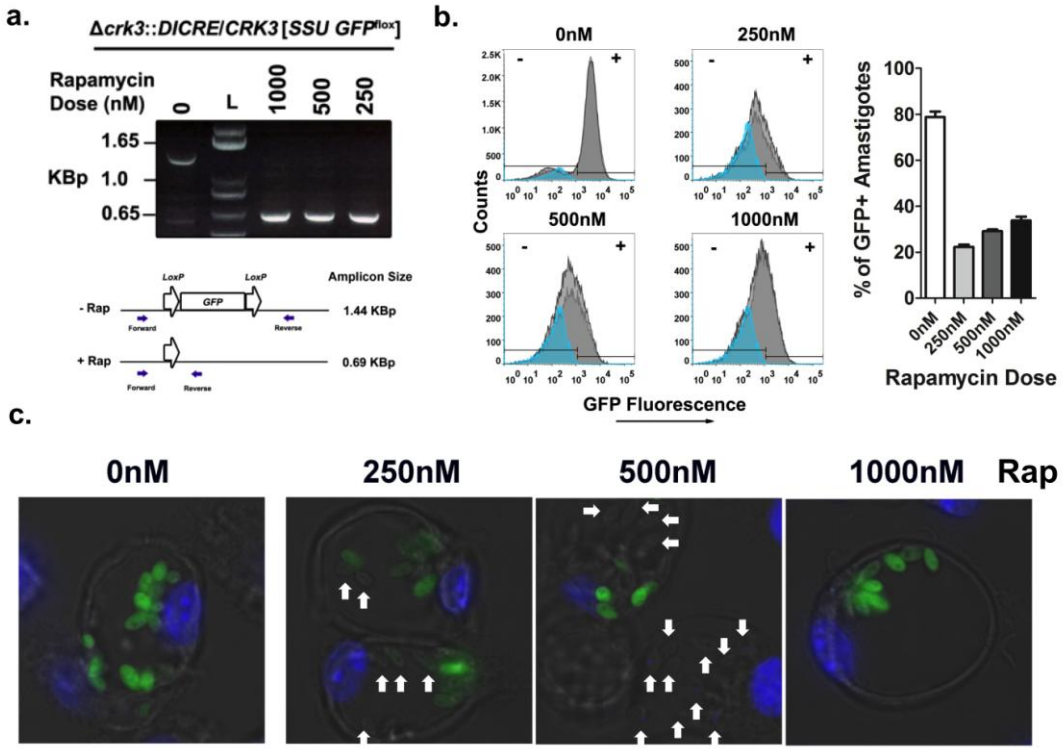


Figure 3:6- DiCre induced loss of GFP in amastigotes. a. PCR amplification of gDNA purified five days post macrophage infection to detect floxed *GFP* excision. Samples run alongside a 1Kb+ DNA ladder (L). **b**. Flow cytometry analysis of GFP fluorescence intensity loss; (left) events were pre-gated on an amastigote gate based on forward (size) and side (granularity) scatter. Blue plots represent the contaminant cellular 'debris' in this gate as a result of macrophage lysis following infection and amastigote preparation. A gate was drawn based on the untreated sample green fluorescent expression and used to quantify loss of GFP as a % (right) based on two biological replicates (Error indicates SD). **c.** Representative images of live cell imaging at days 5 post infection with GFP and DAPI nuclear DNA staining. Non-fluorescent amastigotes are indicated with white arrows.

3.2.7 Stationary phase induction of *GFP* ^{FLOX} loss

A potential utilisation of the diCre system is to induce deletion of a virulence factor or essential gene in promastigotes by rapamycin treatment for a short period of time, followed by infection into murine hosts to investigate the host-pathogen interactions or resulting parasite burden. To establish whether such an '*ex vivo*' strategy would be experimentally viable, a comparison between the

dynamics of *GFP* loss over time in replicating, log-phase and quiescent, stationary phase promastigotes was established (Figure 3:7a). Genomic DNA was extracted following between 1 and 20 hours 50nM rapamycin treatment and PCR analysis conducted to investigate the rate of floxed GFP excision. Amplification of a pronounced 1.44kBp fragment demonstrates that the loss of *GFP* is inefficient at this dose after 20 hours for stationary cells and requires a higher dose or incubation time. In contrast, in the dividing population floxed *GFP* efficiency of excision was higher, with amplification of a 0.69kBp fragment at 20 hours post treatment indicative of highly efficient rates of floxed *GFP* loss. The higher cell density of the stationary population is a likely contributing factor to this reduced rate of excision, with the high concentration of cells and therefore diCre complexes diluting the rapamycin ligand relative to the sparser logarithmic cultures. With this consideration, an '*ex vivo*' pilot trial was conducted to establish the stability of diCre inducible gene loss over the course of an infection. Inducible deletion of *GFP* was induced by a higher dose rapamycin treatment (150nM) of stationary phase promastigotes for 24 hours, followed by inoculation into the ear pinna of Balb/c mice with an uninduced control sample. Amastigotes were recovered from the draining, cervical lymph nodes 6 months post infection, grown as promastigotes in culture for genomic DNA extraction. Analysis shows highly efficient deletion, as confirmed by PCR (Figure 3:7b) and a 90% loss of GFP fluorescence compared with the uninduced control (Figure 3:7c). These preliminary data establish the potential of conditional deletion in promastigotes resulting in loss of the encoded protein during *in vivo* infection. This study establishes that 50nM is sufficient to induce efficient levels of gene deletion in cells seeded at between $5x10^5$ to $7.5x10^6$ cells/ml in 20 hours, with no deleterious effect on growth.

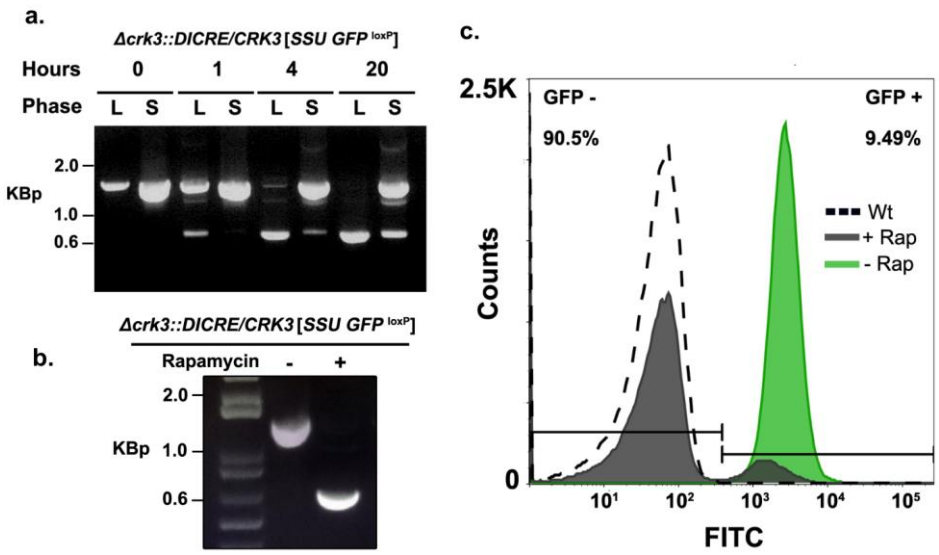


Figure 3:7- '*Ex vivo*' diCre conditional deletion of *GFP.* a. Floxed *GFP* gene loss in log-stage (L) or stationary (S) promastigotes following treatment with 50nM rapamycin over the course of hours. Panel b. *GFP* deletion retention after 6 months murine infection by PCR amplification of gDNA extracted from lesion derived parasites at the *GFP* locus. Panel c. shows % loss of green fluorescent protein following diCre induction and *in vivo* infection by FACS (between 50,000 and 100,000 events each population).

3.3 Discussion

Development of a robust, flexible and conditional method to conduct gene deletion in *Leishmania* has been established. Conditional deletion of the green fluorescent protein coding sequence demonstrates the efficacy of the diCre system in both the promastigote and amastigotes lifecycle stages, with induction stringently controlled in the absence of the rapamycin ligand. The efficiency of the system and our targeting strategy represents an advantageous method for essential gene targeting in both the *in vitro* and *in vivo* contexts.

3.3.1 diCre in *L. mexicana:* efficacy and advantages over current molecular tools

There have been no previous reports of Cre recombination in *Leishmania*. Previous attempts to utilise constitutive or conditionally expressed Cre has resulted in toxicity and 'leakiness' in *T. brucei* (Barrett et al. 2004), so a similar method of conditional Cre expression in *Leishmania* may also result in deleterious side effects resulting from leaky expression. DiCre expression in *Leishmania mexicana* has no effect on promastigote growth or amastigote infectivity before or after induction with rapamycin and is therefore a practical

method to regulate Cre-mediated recombination. In addition, active diCre mediates levels of excision >90% by analysis of the reduced GFP intensity over the course of *in vitro* (**Figure** 3:5) or '*ex vivo*' (Figure 3:7) induction. Efficient excision of the *GFP* transgene is a proof of principle for development of inducible deletion of endogenous genes encoding important proteins for survival and virulence. A key aspect of making the diCre cloning process conducive for targeting such genes is the use of Gateway recombination to add homologous flanks and facilitate target gene replacement with the *diCre* sequence. The expression of the diCre subunits from this endogenous gene locus must therefore be sufficient to induce loss of the target gene. Importantly, these experiments show that replacement of one CRK3 gene with the *diCre* sequence confers expression of the proteins at a level sufficient for inducible deletion in both promastigote and amastigote life cycle stages. The method to replace both copies of a gene with a loxP flanked gene for complementation and the second by *diCre* integration remains a viable and flexible strategy, one which was utilised in the following chapter. These findings are promising as they address a limitation in the current molecular toolkit; the ability to conditionally induce loss of an essential gene.

The induction of diCre mediated gene loss is rapid and efficient, however the subsequent time required for protein and transcript degradation may be a factor in slowing the rate at which a phenotype can be detected following essential gene deletion. A more rapid approach to regulate protein expression involves linkage of a target protein with a regulatory destabilisation domain (DD) based on a modified FKBP domain, whereby protein stability and therefore activity is conferred by Shld-1 addition (Banaszynski et al. 2006). An advantage of this method is the rapid nature of induction, with DD-YFP stability by Shld-1 ligand treatment occurring within 8 hours, and conversely rapid protein degradation within around 2 hours occurs once the ligand is removed (Madeira da Silva et al. 2009). Loss of ddGFP is therefore far more rapid than by diCre mediated gene deletion, as cells must be left for five days to ablate the protein despite undergoing rapid floxed *GFP* deletion. The authors used the DD method to regulate stability of UDP-galactopyranose mutase (UGM), a protein encoded by *GLF* involved in linkage of the membrane bound surface protein liphophosphoglycan (LPG). By complementation of a *GLF* deletion line with a

ddGLF vector, the authors were able to regulate LPG expression in a tightly controlled manner, resulting in sensitivity to complement mediated lysis in the absence of Shld-1 ligand. However, *GLF* and *LPG* null mutants are viable as promastigotes *in vitro* and as this study is the only publication where the dd domain is used in *Leishmania* it appears that the method is not conducive to essential gene regulation. Personal communication with colleagues who have used the system for regulation of essential *Leishmania* proteins reveals that there remains background retention of a non-regulated, wild-type protein. This is likely due to retention of an extra copy of the gene which does not contain the domain indicates that linkage of the FKBP regulatory domain to the encoded protein may ablate the activity of the product, even in the presence of the stabilising agent. As a result, there may be strong selective pressure on those parasites retaining an additional copy of the gene, leading to drug resistant *Leishmania* with only partial regulation of protein expression. An advantage of floxed gene complementation is that it may circumvent this selective pressure due to the lack of any FKBP-like exogenous protein domain. By this method there will be weaker selective pressure on parasites with altered ploidy, resulting in the majority of transfectants expressing the protein from the floxed gene resulting in more stringent regulation. The diCre strategy may therefore represent an improved method to regulate gene and down-stream protein expression, albeit with a delayed deletion phenotype.

High-throughput methodologies for regulating the expression of whole families of proteins is a desirable and powerful tool. We have endeavoured to make the diCre/loxP cloning process as amenable as possible, however the necessity to integrate these constructs is a limiting factor in terms of the time required to generate and select clonal lines. The lack of necessary Dicer-like and argonaute proteins prevents the small inhibitory RNA mediated degradation of transcript and the use of RNA interference in most species of *Leishmania*. Since the discovery of a functional RNA interference pathway in *T. brucei* (Ngô et al. 1998) and the development of an inducible method for generating stem-loop construct to generate double stranded RNA guides for siRNA targeting of specific genes (Shi et al. 2000), this method has been used extensively to study gene function. There are no limitations based on gene copy variation by this method, as the siRNA mediates targeted degradation of mRNA to knockdown expression,

therefore one round of transfection is sufficient to generate a conditional line. The development of Gateway cloning methods has accelerated the rate at which RNAi stem-loop constructs can be cloned to target gene families, as exploited by application to the 190 protein kinase 'kinome' (Jones et al. 2014). The pooling of such clones produces specific protein family libraries for high-throughput screening to identify 'hits' based on loss or prevalence of particular genes in a population following altered environmental conditions such as differentiation (Jones et al. 2014; Mony et al. 2014) or *in vivo* infection. DiCre mediated deletion represents a system which is far less conducive to such high-throughput application, however there is an absence of a conditional RNAi method in *Leishmania*. The absence of the RNAi pathway in the *L.* (*Leishmania*) subgenus is an impediment to the utilisation of RNAi to dissect gene function. Despite the retention of a functional RNAi pathway in the *L.* (*Viannia*) subgenus, there is no method for conditional regulation of RNAi mediated transcript degradation as current 'stem-loop' constructs lack any inducible promoters for regulation.

The average time frame to generate such RNAi lines of ~1 week is very rapid compared with the roughly two and a half months required for the generation of a diCre inducible line. However, the relatedness of kinetoplastids is such that 75% of coding gene sequences are shared between African trypanosomes and *Leishmania* (Subramaniam et al. 2006). Therefore any 'hits' derived from such tractable, high-throughput studies can be used to form concise hypotheses with which to utilise diCre in the study of the *Leishmania* homologues, and would be particularly attractive when knockdown results in a severe phenotype associated with essential protein loss. RNAi is not a perfect tool, indeed a limitation of the system is the variation in transcript down-regulation, and also knockdown in the absence of tetracycline due to the 'leakiness' of TetR mediated control (Tu & Wang 2004). DiCre has no such background activity as excision in the absence of rapamycin was not detected and consistently induces loss of *GFP* through efficiently induced site-mediated recombination and represents a useful strategy with which to dissect the function of genes and their encoded protein in a tightly regulated manner.

3.3.2 The use of rapamycin and *Leishmania* TOR

Rapamycin is the ligand necessary for diCre induction, but genomic analysis reveals that *Leishmania* have three homologues of the mammalian target of rapamycin (mTOR) gene (Madeira da Silva & Beverley 2010). mTOR is a serine/threonine kinase functioning as a master regulator of many cellular processes through separate signal transduction pathways. An important function of mTOR is in complex formation with Raptor and mLST8 (mTORC1) to mediate biological processes such as translation and autophagy (Ballou & Lin 2008). Studies of the processes controlled by the mTORC1 complex have been amendable through rapamycin treatment, whereby the compound binds the FKBP12 protein. This rapamycin-FKBP12 complex then binds the FRB region of the mTOR protein, obstructing active site mediated phosphorlyation of substrate and 'weakening' of the mTORC1 complex (Yip et al. 2010). This allosteric inhibition has enabled the study of pathways down regulated as a result of mTORC1 complex inhibition, however other mTOR activities are not inhibited by rapamycin such as mTORC2 complex formation (Ballou & Lin 2008). The presence of mTOR homologues in *Leishmania* with conserved motifs such as HEAT and FRB domains raises the issue of rapamycin as a ligand for diCre induction. mTOR binding by rapamycin is dependent on a Serine residue at position 2035, with mutagenesis of this site to any residue other than an Alanine preventing FKBP12-rapamycin binding (Chen et al. 1995). *L. major* and *L. mexicana* TOR1 (LmjF.36.6320 and LmxM.36.6320) contain tryptophan residues at this site, thereby conferring resistance to rapamycin inhibition. The two remaining TORs, TOR2 (LmjF.34.4530 and LmxM.34.4530) and TOR3 (LmjF.34.3940 and LmxM.34.3940) both contain functional alanine residues at this site, however little is known about the activity of these mTOR homologues. Gene deletion of *TOR3* results in normal *Leishmania major* promastigote growth and differentiation but attenuation *in vivo*, and a null mutant of *TOR2* could not be generated as indicative of essentiality (Madeira da Silva & Beverley 2010). In addition to mTOR homologues in *Leishmania*, a BLAST search identifies three FKBP12 homologues encoded by LmxM.22.1430, LmxM.19.1530 and LmxM.36.0230. A tryptophan residue at position 59 is associated with binding of rapamycin to FKBP12 (Choi et al. 1996), and interestingly this residue is conserved in all encoded *Leishmania* FKBP12. The effect of rapamycin treatment

on each of these proteins is a potential concern in the context of deletion activity, but in contrast rapamycin treatment has been shown to enhance amastigote proliferation during intracellular infection of macrophage (Pinheiro et al. 2009; Jaramillo et al. 2011). Considering the relatively low dose used for rapid diCre induction (100-200nM), and the absence of cell cycle defects and only a marginal growth reduction with a high (1μM) dosage (**Figure** 3:4), rapamycin treatment does not result in any detectable, deleterious side-effects in promastigotes.

3.3.3 Implications for *in vivo* conditional deletion

Previous studies on genes associated with virulence and *in vivo* maintenance of parasite survival rely on the survival of null mutant parasites as promastigotes *in vitro*, therefore if a protein is essential for survival in both promastigotes and amastigotes the study of *in vivo* infectivity is severely limited. Currently, the use of plasmid shuffle has allowed the N-Myristoyltrnsferase (NMT) protein to be identified as essential to *L. donovani* amastigotes survival *in vivo* by retention of the plasmid encoding NMT and a toxic TK in array throughout murine infection (Paape, personal communication). The lack of regulation of this system is a limitation which prevents NMT expression from being manipulated to investigate further host-parasite dynamics, such as the resulting immune response to infection with *null* mutant parasites. Proteins such as NMT are potential targets for drug inhibition, therefore diCre mediated '*ex vivo*' excision may represent a method to study the immune response and outcome of infection with conditional *null* mutants. The nature of diCre deletion is such that a gene is ablated but transcript and protein can remain as a pool in the population; this is exemplified by the retention of GFP in many of the lesion derived amastigotes despite efficient *GFP* loss. This *in vitro* caveat may benefit the '*ex vivo*' deletion strategy as deletion will not result in reduced protein expression immediately, thereby allowing infection to be established by metacyclic promastigotes with appropriate protein retention and translation from mRNA, but which are no longer able to transcribe the gene following differentiation and expansion as amastigotes. If inducible loss of the gene prevents establishment of infection, the protein expressed is therefore essential for amastigotes survival. A limit of this approach will be the efficiency of floxed gene deletion over the 24 hour time period; if the target gene is essential, there will be an outgrowth of cells

retaining the gene. As demonstrated in this study, when a gene is not essential for survival the rate of deletion remains constant over the course of infection (Figure 3:7b. & c.), therefore if the subsequent outgrowth population retain the full 'floxed' gene this is indicative of the selective pressure on this population *in vivo*. Gene deletion by active diCre occurs rapidly and consistently at treatments above 25nM, however the starting cell concentration must be taken into account when calculating excision as a similar dose in stationary cells results in poor excision. This study establishes that 50nM is a sufficient concentration for induction of gene deletion within 20 hours by seeding promastigotes to $5x10^5$ to $7.5x10^6$ cells/ml (Figure 3:7).

A desirable strategy for the validation of *Leishmania* proteins as therapeutic targets is the regulation of proteins essential to parasitic survival during infection. This is particularly important when a gene has been identified as essential *in vitro* thereby hindering research into its role in immune evasion or host manipulation in an *in vivo* context (Barrett et al. 1999). RNAi has been utilised to carry out such investigation in *T. brucei*, whereby knockdown is induced during murine infection by doxycyclin treated drinking water to induce degradation of the target transcript. By monitoring the resulting parasitaemia the outcome of knockdown and therefore essentiality of the target protein can be established (Abdulla et al. 2008). Direct *in vivo* regulation of *Leishmania* essential genes would be a significant tool for identifying proteins which would represent useful therapeutic targets; inducible deletion would result in loss of protein expression as a proxy of drug inhibition. Studies into host-*Leishmania* interactions would also benefit greatly by inducible deletion to bypass the early immune response to infection of *null* mutant metacyclics and examine the influence of the target virulence or regulatory factor in amastigotes directly. By deletion induction, analysis of the host immunobiology such as immune cell recruitment, activation and cytokine expression would lend itself to establishing the outcome of gene deletion in the context of the immune response. The dissection of host pathogen interactions by this method would expand our working knowledge and aid vaccine design (Kumar & Engwerda 2014).

4 Inducible deletion of the gene encoding the essential cdc2-like kinase, CRK3

4.1 Introduction

4.1.1 Essential genes as promising drug targets

Since the establishment of transfection and homologous recombination to conduct gene deletion in *Leishmania* (Cruz & Beverley 1990), proteins have been identified which are essential to the survival of the parasites. Essentiality can manifest itself in a variety of ways; a lethal phenotype in all life-cycle stages, a conditional lethal phenotype whereby cell death is induced upon an alteration in growth conditions such as nutrient availability, or essentiality in one life cycle stage but not the other (Barrett et al. 1999). The identification of the latter is currently limited to the generation of viable, *null* mutant procyclic promastigotes which are subsequently unable to establish infection as amastigotes in a mouse or *in vitro* macrophage. By adhering to one of these conditions, a gene can be established as essential to virulence and therefore provisionally identified as a drug target. Barrett et al (1999) expand these criteria further by highlighting the fact that although removal of a gene results in a lethal phenotype, drug inhibition of the target protein may have no curative effect in a disease context. This is an important consideration if the encoded protein functions as a structural rather than catalytic component of an active complex, for example.

By these criteria, the inherent essentiality of the protein is not sufficient to establish it as an appropriate drug target until active and catalytic sites confirm the protein as suitable for inhibition by chemicals or small molecules. In the absence of a conditional method to regulate gene expression, the analysis of genes as potential drug targets has relied on progressively advanced techniques such as recombinant protein activity assays (Gomes et al. 2010), mutant yeast complementation (Wang et al. 1998) and plasmid shuffle (Morales et al. 2010; Dacher et al. 2014). Recombinant protein assays are particularly useful for inhibitor screens because of their application in high-throughput inhibitor screens to identify loss of enzymatic activity (Walker et al. 2011) to enable testing of more specific inhibitors in intracellular parasite assays (Rachidi et al.

2014). Through mutagenesis of the encoding DNA sequence, particular residues can be identified as active sites required for the correct function of the protein, thereby validating such regions for targeted inhibition of catalytic function by pharmacological inhibition. In an established recombinant system yielding high concentrations of active protein, the mutation of multiple residues is an amenable method to establish residues which compose active sites that represent targets for drug inhibition. Yet in such large scale screens, there can be issues with relating loss of enzyme activity with parasite killing once these compounds are applied to *in vitro* cultures due to off-target or non-specific effects of the inhibitors (Paape et al. 2014). There is no guarantee that enzymatic inhibition of recombinant complexes directly translates to *in vitro* efficacy of the same compound once applied to an intracellular screen. This dichotomy between assays represents a limitation with such studies, and ideally a more targeted means to uncover the phenotype by loss of the protein *in vitro* would better establish that protein as a therapeutic candidate.

We are compromised in our ability to study and validate drug targets encoded by essential genes by the lack of a method to conditionally regulate their expression in *Leishmania*. Functional analysis of proteins encoded by essential genes by inducible gene deletion is a desirable tool in *Leishmania*, as it would allow the phenotypic validation of proteins as drug targets, particularly if utilised in a disease context. In conjunction with existing screening methods, knowledge of the phenotype resulting from inducible deletion would facilitate the testing of lead compounds and their validation as specific inhibitors; in this context, drug inhibition of a target protein in viable *Leishmania* should mimic the phenotype manifest by inducible gene deletion, such as arrest at a defined cell cycle checkpoint. By this method, the specificity of the inhibitor and the necessary dose required to elicit inhibition at a level equivalent to gene loss could be identified.

4.1.2 CRK3 as a drug target in *Leishmania*

Protein kinases mediate regulation of cellular function through phosphorylation of a diverse range of proteins to mediate cellular processes necessary for survival. As a result of this powerful regulatory activity, protein kinases represent useful therapeutic targets in a disease context; in mammalian cells,

kinase inhibition is particularly associated with anti-cancer treatment, whilst in parasitic protozoans the ability to repress kinase activity represents a promising avenue in preventing cell division, differentiation and survival (Naula et al. 2005) during infection. A subset of protein kinases with particular importance in mediating the cell cycle are the cyclin dependent (CDK) protein kinases. Studies into the role of CDKs, such as cdc2 in *S. pombe* and mammalian cells demonstrate the essentiality of active CDK-cyclin complexes in regulating regimented cell cycle progression through mitosis (Gutiérrez-Escribano & Nurse 2015). There is an established repertoire of approved, therapeutic kinase inhibitors for human use (Jänne et al. 2009), with CDK inhibition representing a promising therapeutic route to prevent tumour growth in human cancer. Compounds such as flavopiridol, olomoucine, roscovitine and kenpaullone exhibit a range of CDK inhibitory activities, and subsequent testing as anti-cancer agents for human use has been conducted (Cicenas & Valius 2011).

Compounds which inhibit CDK activity such as indirubins function by competitive binding in the ATP binding pocket (Leclerc et al. 2001), with application of the use of such compounds in humans laying the foundation for similar CDK inhibition in trypanosomatids; the basis of such work was the discovery of homologues of mammalian CDKs, termed CDK related protein kinases (CRKs). These were first identified in *L. mexicana* by immunoblotting of cell extracts with a monoclonal antibody raised against the cdc2 conserved, 16 amino acid PSTAIR box (Mottram et al. 1993) implicated in cyclin binding. Western blotting detected the presence of multiple proteins in a range of molecular weights, suggestive of the expression of multiple CRKs. By cloning and sequencing, the LmmCRK1 protein kinase was identified by conserved structural components such as the PSTAIR domain and conserved phosphorylation sites found in CDC2. Protein kinase activity was confirmed in promastigote forms by immunoprecipitation and subsequent kinase activity assay. Later investigation revealed the essential role of CRK1 in mediating the cell cycle of *L. mexicana* by the failure to generate a *null* mutant cell line (Mottram et al. 1996), however the inability to complement a yeast cdc2 mutant by expression of *lmmcrk1* was indicative of diversity from the yeast homologue (Mottram et al. 1993). In contrast, yeast complementation by a second CRK discovered in both *L. mexicana* (Grant et al. 1998) and *L. major* termed CRK3 was able to recover a yeast cdc2 mutant (Wang et al. 1998),

indicative of a closer homologous function to cdc2. However further analysis of LmxCRK3 demonstrates the diverged function of this CRK relative to its yeast homologue. Sequence analysis showing a 54% identity with mammalian cdc2 implicates a sufficient divergence for specific targeting of the *Leishmania* CRK (Grant et al. 1998). LmxCRK3 is essential for growth of promastigotes by the selection of drug resistant cells with altered ploidy following replacement of the coding sequence with drug resistance markers.

To be active, CRK3 must form a complex with a regulatory subunit cyclins (CYCs) and be de-phosphrylated at the ATP binding pocket and phosphorylated at the T-loop residue (Grant et al. 2004). In the absence of a reverse genetic technique to conditionally delete *CRK3* in *L. mexicana*, the use of CDK inhibitors, in this case flavopiridol, is an appropriate method to inhibit CRK3 activity. Flavopiridol treatment of promastigotes results in cell cycle arrest in G2/M (Hassan, 2001). RNAi of TbCRK3 which has ~78% sequence identity to LmxCRK3 in the closely related *T. brucei* results in arrest at the G2/M stage, however the resulting phenotypes differ depending on whether induction is carried out in bloodstream or procyclic forms (Tu & Wang 2004). A cell cycle arrest in G2/M resulting from flavopiridol treatment of *L. mexicana* procyclic promastigotes may be indicative of a shared function of active CRK3 in mediating the transition over mitosis between different species of kinetoplastids. Yet the inhibition of the mitotic activity of LmxCRK3 by competitive binding of flavopiridol to the ATP binding site may not be specific, as the presence of 12 predicted CRKs this compound may not be inhibitory to CRK3 alone.

To be a useful drug target, the activity of a *Leishmania* protein must be inhibitory in the mammalian infective amastigote stage. Promisingly, treatment of *L. donovani* and *L. mexicana* infected murine macrophage with CDK inhibitors from an indirubin-based chemical library resulted in impaired parasite viability (Grant et al. 2004). Five indurubin kinase inhibitors which also showed inhibitory activity towards $CRK3^{His}$ were validated by this screen and subsequently tested against *L. mexicana* axenic amastigotes and promastigotes by *in vitro* growth curve analysis. However, only two of these compounds resulted in impaired growth as a result of impaired cell cycle regulation. CRK3 is active in both life cycle stages (Grant et al. 1998), but the absence of a mechanism to regulate

essential gene expression renders phenotypic assessment of gene deletion in both life cycle stages unfeasible. The cell cycle arrest phenotype by indurubin treatment is indicative of CRK inhibition in both life cycle stages, however the study was unable to identify the selectivity of such compounds towards CRK3. To facilitate a larger screen of possible anti-leishmanial compounds specifically targeting CRK3, recombinant CRK3:CYC complexes were expressed and used for functional analysis of protein kinase activity (Gomes et al. 2010) and high-throughput inhibitor screening (Walker et al. 2011).

A variety of cognate cyclins of have been identified in eukaryotic organisms, with 11 identified in *L. major* (Naula et al. 2005). Of these, CYC6 (Walker et al. 2011) and the *Leishmania* specific CYCA pair with LmxCRK3 to form active protein kinase complexes which phosphorylate downstream mitotic regulators (Gomes et al. 2010). Active, recombinant *L. major* CRK3:CYCA complexes are phosphorylated at the T-loop residue T178 by a CDK activating kinase (CAK). Use of a yeast derived CAK, Civ-1 was utilised in activity assays, however Civ-1 mediated phosphorylation at this site enhances but is not essential for kinase activity as measured by phosphorylation of histone-H1 substrate (Gomes et al. 2010). Mutation of this T-loop residue to a glutamic acid in the *P. falciparum* homologue of cdc2 (PfPK5) mimics phosphorylation and increases histone-H1 and casein phosphorylation 5-10 fold (Graeser et al. 1996), but interestingly in *Leishmania,* T178E mutagenesis of LmxCRK3 inhibits functional rescue in *S. pombe* (Wang et al. 1998) and ablates CRK3:CYCA recombinant protein kinase activity (Gomes et al. 2010). These findings are in partial agreement with cdc2 mutant yeast recovery experiments with *S. pombe* (Gould et al. 1991), whereby mutagenesis of the corresponding T-loop residue at position 167 to glutamic acid results in an intermediate recovery of colony growth. These studies highlight the importance of this T-loop residue in the mediating protein kinase activity and implicate it as potentially viable site for targeted chemical inhibition in *Leishmania*. Importantly, treatment of recombinant CRK3:CYCA with CDK inhibitors flavopiridol and indirubin-3'-monoxime were shown to inhibit histone-H1 kinase activity at similar IC_{50} concentrations as previously determined by the same assays using purified $CRK3^{His}$ (Hassan et al. 2001, Grant et al. 2004). These concurrent data provide further evidence that the cell cycle arrest and growth

attenuation resulting from CDK inhibitor treatment of *L. mexicana* is a result of impaired CRK3 activity.

The role of CRK3:CYC6 in mediating the *Leishmania* cell cycle transition through mitosis is implicated by RNAi of CYC6 in *T. brucei* (Hammarton et al. 2003). As established by TbCRK3 RNAi (Tu & Wang 2004), there are differences in the phenotypic outcome by RNAi of CYC6 between bloodstream and procyclic forms, indicative of stage specific protein kinase activity. Again, the absence of a method to regulate CRK3 expression in *L. mexicana* prevents such stage-specific dissection, however the block in the cell cycle at G2/M suggest a similar role in cell cycle regulation in the related *Leishmania* as shown by CDK inhibitor treatment.

The preparation of active, recombinant CRK3:CYC6 kinase complexes enabled a high-throughput screen to identify selective inhibitors of kinase activity with no such inhibition of a range of recombinant, human CDK:CYC complexes (Walker et al. 2011). High throughput chemical screens were conducted using two separate compound libraries; the first screen utilised a diverse, 25,000 compound Lexicon library, with initial screening confirming CRK3 specificity by counter screening against mammalian CDK2:Cyca. This yielded 12 potent azupurines (IC_{50} values <11μM) against CRK3:CYC6, but further counter screening against a range of mammalian CDK:CYC complexes revealed inhibitory activity against the mammalian protein kinase CDK4:CYCD1 complex at IC_{50} values <30μM. The lack of *Leishmania* selectivity is an impediment in terms of therapeutic use, with the additional caveat of low potency against *L. major* amastigotes (IC_{50} values >50μM). Despite this, these results allowed structural modelling of the binding site of these azupurines to elucidate this lack of selectivity. Interestingly, the authors describe an acceptor-donor-acceptor (A-D-A) binding motif, where hydrogen bonds are formed between the azupurines and backbone residues present in both CRK3 and CDK4. The potency of these compounds to either the human or *Leishmania* CDKs was hypothesised to be a result of structural differences within the azupurine scaffold.

To explore this further, an additional 23 azupurine derivatives were synthesised with altered chemical structure of the azupurine backbone, and screened against recombinant CRK3:CYC6. Such changes varied the efficacy of CRK3:CYC6

inhibition, with the likelihood that the presence of a benzene ring results in complimentary binding to the hydrophobic pocket present in CRK3. Screening of these compounds against promastigotes and amastigotes showed little growth inhibition, and despite the employment of a second BioFocus SFK48 kinase-focused inhibitor screen there remained a disagreement between recombinant protein inhibition and anti-parasite activity as commonly seen in such screening experiments (Paape et al. 2014). Despite an absence of potent anti-parasite compounds, such work demonstrates how a high-throughput screening process is important for elucidating specific structure-activity relationships (Bell et al. 2012). An additional screen using multiple chemical series aimed at finding specific CRK3 inhibitors was conducted in parallel with this one (Cleghorn et al. 2011), with the data gathered from such study lending itself to practical design of *Leishmania* specific inhibitors of CRK3 and other potential therapeutic targets (Hutton et al. 2014).

A common disparity arising from such screens is specific inhibition of recombinant complexes but inefficient anti-parasitic activity when utilising the same compound. This can also be the case in reverse, where parasite killing is efficient at low doses of compound but recombinant activity is unaffected. The difficulty in establishing and validating an essential protein by chemical inhibition is exemplified by this duality. In order to establish a protein as a 'useful' drug target, we must utilise reverse genetic techniques to conditionally regulate expression of the parasitic gene. An important consideration is that the treatment of leishmaniasis is initiated only once the disease manifests itself in the form of a cutaneous lesion, or following diagnosis of the visceral form following symptoms such as fever or cachexia (Herwaldt 1999), at which stage the parasites have differentiated into replicative amastigotes. Demonstration of the essentiality of a protein during this life cycle stage is crucial. However, current molecular techniques to regulate the expression of essential genes such as *CRK3* in any life cycle stage do not currently exist. In addition, downstream analysis of the resulting immune response yields useful information into the outcome of disease clearance following inoculation of *null* mutant *Leishmania* lines into appropriate murine models (Alexander et al. 1998, Buxbaum et al. 2003). The resolution of infection by development of an appropriate Th1 immune response to *Leishmania null* mutants has implications on the development of

protective immunity from subsequent infection. These experiments are dependent on the deletion of genes which are not essential in promastigotes; therefore an absence of a conditional system to regulate genes which are essential at all life cycle stages prevents such analysis. An *in vivo* infection model which encompasses the inducible deletion of an essential gene to monitor the resultant parasite burden by bioluminescence expression, in addition to phenotyping of the resulting immune response to inducible *null* mutant parasite infection represents a crucial model for drug target validation.

4.1.3 Research Aims

The body of work discussed in this chapter provides strong evidence that CRK3 represents a useful target for pharmacological inhibition. To further establish if this is the case, we aim to utilise the diCre inducible deletion system to conditionally regulate *CRK3* expression in order to:

1. Generate a conditional deletion *L. mexicana* line to enable phenotypic analysis by of *CRK3* deficient promastigotes.

2. Test functional recovery and specificity of floxed CRK3 deletion by complementation with a *CRK3* transgene, and conduct complementation to define the activity of a *CRK3*^{T178E} mutant in promastigotes.

3. To investigate the essentiality of protein kinase activity by conditional deletion of floxed *CRK3* in the mammalian infective, amastigote stage.

4. To investigate the essentiality of protein kinase activity during murine infection by conditional deletion of floxed *CRK3* in stationary phase, bioluminescent reporter promastigotes and subsequent infection of BALB/c mice.

5. Develop a model to study the recruitment of innate immune cell populations to both infection site and draining lymph node by infection with conditional *null* mutant, bioluminescent reporter *L. mexicana*.

4.2 Results

4.2.1 Generation of a *CRK3* conditional deletion cell line

Application of the diCre system for targeted gene replacement of *L. mexicana* was designed to be as flexible as possible in order to target a variety of genes, without laborious and time-consuming preparatory cloning. The diCre construct and loxP vectors were therefore sub-cloned into the pDONR221 Gateway vector containing *att* recombination sites to facilitate the flanking of appropriate 5' and 3' homologous flanking regions of a target gene of interest through LR clonase mediated recombination (see section 3.2.3 for more information). By Gateway recombination, the constructs were flanked by 500bp *CRK3* flank amplicons containing the appropriate *att* sites and *Pac*I and *Pme*I restriction sites for restriction digest to linearise the integration cassettes for homologous recombination.

Following generation of vectors flanked by these homologous sequences, the *CRK3* coding sequence was inserted into the loxP vector by PCR amplification from wild-type genomic DNA using oligonucleotides containing 18bp of gene homology and conferring a *NdeI* restriction enzyme site at the 5' end, a *SpeI* site at the 3' end in the absence of a 'Stop' codon. Integration of this amplicon upstream of a GFP coding sequence by restriction digest mediated ligation into pGL2315 resulted in a floxed, *CRK3-GFP* expression cassette (**Figure** 4:1a). CRK3 expressed with a C-terminal protein fusion tag is enzymatically functional (Hassan et al. 2001, Grant et al. 2004, Gomes et al. 2010), therefore the expression of the floxed CRK3 linked to a C-terminal GFP was utilised to examine protein loss by immunoblotting in the absence of an available CRK3 antibody. Each *CRK3* copy was replaced by consecutive transfection and selection using the diCre construct and the floxed, *CRK3* expression cassette respectively (**Figure** 4:1a). Each vector contains an encoded drug resistance marker conferring either blasticidin or puromycin resistance to enable drug selection of integrated clones following transfection. Generation of a *CRK3* heterozygote, termed Δ*crk3*::*DICRE,* was identified by PCR amplification of genomic DNA extracted from six blasticidin resistant clones (**Figure** 4:1b). 5' and 3' 940b and 950bp amplicons were obtained by amplification with diCre specific primers and primers binding outwith the integration site, demonstrating replacement of the

first *CRK3* allele. Clone F was used for replacement of the second *CRK3* allele by transfection with the floxed *CRK3* construct generated multiple double drug resistant clones. Genomic DNA was extracted from eight clones, and PCR amplification of the 5' and 3' *CRK3* flanking regions of expected size was conducted using pairs of oligonucleotides which bind outside the locus integration site, and within the *diCre* sequence, floxed *CRK3* vector or the *CRK3* gene in the opposite orientations (**Figure** 4:1b). By this method, clones 2 and 8 were demonstrated as lacking both *CRK3* copies by replacement. By conventional nomenclature for *Leishmania* (Clayton et al. 1998), this cell line was termed Δ*crk3*::*DICRE*/Δ*crk3*::*CRK3*^{FLOX} .

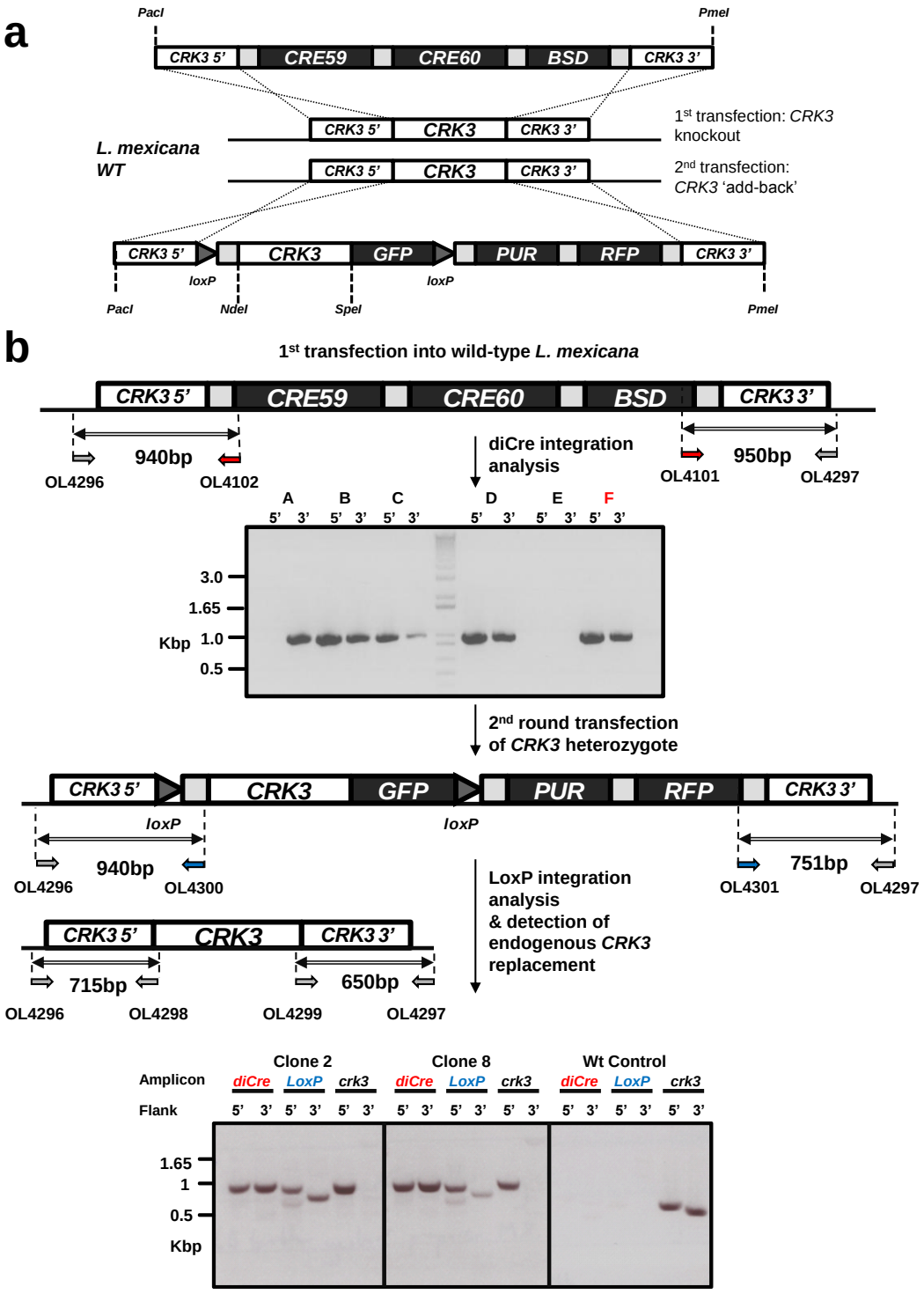


Figure 4:1- Establishment of a CRK3 inducible deletion *L. mexicana* cell line. a. Schematic representing the diCre and floxed CRK3 replacement strategy. Homologous recombination was facilitated by Gateway flanking of both diCre and loxP vectors with *crk3* 5' and 3' 500bp homologous regions to mediate transfection and replacement of both alleles **b**. Transfection of wild-type L. *mexicana* with the diCre construct; integration was confirmed by PCR amplification of genomic DNA extracted from six clones with primers (OL) binding outwith the integration site (grey arrows) and within the diCre coding sequence (blue arrows) to amplify 950bp amplicons. A single blasticidin (BSD) resistant clone F with *diCre* integrated at the *crk3* locus was subsequently transfected with the loxP construct to replace the remaining endogenous *crk3* allele with a floxed *CRK3* fused to a 3' *GFP* tag, thereby generating a diCre mediated conditional deletion line: Δ*crk3*::*DICRE*/ Δ*crk3*::*CRK3*$^{FLOX.}$ PCR amplification of genomic DNA extracted from two blasticidin and puromycin (PUR) clone 2 and 8 drug resistant lines with oligonucleotides binding out with the integration site (grey arrows), within the *crk3* coding sequence (grey arrows), within the loxP vector (blue arrows) and diCre sequences (red arrows). See materials and methods for OL sequences.

4.2.2 Conditional deletion of *CRK3* in promastigotes using inducible diCre

Based on our previous data demonstrating the efficient loss of floxed *GFP* following diCre induction by treatment of logarithmically growing promastigotes with 50nM rapamycin, we deemed this dose sufficient to induce efficient excision of floxed *CRK3*. The loss of an essential gene should result in the cessation of growth, therefore to investigate this growth curves were generated by seeding promastigote cultures at $5x10^5$ cells/ml and gene deletion induced by 50nM rapamycin treatment alongside an untreated control for each clone. Growth was measured over the course of 7 days, demonstrating a severe growth defect in those cells treated with rapamycin (**Figure** 4:2). By day 7, growth of the induced cells had recovered, therefore to investigate whether this was a result of selective pressure for cells retaining an extra copy of *CRK3* or as a result of inefficient diCre induction, the cultures previously treated with rapamycin were seeded once more at $5x10^5$ cells/ml concentrations and either treated with rapamycin once more (redose) or allowed to grow (recovery).

A pronounced growth defect was achieved by re-dosing, indicating that the recovery population was likely a result of inefficient diCre activation. Subsequent conditional gene induction experiments were conducted with 100nM rapamycin to address this possible issue of inefficient diCre mediated gene excision.

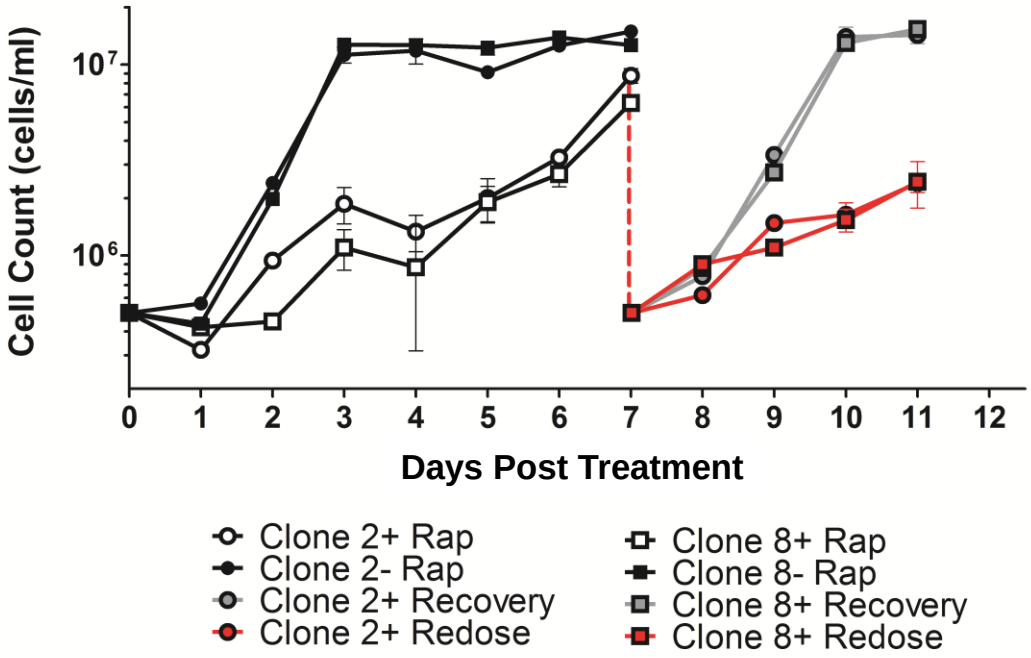


Figure 4:2- Promastigote growth following diCre mediated floxed *CRK3* excision. Clones 2 and 8 were seeded as promastigotes at 5x10^5 cells/ml cultures and grown in the presence or absence of 50nM rapamycin (Rap) for 7 days. Cell counts were obtained at 24 hour intervals to monitor growth and a representative growth curve was plotted. After 7 days growth the rapamycin treated cells were seeded at 5x10^5 cells/ml as indicated by the red, dotted line in the presence (Redose) or absence (Recovery) of 50nM rapamycin and growth monitored by cell counting every 24 hours for four days. Error shown as standard deviation of the mean between 1 and 3 replicates dependent on the concentration of cells due to the haemocytometer detection limit of 1x10^5 cells/ml.

Data in the previous chapter showed no promastigote toxicity of rapamycin even at 500nM treatment indicating that promastigotes were insensitive to treatment with rapamycin, which is in agreement with other studies (Madeira da Silva et al. 2009). The growth arrest phenotype is therefore likely due to the loss of floxed *CRK3*. To investigate the rate of floxed *CRK3* excision, cells were once more seeded at 5x10^5 cells/ml in either the presence or absence of 100nM rapamycin, and genomic DNA extraction performed at 24 hour and 48 hour time points. The genomic DNA was analysed by PCR amplification using oligonucleotides binding either side of a 3.4Kbp region outwith the floxed site (**Figure** 4:3a upper panel). Once diCre activity is induced and the floxed fragment excised, the resulting amplicon is 1.36Kbp in size, therefore facilitating a rapid analysis of gene loss. Gene deletion as detected by this method occurred efficiently within 24 hours for both clones, with no larger fragment being visible by agarose gel separation (**Figure** 4:3a lower panel). In addition, the absence of a 1.36Kbp amplicon in either of the untreated clones reveals the tight regulation of diCre mediated, floxed *CRK3* excision.

To confirm the PCR analysis that loss of floxed *CRK3* was efficient within 24 hours post rapamycin induction, Southern blotting was conducted using genomic DNA extracted from clone 2 procyclic promastigotes grown in the presence or absence of rapamycin for 24 and 48 hours. A wild-type *L. mexicana* genomic DNA control was included. Restriction digest with *NruI* resulted in excision of a 6.2Kbp fragment containing the floxed *CRK3* sequence, or a 2.4Kbp wild-type fragment containing endogenous *CRK3*. Digested DNA was subsequently resolved by agarose gel electrophoresis and hybridised with a chemiluminescent *CRK3* open reading frame probe. A 6.2Kbp fragment containing the *CRK3* ORF was detected in the untreated control at 24 hours, and with the rapamycin treated group showing partial retention of the gene at this time point. The 6.2Kbp fragment was not detected by Southern blotting of the 48 hour rapamycin treated genomic DNA (**Figure** 4:3b). The inclusion of a Wt gDNA control demonstrates the specificity of the probe, however the presence of a band at >2.4Kbp is likely a result of incomplete restriction digestion.

Interestingly, Southern blotting of the 24 and 48 hour rapamycin treated samples yields a ~3Kbp *CRK3* retained fragment; diCre recombination results in the circularisation and therefore loss of a floxed target, therefore detection of a fragment at this size may be an indication that the floxed *CRK3* coding sequence is retained as an episome. The circular DNA should be around 2Kbp in size, therefore the detection of a larger ~3Kbp fragment may be due to poor gel resolution due to its circularisation. The lower chemiluminescent signal of the equally sized fragment at the later, 48 hour time point relative to the 24 hour induced line suggests that this ‘episome’ is lost over time.

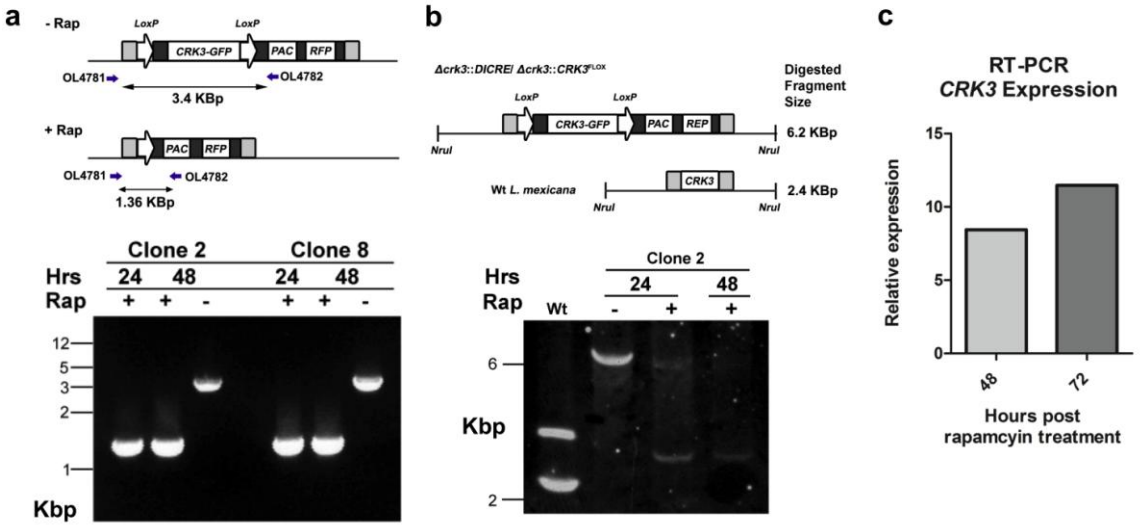


Figure 4:3- DNA and transcript analysis of floxed *CRK3* excision. a. (upper) A schematic representation of the strategy utilised to investigate *CRK3* loss by a change in size following PCR amplification with primers binding upstream of the 5' *CRK3* integration flank (grey) and within the puromycin resistance cassette. (lower) Genomic DNA extracted from clones 2 and 8 at 24 and 48 hours after rapamycin treatment was used for PCR amplification and amplicons resolved on an agarose gel. **b.** (upper) A schematic representation of the size change of the *NruI* restriction digest fragment upon integration and replacement of the endogenous wild-type *L. mexicana* (Wt) locus by the floxed *CRK3* cassette. (lower) Southern blot analysis of wild type and clone 2 cell lines following growth in the presence or absence of rapamycin. 5µg of DNA was digested with *NruI* and resolved by agarose gel electrophoresis, transferred to a nylon membrane and hybridised with a chemiluminescent *CRK3* open reading frame probe. **c.** Real-time PCR analysis of cDNA generated from RNA extracted at 48 and 72 hours post rapamycin treatment of clone 2 promastigotes. Fold increased expression of transcript is quantified relative to a 0 hour treatment control.

Inducible deletion of floxed *CRK3* is rapid and tightly regulated based on these data, therefore the distinct growth arrest phenotype exhibited 48 hours post rapamycin mediated induction is attributed to a loss of active CRK3. Western blotting of promastigotes cell extracts grown in the absence or presence of rapamycin with an anti-GFP antibody were problematic due to the aberrant growth and morphology of the induced cells. Real-time PCR was instead utilised to compare the level of *CRK3* transcript expressed 48 and 72 hours post treatment between induced and uninduced lines showed a 7 and 11 fold increase in *CRK3* specific transcript in the induced lines respectively (**Figure** 4:3c). Such a contrary finding suggested the possibility of CRK3 overexpression by circularisation as an episome. This was assessed by conditional complementation of this line with a *CRK3* as a transgene, yielding a cell line insensitive to floxed *CRK3* gene deletion and establishing gene deletion as the reason for growth arrest (**Figure** 4:6).

The pronounced growth arrest phenotype upon *CRK3* conditional deletion was investigated further by DNA content analysis. Δ*crk3*::*DICRE*/Δ*crk3*::*CRK3*^{FLOX} clone 2 promastigotes were grown in the presence or absence of 100nM rapamycin for 120 hours, resulting in a reduced growth rate by 48 hours post diCre induction and growth arrest by 96 hours (**Figure** 4:4a). Cells were then extracted at 72 and 96 post treatment, fixed with methanol and stained with propidium iodide (PI) and DNA content analysis conducted by flow cytometry to identify the proportion of parasites at each cell cycle stage (**Figure** 4:4b). As anticipated, loss of *CRK3* resulted in the accumulation of cells in the G2/M phase (4N) of the cell cycle by 72 hours, with an increasing proportion of >4N by 96 hours in which population multinucleated cells were identified (**Figure** 4:4c). Taken together, these demonstrate a block in G2/M and the accumulation of cells with a high DNA content which are unable to undergo division, confirming the requirement of active CRK3 in mediating the transition through G2/M. Interestingly there is an accumulation of a <0N population, usually indicative of a population of anucleated 'zoids' which lack a nucleus yet retain a kinetoplast. The proportion of <0N cells with low DNA content increased from 72 to 96 hours, and due to the defined population peak it is likely this population represents anucleated zoids rather than a result of cell death and DNA degradation as demonstrated previously (Grant et al. 2004).

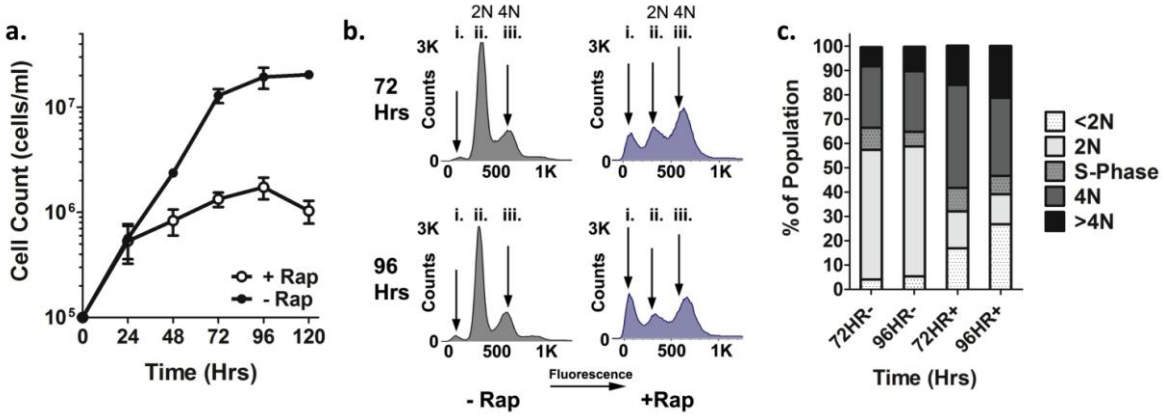


Figure 4:4- DNA content analysis of *CRK3* inducible deletion promastigotes. a. Clone 2 cells were seeded as promastigotes at 1x10^5 cells/ml cultures and grown in the presence or absence of 100nM rapamycin (Rap) for 5 days (error shown as SD, N=3). Cell counts were obtained at 24 hour intervals to monitor growth and a representative growth curve was plotted. **b.** At 72 and 96 hours, cells were fixed and stained with propidium iodide for FACS analysis of 100,000 cells on a MacsQuant flow cytometer. Arrows indicate the positions of the 2N content of cells representing G_1 phase cells (ii) and 4N content representing G_2 (iii) peaks in untreated controls. An additional peak of low DNA content associated with increased incidence of <2N zoids (i) in the population **c.** Graphical representation of the DNA content of each population based on the flow cytometry plots

To confirm the accumulation of multi-nucleated, G2/M stalled cells and anucleated zoids by floxed *CRK3* deletion, DNA staining by DAPI treatment and microscopic analysis was performed on cells 96 hours post rapamycin treatment (**Figure** 4:5). Conditional deletion of *CRK3* in promastigotes resulted in cells that had undergone repeated rounds of flagellar synthesis in the absence of cytokinesis. In addition, these cells were generally larger than the uninduced control cells and some are observed as multi-nucleated (>2N1K), therefore it appears that the cells continued to undergo the growth and flagellar growth normally associated with synthesis and G2 stage of the cell cycle, but were blocked in their ability to undergo mitosis, preventing appropriate cell division.

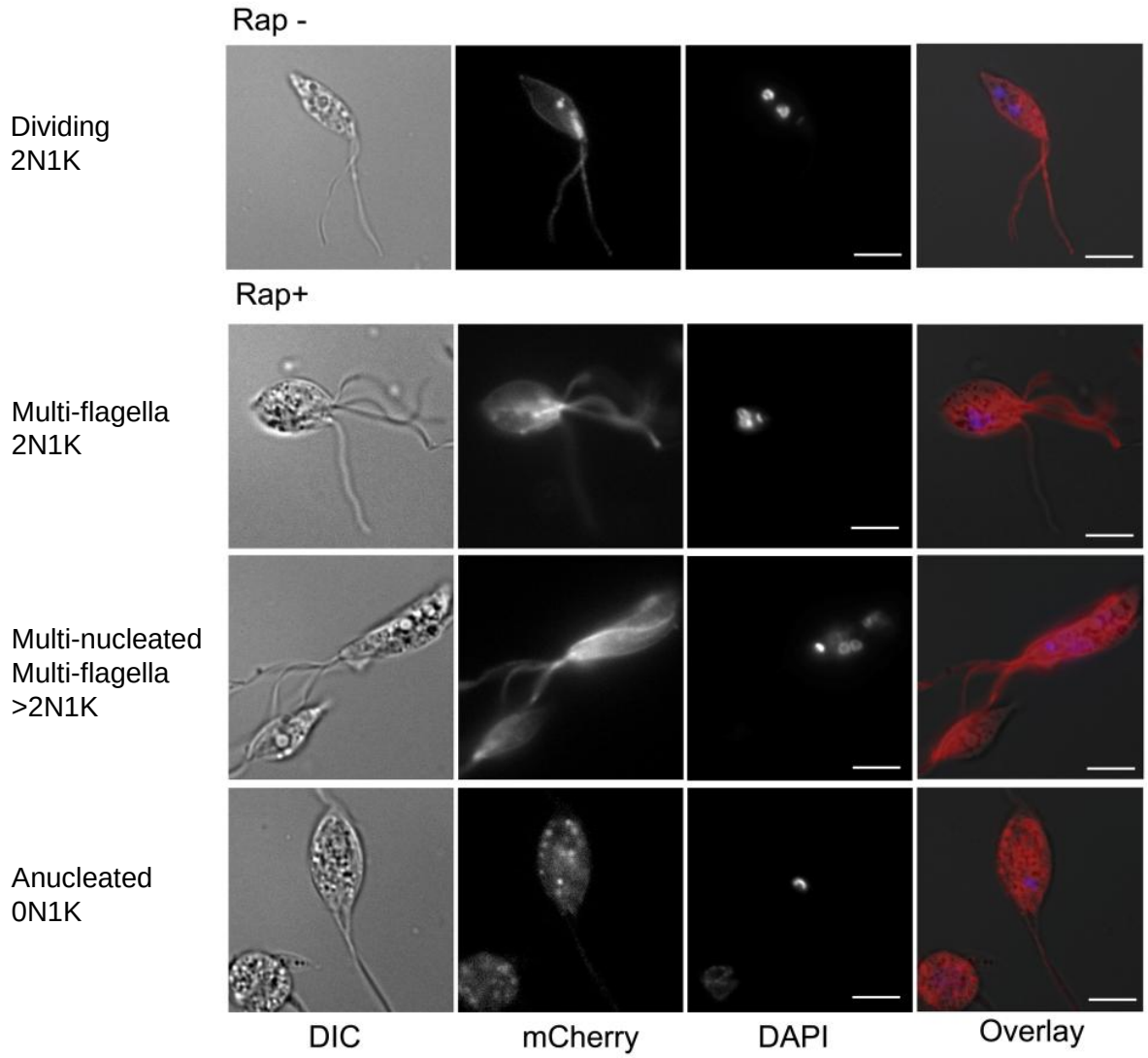


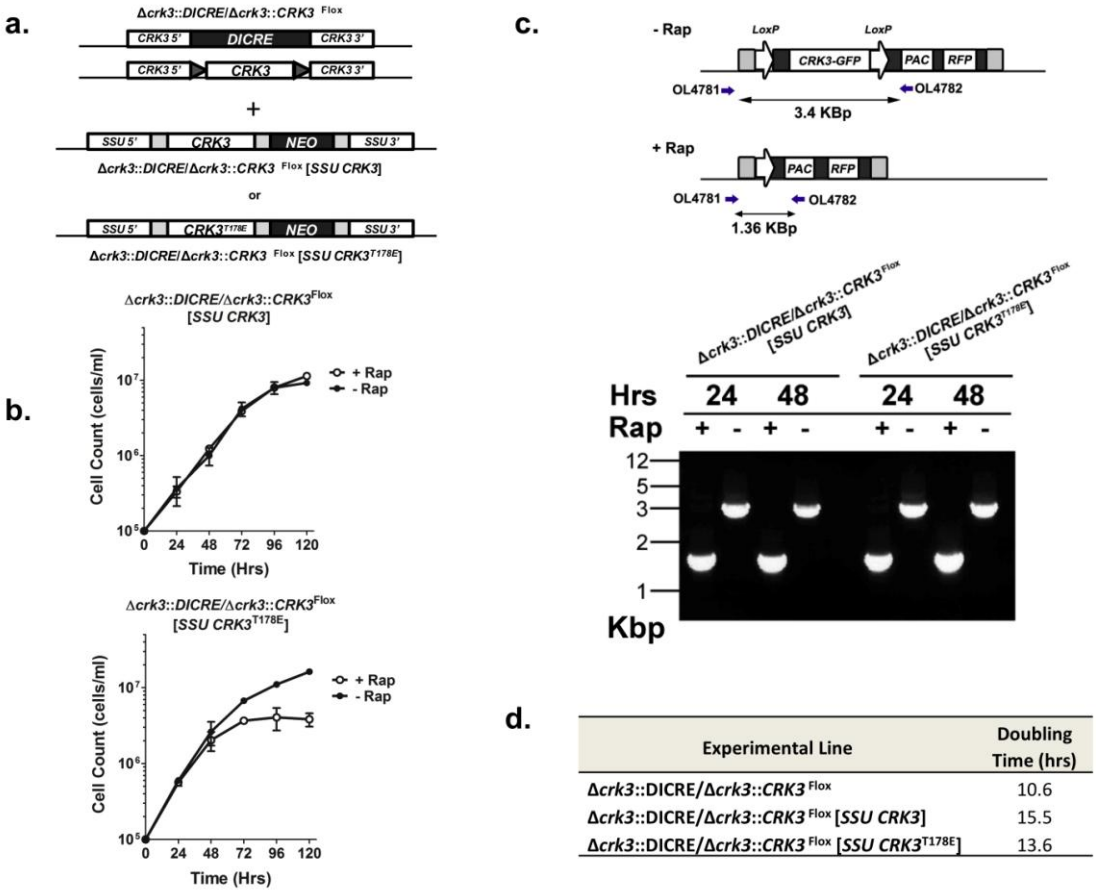
Figure 4:5- Morphology and nuclear staining of promastigotes after *CRK3* deletion. Images of cells grown in the presence (top) or absence (bottom three rows) of 100nM rapamycin for 96 hours. Cells (clone 2) were fixed and stained with DAPI in Fluoromount solution to detect nuclear and kinetoplast content. The expression of HASPB-mCherry red fluorescent protein (RFP) from the loxP vector backbone allowed red-fluorescence detection from the membrane and flagella. Scale bar 5µm.

The presence of anucleated *L. mexicana* promastigotes following indirubin treatment has been demonstrated previously (Grant et al. 2004), and cells with a similar phenotype were also identified by microscopy following inducible deletion of *CRK3* (**Figure** 4:5). The presence of such cells indicates that cytokinesis is not entirely blocked in the absence of mitosis, an explanation for the expanding <2N population resulting from loss of *CRK3*. These data show that loss of CRK3 results in the blocking of cells in the G2/M stage of the cell cycle, resulting in continued protein synthesis as demonstrated by the accumulation of multiple flagella on individual cells, as well as an increased cell size. This cell cycle arrest and resulting phenotype is indicative of the efficacy of diCre mediated gene excision, and forms the basis for further probing of CRK3 function in promastigotes.

4.2.3 Inducible complementation assays by *CRK3* deletion

The ability to conditionally regulate expression of CRK3 enables the identification of residues that are essential for protein kinase activity. This approach involves complementation of the inducible deletion line with an exogenous gene copy, which can be mutated to identify active sites once expressed as protein. Conditional gene deletion of the floxed gene copy induces expression of only the mutant transgene, enabling phenotype dissection. By application to CRK3, the floxed *CRK3* copy should be sufficient to maintain progression through the cell cycle until excised by rapamycin activation of diCre, probing whether mutated CRK3 protein activity functionally recovers growth, or results in a block in G2/M as a consequence of impaired protein kinase activity. Cell lines were generated by transfection of the Δ*crk3*::*DICRE*/Δ*crk3*::*CRK3*^{FLOX} cell line (now referred to as the 'parental flox' line) with a wild-type *CRK3* sequence (Δ*crk3*::*DICRE*/Δ*crk3*::*CRK3*^{FLOX} [*SSU CRK3*]) or a T178E mutated version (Δ*crk3*::*DICRE*/Δ*crk3*::*CRK3*^{FLOX} [*SSU CRK3*^{T178E}]) into the 18S ribosomal small subunit (SSU) using a modified version of the pRib vector conferring neomycin resistance. Previous studies generating CRK3 mutants for use in yeast recovery (Wang et al. 1998) and recombinant protein activity assays (Gomes et al. 2010) show loss of kinase function by mutation of this T-loop residue. Previous attempts to investigate the essentiality of this residue *in situ* were unfeasible due to the essentiality of CRK3, therefore we utilised conditional complementation to conduct such work.

Three clones per transfection were selected that were resistant to puromycin, blasticidin and G418 and tested for their sensitivity to floxed *CRK3* deletion by growth in the absence or presence of 100nM rapamycin by cell counting over the course of 5 days. Growth curves in the absence or presence of rapamycin were analogous between each clone tested, therefore representative growth curves were prepared from a single clone from each cell line (**Figure** 4:6b). Loss of floxed *CRK3* in the mutant T178E expressing line resulted in a growth arrest by 72 hours, a delay comparable with that previously seen in the parental flox line where a pronounced difference between rapamycin treated and untreated cells is evident by 48 hours. In contrast the growth arrest phenotype induced by loss of floxed *CRK3* in the Δ*crk3*::*DICRE*/Δ*crk3*::*CRK3*^{FLOX} line was recovered by complementation with the wild-type *CRK3* transgene, as evidenced by growth in the presence of rapamycin. Recovery of growth by complementation substantiates the previous evidence that CRK3 activity is essential for cell cycle progression in promastigotes. To ensure the resultant phenotypes were as a result of floxed *CRK3* loss, genomic DNA extraction and subsequent PCR amplification of the floxed *CRK3* gene locus was conducted to confirm gene loss, with amplification of a smaller 1.36Kbp fragment following 24 hours post rapamycin treatment for both cell lines (**Figure** 4:6c).



Experimental Line	Doubling Time (hrs)
Δ*crk3*::DICRE/Δ*crk3*::*CRK3* Flox	10.6
Δ*crk3*::DICRE/Δ*crk3*::*CRK3* Flox [*SSU CRK3*]	15.5
Δ*crk3*::DICRE/Δ*crk3*::*CRK3* Flox [*SSU CRK3*^{T178E}]	13.6

Figure 4:6- Generation of mutant and wild-type *CRK3* inducible complementation lines. a. Schematic representation of the process of generating complementary lines by integration into ribosomal locus of the *CRK3* inducible deletion line with constructs containing a wild-type or T178E mutant *CRK3* transgene in a vector conferring resistance to G418. **b.** 3 clones per cell line were confirmed as G418 resistant. A single clone for the T178E mutant (lower) and wild-type complementary line (upper) were seeded as promastigotes at 1x10^5 cells/ml cultures and grown in the presence or absence of 100nM rapamycin (Rap) for 5 days (error shown as SD, N=3). Cell counts were obtained at 24 hour intervals to monitor growth and a representative growth curve was plotted. **c.** PCR validation of floxed *CRK3* gene deletion; (upper) schematic for amplification of the CRK3 locus, with expected sizes before and after excision. (lower) Amplification of gDNA extracted from cells grown in the presence or absence of rapamycin at 24 and 48 hours for each cell line. **d.** Doubling time values in hours of each cell line during the first 48 hours of logarithmic growth.

These data confirm that the growth phenotype resulting from floxed *CRK3* excision in the parental flox line is a result of loss of protein expression, as opposed to an overexpression from a functional episome. In the absence of rapamycin treatment, both wild-type and mutant complemented cell lines grow at reduced rates compared with the parental flox cell line (**Figure** 4:6d). A growth defect might be anticipated following overexpression of the inactive CRK3^{T178E} mutant as this could have a dominant-negative phenotype, however the more severe growth reduction of the wild-type complemented line suggest overexpression of CRK3 from the ribosomal small subunit is deleterious to the cells. Despite a less pronounced effect on growth, the *CRK3^{T178E}* complemented promastigotes appeared reduced in cell size and lack flagella prior to rapamycin

treatment. This curious phenotype may be indicative of a metabolic stress from overexpression of the inactive T178E, with loss of flagellum and reduced cell size compensating for a dominant negative stress. The loss of growth by 72 hours post induction compared with 48 hours in the parental flox line was likely a result of the reduced growth rate of the cells, resulting in the growth arrest phenotype to take longer to manifest.

DNA content analysis was performed to establish whether expression of the inactive CRK3^{T178E} mutant results in arrest at G2/M, and to confirm the standard progression of the *CRK3* complemented line through the cell cycle (Figure 4:7a-d). Both cell lines were grown in the presence or absence of rapamycin for 96 hours, with cells fixed and stained with PI at hours 72 and 96 for DNA content analysis by flow cytometry. In the wild-type complementation line, floxed *CRK3* excision had no detectable effect on the cell cycle compared with the uninduced control (Figure 4:7a & b). However, a standard flow cytometry profile with a large proportion (~70%) of cells at 2N was not seen; instead there was a large proportion (25-30%) of >4N cells, with a roughly equal 4N population independent of time or treatment. The slow growth of this cell line as a result of *CRK3* overexpression may be influencing the flow cytometry profile. Despite this, these data demonstrate that the attenuated growth obtained by inducible Cre excision of floxed *CRK3* is complemented by expression of wild-type *CRK3* as a transgene.

Analysis of the *CRK3*^{T178E} complementation line yielded a DNA content profile which better fitted the established cell cycle distribution, with a larger proportion (~40%) of 2N cells at both time points (Figure 4:7c & d). Floxed *CRK3* deletion resulted in the reduction of this population to ~15%, with an increased abundance of 4N and >4N cells (~70% in total) at both time points. These populations represent cells which are cell cycle arrested at G2/M, and these data are in agreement with the proportion of parental flox cells blocked in G2/M as indicated by the 4N and >4N populations (~60% in total) at 72 hours post induction (**Figure** 4:4c). These data indicate that the CRK3^{T178E} mutant is unable to mediate cell cycle progression. Inducible deletion of floxed *CRK3* in the Δ*crk3*::*DICRE*/Δ*crk3*::*CRK3*^{FLOX} parental strain resulted in a <0N population of ~25% by 96 hours post induction, whereas the mutant complementation line had

a <0N population of around 10%. This discrepancy may explain the reduced proportion of 4N and >4N cells blocked in G2/M as the induced parental flox line, as the production of anucleated zoids by cytokinesis in the absence of mitosis is reduced by the slower growth rate of these CRK3^{T178E} expressing cells.

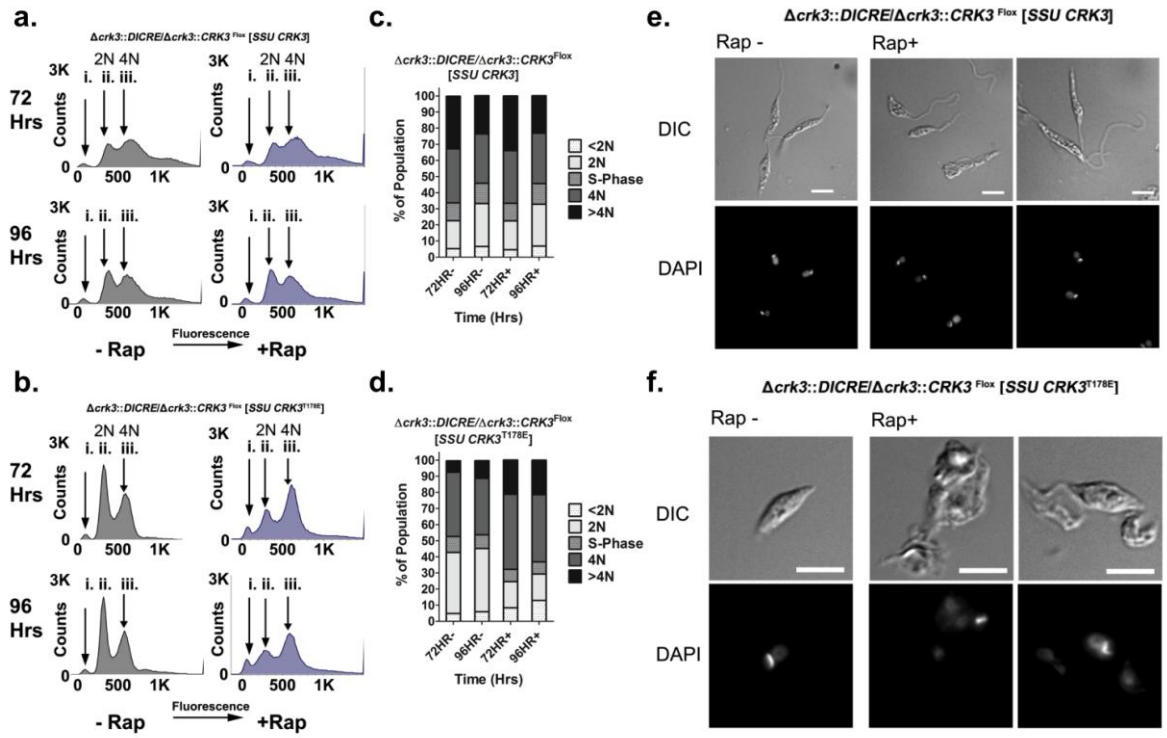


Figure 4:7- DNA content analysis of inducible complementation lines. a. Complemented CRK3 and b. CRK3T178E mutant cells were seeded at 1x105 cells/ml in the presence or absence of 100nM rapamcyin. After 72 and 96 hours, cells were fixed and stained with propidium iodide for flow cytometry analysis of 100,000 cells on a MacsQuant flow cytometer. Arrows indicate the positions of the 2N content of cells representing G1 phase cells (ii) and 4N content representing G2 (iii) peaks in untreated controls. An additional peak of low DNA content associated with increased incidence of <2N zoids (i) in the population Graphical representation of the DNA content of c. CRK3 and d. CRK3T178E complemented cell lines based on the flow cytometry plots. e. CRK3 and f. CRK3T178E complemented cell lines were fixed and stained with DAPI in Fluoromount solution to detect nuclear and kinetoplast content. Scale bar 5µm.

To further investigate the morphology and DNA content of these complemented cells, fluorescence microscopy was performed on cells stained with DAPI after growth in the absence or presence of 100nM rapamycin for 96 hours. Analysis of wild-type complemented cells revealed no difference between induced and uninduced, with cells mainly exhibiting 1N1K karyotypes (Figure 4:7e). In contrast, loss of floxed *CRK3* in the CRK3^{T178E} expressing line results in the appearance of aberrant cells with increased cell size and multi-nucleation (Figure 4:7f). Such morphology was indicative of a block in G2/M and impaired cytokinesis, with an overall increased cell size indicative of the continued

growth and protein expression in preparation for mitosis. These data demonstrate that the growth cycle defect following loss of floxed *CRK3* can be recovered by expression of a *CRK3* transgene, and that mutation of the T-loop residue at position 178 to a glutamic acid ablates kinase function in *L. mexicana* promastigotes.

4.2.4 Conditional deletion of *CRK3* in lesion-derived amastigotes

The lack of a molecular tool for conditional regulation of essential genes in the replicative, mammalian infective amastigote stage *Leishmania* is a major impediment to the validation of molecular targets which represent useful drug targets, as the inability to generate a *null* mutant in promastigotes is not necessarily an indication that the activity of the encoded protein is essential for amastigotes. DiCre is functional in amastigotes as determined in chapter 3 by conditional deletion of floxed *GFP*. To establish whether CRK3 activity is essential for amastigote replication, the Δ*crk3*::*DICRE*/Δ*crk3*::*CRK3*^{FLOX} clone 2 cell line was isolated from the footpads of infected BALB/c mice two months after inoculation. An amenable property of *L. mexicana* over other species is the ability to culture the parasite as axenic amastigotes (Bates 1994) in acidic, serum supplemented Schneider's Drosophila medium, therefore the isolated amastigotes were cultured in the presence or absence of 200nM rapamycin and growth measured by cell counting at 24 hours post treatment, then twice more at 48 hour intervals (**Figure** 4:8a). Floxed *CRK3* deletion by treatment with rapamycin resulted in a dramatically reduced growth rate relative to uninduced amastigotes. To investigate whether these cells were arrested in the cell cycle at G_2/M as observed following conditional deletion of *CRK3* in promastigotes, DNA content analysis was conducted at 72 hours (**Figure** 4:8b). In contrast to G2/M stalling, the resulting profile was instead indicative of arrest in the G1 phase of the cell cycle. To determine the effect on cellular morphology and nuclear content, DAPI staining of live cells (**Figure** 4:8c) showed aberrant amastigotes with rounded morphology and fragmented DNA content.

To demonstrate that rapamycin treatment was inducing excision of floxed *CRK3*, the experiment was repeated by isolation of Δ*crk3*::*DICRE*/Δ*crk3*::*CRK3*^{FLOX} lesion-derived amastigotes and subsequent growth measured in the presence or absence of 200nM rapamycin for 5 days (**Figure** 4:8d). Antibiotic selection with

puromycin and blasticidin was included as an additional factor to investigate whether diCre activity and therefore the efficiency of gene excision was enhanced by selection for those parasites retaining the diCre and floxed gene cassettes. A distinct growth arrest phenotype was manifest by cell counting at day 5 post treatment, with little difference in terms of antibiotic selection between treatment regimes. The growth rate of both induced and uninduced was dramatically reduced compared with the previous experiment (**Figure** 4:8a), with lesion derived amastigote growth rate highly variable between four independent experimental replicates. By genomic DNA extraction at day 5 post treatment, PCR analysis indicated the loss of floxed *CRK3* and the retention of diCre activity in the absence of antibiotic selection (**Figure** 4:8e). Such findings have important implications for the utilisation of diCre inducible deletion of essential genes *in vivo*, as the application of antibiotics in mice for parasite selection is not feasible. A previous experiment utilising lesion-derived Δ*crk3*::*DICRE*/Δ*crk3*::*CRK3*^{FLOX} amastigotes extracted from a different mouse also exhibited growth arrest and conditional deletion of floxed *CRK3* by PCR analysis following treatment with 100nM rapamycin for 5 days (data not shown). Together, these data showed that integration of the diCre and floxed gene into the endogenous gene locus confers sufficient floxed gene expression for the maintenance of infection, whilst continuing to express the diCre molecular machinery at a level sufficient for inducible gene deletion throughout infection.

Despite conditional deletion of *CRK3* in at least two independent experiments, the growth arrest at G_1, coupled with the aberrant morphology by rapamycin treatment of Δ*crk3*::*DICRE*/Δ*crk3*::*CRK3*^{FLOX} cannot solely be attributed to loss of *CRK3;* rapamycin treatment inhibits the growth of wild type, lesion-derived *L. mexicana* amastigotes (**Figure** 4:8d). This inherent sensitivity to rapamycin necessitates an alternative dosage regime of amastigotes, with a 24 hour dosage period a potential alternative to dissect the outcome of gene deletion from the effect on growth by rapamycin treatment.

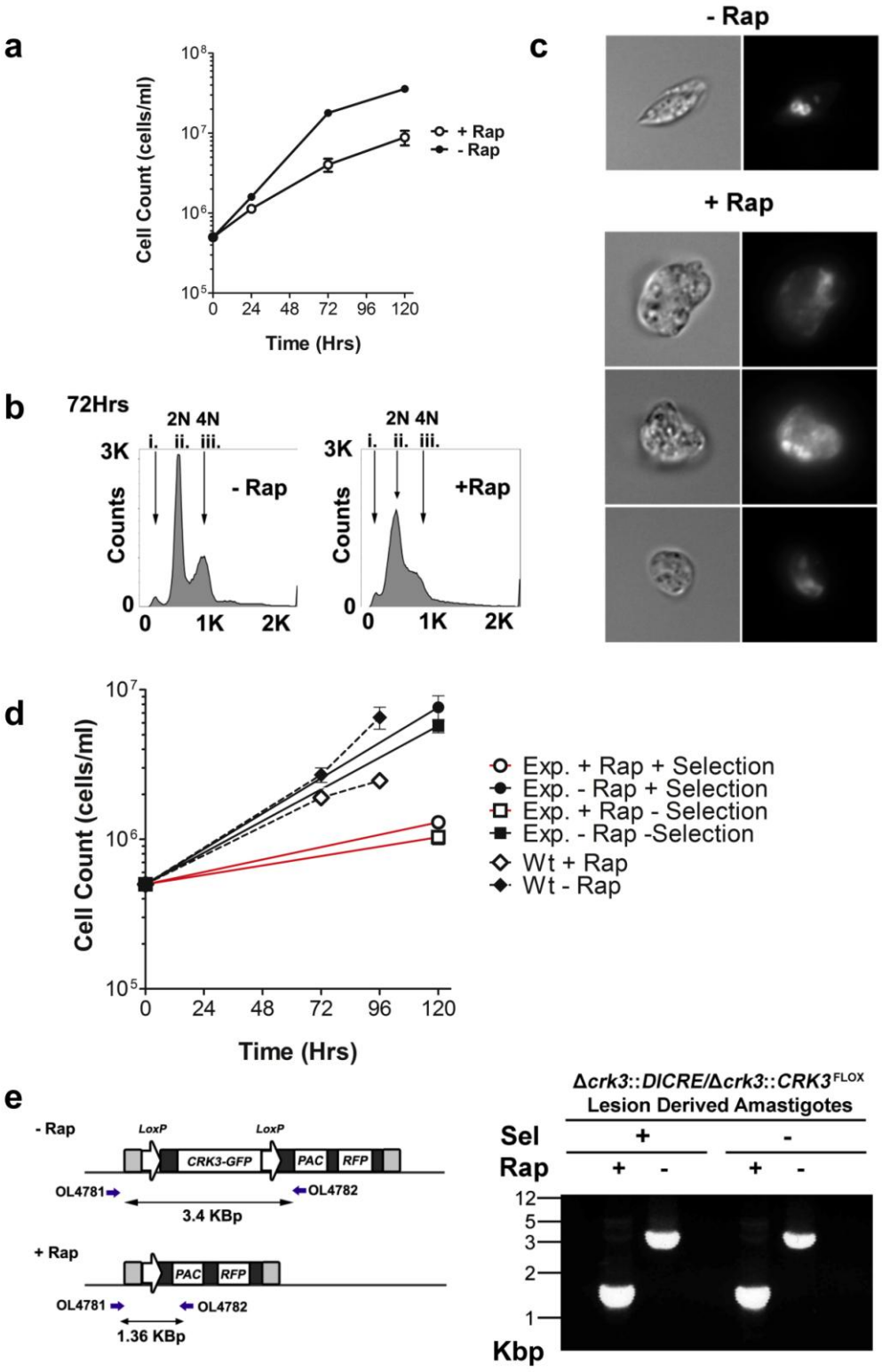


Figure 4:8- Conditional deletion of *CRK3* in amastigotes and the increased sensitivity to rapamycin treatment. a. Δ*crk3*::*DlCRE*/Δ*crk3*::*CRK3*^{FLOX} amastigotes were seeded at 1x10^{6} cells/ml in the presence or absence of 200nM rapamycin and counted at 24, 72 and 120 hours post treatment (SD, N=3). **b**. DNA content analysis on 100,000 cells at 72 hours post treatment; i. represents <2N population, ii. indicates cells 2N content cells in G_1 and iii. cells with 4N content at G_2 prior to mitosis. **c**. DAPI staining microscopy of live amastigotes at 72 hours post treatment. **d**. The experiment in panel (a) was repeated with the Δ*crk3*::*DlCRE*/Δ*crk3*::*CRK3*^{FLOX} line (Exp) grown in the presence or absence of 10µg/ml puromycin and blasticidin for antibiotic resistance selection (Selection) with a seperate wild-type (Wt) *L. mexicana* rapamycin treatment control. Growth curves were prepared by counting the experimental line at 120 hours post induction, whilst the wild-type cells were cunted at 72 and 96 hours post treatment. **e**. PCR amplification of gDNA extracted at 120 hours post treatment (right) using the strategy detailed in the schematic (left).

4.2.5 Conditional deletion of *CRK3* in stationary phase promastigotes to assess activity *in vivo*

A major obstacle in drug target identification is our inability to conduct inducible deletion of an essential gene to establish the requirement of the expressed protein during infection of a murine host. Active CRK3 is essential to mediate cell cycle progression through mitosis in dividing promastigotes, however to further identify CRK3 as a potential drug target we must conduct deletion of *CRK3* in an *in vivo* context. The use of an *in vivo* imaging system (IVIS) is a longitudinal, non invasive method to track the burden of transgenic *L. mexicana* expressing a bioluminescent protein, therefore floxed *CRK3* conditional deletion promastigotes were generated which express a more sensitive red-shifted luciferase (RE9H) compared with renilla and conventional firefly luciferases (McLatchie et al. 2013).

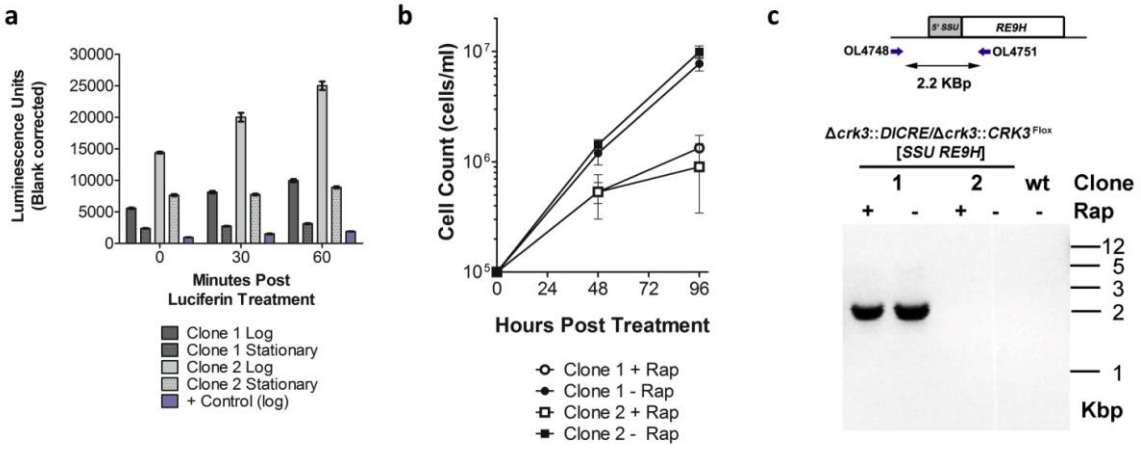


Figure 4:9- Generation of a *CRK3* inducible deletion line expressing red-shifted luciferase. a. Red-shifted luciferase expression was measured in two G418r clones as logarithmically (log) growing and stationary phase with a [*SSU RE9H*] transgenic *L. mexicana* line as a positive control (+Control). Cells were washed in 1xPBS and $1x10^6$ cells/well added to individual wells in a 96 well plate in duplicate (N=2, error is SE of the mean). Bioluminescence was detected using a Pherestar plate reader at 0, 30 and 60 minutes post luciferin addition. **b.** Sensitivity to CRK3 deletion is exhibited by both clones as measured by growth curves prepared by seeding promastigotes at $1x10^5$ cells/ml cultures and grown in the presence or absence of 100nM rapamycin (-/+Rap) for 5 days (error shown as SD, N=3). Cell counts were obtained at 48 hour intervals to monitor growth and a representative growth curve was plotted. **c.** Integration of the RE9H expression construct at the ribosomal locus was investigated by PCR amplification using primers binding upstream of the 5'*SSU* integration site and in the reverse orientation from the RE9H coding sequence to amplify a 2.2KBp fragment (upper). PCR amplification of genomic DNA extracted from both clones and a wild-type *L. mexicana* negative control (wt) was conducted and the amplified products resolved on an agarose gel.

The parental flox, inducible *CRK3* deletion line (Δ*crk3*::*DICRE*/Δ*crk3*::*CRK3*^{FLOX}) was transfected with the pRib vector previously utilised for *CRK3* integration into the 18S ribosomal RNA locus (**Figure** 4:6a) to generate the

Δ*crk3*::*DICRE*/Δ*crk3*::*CRK3*^{FLOX} [*SSU RE9H*] cell line (subsequently referred to as 'bioluminescent parental flox line'). Transfection of this construct yielded two G418 resistant clones which were tested for bioluminescent protein expression and the retention of inducible deletion of *CRK3* by diCre mediated recombination following rapamycin treatment. Both clones exhibited high levels of red-shifted luciferase expression relative to an *L. mexicana* line expressing RE9H from the SSU ([*SSU RE9H*) which was included as a positive control. Clone 1 had ~5 fold higher expression and clone 2 had >10 fold higher expression compared with the positive control during logarithmic stage growth at all time points. The clones were then tested for their ability to undergo inducible deletion of *CRK3* by growth in the presence or absence of 100nM rapamycin for 96 hours (**Figure** 4:9b), with rapamycin resulting in growth arrest by 48 hours post treatment as previously demonstrated in the parental flox line (**Figure** 4:2). Integration of the bioluminescent gene into the 18S locus was checked prior to use in vivo. To confirm integration of these constructs, genomic DNA was extracted from each clone and PCR amplification using primers binding upstream of the *5'SSU* flank and within the *RE9H* coding sequence were used to amplify a 2.2Kbp fragment (**Figure** 4:9c). Such an amplicon was absent in clone 2, and in conjunction with the extremely high expression of RE9H by plate reader assay these data implicate the expression of the gene from an episome, or by integration elsewhere in the genome. Expression of the reporter cassette from an episome is retained during *in vitro* culture by the presence of drug selective pressure, however once inoculated into mice the episome and therefore gene expression can be lost. In contrast, amplification of clone 1 genomic DNA resulted in the expected size of amplicon, thereby confirming integration and establishing the suitability for this cell line to be utilised for *in vivo* measures of parasite burden.

To circumvent the sensitivity of amastigotes to rapamycin treatment and test the hypothesis that CRK3 activity was essential for amastigotes during murine infection, a strategy was conducted which involved the induction of diCre activity in stationary phase promastigotes for 24 hours followed by subcutaneous infection of BALB/c footpads. Previous attempts to induce diCre activity in stationary phase promastigotes for deletion of floxed GFP by treatment with 50nM rapamycin for 20 hours was inefficient (Figure 3:7a), with the higher

number of cells in stationary phase cultures necessitating a higher rapamycin concentration to deliver an adequate dose of rapamycin to each cell. Therefore a concentration of 1μM was used. This high dose of rapamycin had no detectable effect on the cell cycle profile of log-stage promastigotes, and only a marginal effect on the overall growth of wild-type *L. mexicana* promastigotes (Figure 3:4c) and was deemed sufficient to induce deletion in the absence of any deleterious side effects. To ensure that rapamycin treatment had no effect on infectivity, *L. mexicana* [*SSU RE9H*] were cultured to stationary phase and dosed in an identical manner prior to infection to act as a treatment control. Both the bioluminescent parental flox line (experimental) and treatment control lines were grown to stationary phase by seeding cultures at a reasonably high cell density ($2x10^6$ cells/ml) for 4 days. The doubling time of this cell line was such that stationary phase (~$1.6x10^7$ cells/ml) was achieved within two days. After 3 days culturing, experimental and control lines were split and cultured in the presence or absence of 1μM rapamycin for 24 hours. After 24 hours the cells were washed twice in 1xPBS and $2x10^6$ cells inoculated into the footpads of four Balb/c mice per treatment group. Genomic DNA extraction of the stationary phase promastigotes following the 24 hour incubation was conducted to enable PCR amplification of the *CRK3* locus. This showed efficient loss of *CRK3*, with a small percentage retaining the floxed gene (**Figure** 4:10a).

IVIS imaging of the bioluminescence signal from the footpads of infected mice at 5 weekly time points yielded levels of flux far higher than background, with region of interest values (ROI) obtained from those mice infected with the untreated experimental and treatment control groups being higher relative to the rapamycin treated experimental line by week 1 post infection (**Figure** 4:10b). Imaging over the course of 5 weeks shows the steady increase in parasite burden in those mice infected with the treatment control and experimental cell which have not undergone *CRK3* deletion, with the exception of a single Δ*crk3*::*DICRE*/Δ*crk3*::*CRK3*^{FLOX} [*SSU RE9H*] – rap infected mouse which had no detectable signal by week 5 (mouse removed from subsequent analysis in **Figure** 4:10c resulting in a sample size of 3). In contrast to the high bioluminescent signal detected from these mice, a low level of light was emitted from mice infected with cells lacking *CRK3*. However, the flox ROI values obtained were

above the background signal (~$5x10^3$ photons/sec/cm^2) suggesting the survival of a dramatically reduced number of cells compared with the uninduced group.

The steady increase in bioluminescent signal from mice infected with the treatment control group showed that rapamycin alone is not deleterious to the establishment of infection, and instead results from a loss of active CRK3. The low signal obtained from mice infected with the *CRK3* inducible deletion corroborates the evidence obtained by PCR analysis (**Figure** 4:10a) that a small population of cells retain the floxed gene and therefore remain viable. Imaging at 9 weeks post infection revealed an increased bioluminescent signal across all treatment groups, with an increased ROI value of $1x10^6$ photons/sec/cm^2 in the previously low intensity conditional deletion group. A likely explanation for this later recovery was the establishment of infection by those parasites that had not responded to rapamycin induction to undergo *CRK3* deletion. To test this, these 'recovery' amastigotes were purified from each footpad at 10 weeks post infection and cultured *in vitro* as promastigotes to obtain sufficient cell concentrations for genomic DNA extraction and PCR analysis of *CRK3* loss (**Figure** 4:10d). By PCR amplification alongside positive and negative control DNA from a previous floxed *CRK3* deletion experiment and resolution of the resulting amplicons by agarose gel electrophoresis, each extracted population has complete retention of the floxed *CRK3* in the genome. Taken together, the attenuation of infection over 5 weeks by gene deletion and the later establishment of infection by a small population of amastigotes retaining floxed *CRK3* validates that active CRK3 is essential for the maintenance of virulence *in vivo*.

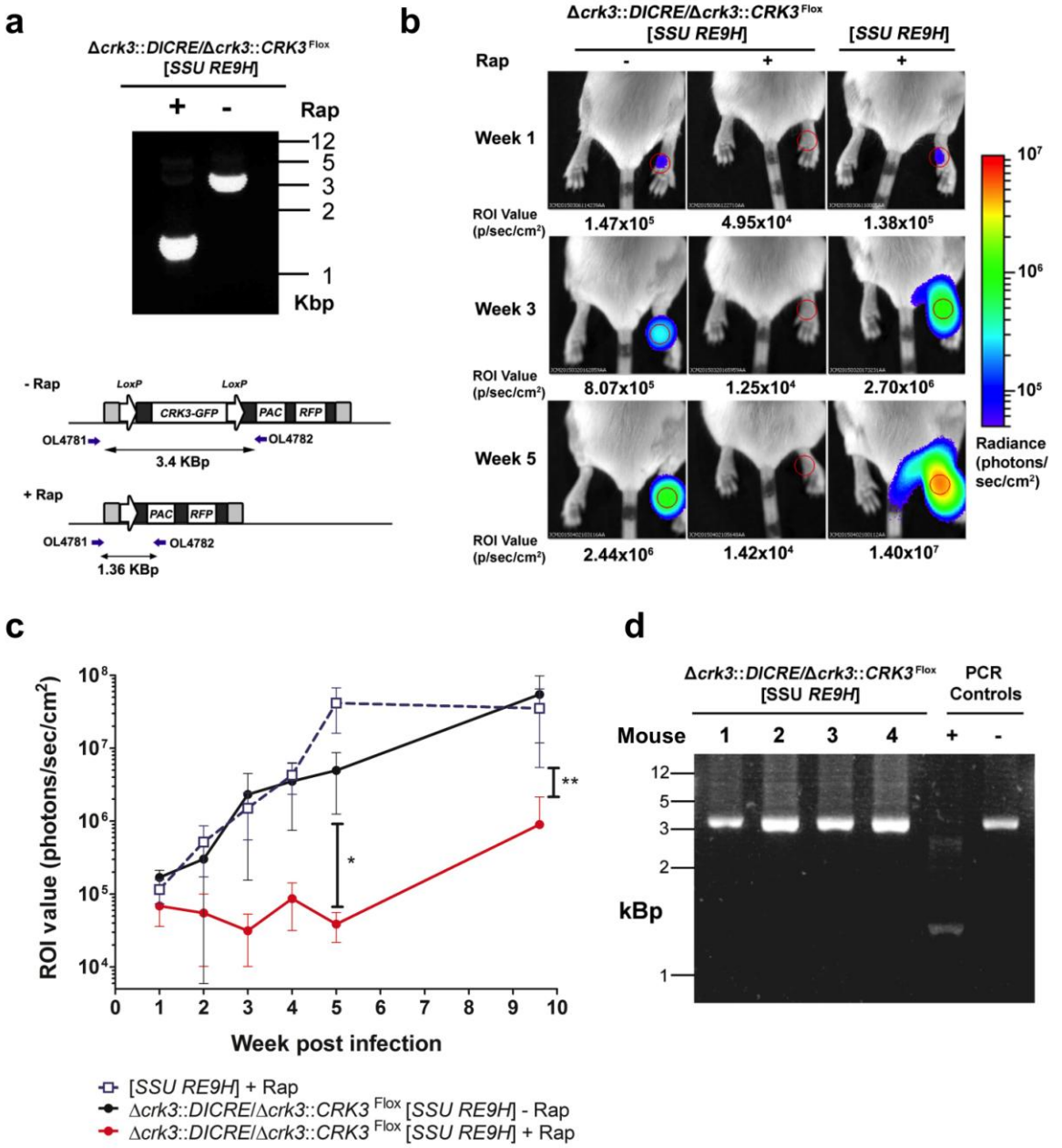


Figure 4:10- Inducible deletion of *CRK3* in stationary phase promastigotes results in attenuated virulence *in vivo*. a. (left) A schematic representation of the strategy utilised to investigate *CRK3* loss by PCR amplification (right) Genomic DNA extracted from rapamycin treated and untreated experimental cells after 24 hours was used for PCR amplification and amplicons resolved on an agarose gel. **b.** Representative images of the bioluminescent signal obtained from the footpads of mice infected with *L. mexicana* retaining *CRK3* (left), deficient in *CRK3* (middle) or bioluminescent control cells pre-treated with rapamycin at 1, 3 and 5 weeks post infection. Region of interest (ROI) values (photons/second/cm^2) are shown below each image. **c.** The mean ROI value for each treatment group (N=3-4, error bars denote the standard deviation around the mean) at each week imaged. A significant difference in bioluminescent signal (*= P-value of <0.05 and **= P-value of <0.005 by unpaired T-test and 2-way ANOVA) is observed between experimental uninduced and induced replicates at week 5 and 9 P.I. **d**. Amastigotes were extracted from the footpads of each mouse (Sample 1-4) infected with the induced experimental group and grown in culture. Genomic DNA was extracted from each expanded promastigote population and used for PCR amplification alongside +/- gDNA as controls for floxed *CRK3* loss or retention (as schematic in **a**). Amplicons were resolved on an agarose gel.

4.2.6 Analysis of immune cell recruitment following *L. mexicana* infection; implications for *in vivo* study of *CRK3*

The use of non-invasive imaging to measure bioluminescent parasites during *in vivo* studies of parasite burden is beneficial both in terms of reducing the number of mice required for conducting experiments, and in the ability to monitor the course of infection longitudinally in a single mouse. The results from the previous section demonstrate that when coupled to an inducible method of essential gene deletion, this combination represents a powerful method to establish the candidate gene as encoding an important drug target for maintaining infection. However, a further aim of this project was to expand this model even further by combining *in vivo* imaging techniques, inducible gene regulation and the subsequent analysis of immune cell recruitment by flow cytometry of the infection site and draining lymph node. By this method, a gene can be identified as essential and the resulting influence on the immune response established. Such study has implications on the resolution of infection, but also on the immunity to subsequent infection. The purpose of the study presented here was to establish a model to investigate immune cell recruitment at the infection site and draining lymph node (dLN) during a late stage ear infection with bioluminescent reporter *L. mexicana*, as a reference point for further study using the diCre inducible system to delete essential or virulence factor genes.

Prior to the adaptation of the diCre conditional deletion system in *L. mexicana*, a model to study innate cell recruitment at the inoculation site and draining lymph node was established by generation of wild-type *L. mexicana* reporter lines. By transfection with pGL2217 or pGL2234, constructs were integrated into the ribosomal locus conferring expression of either firefly luciferase (LUC) or red-shifted luciferase (RE9H) respectively (utilised in section 4.2.4.). Drug resistant clones were selected and bioluminescence expression measured for four clones each per transfection at both logarithmically growing and stationary phase growth (**Figure** 4:11a). LUC bioluminescence was consistently higher than RE9H at both growth stages, however the red-shifted light emitted by RE9H results in more sensitive detection during *in vivo* imaging. A single, highly expressing clone from each group was inoculated into the footpad of a single Balb/c mouse each to allow direct comparison using the IVIS. By 1 month post

infection, the signal detected from the footpad infected with the [*SSU RE9H*] reporter line infected was ~5 fold higher when compared with the footpad infected with the [*SSU LUC*] reporter line (**Figure** 4:11b), with a higher signal also detected from the region corresponding to the draining popliteal lymph node. Parasite burden was not directly measured however, therefore this increased signal may have derived from an increased parasite burden in the footpad. Despite this lack of investigation, the increased detection sensitivity of RE9H over LUC2 (McLatchie et al. 2013) led to the purification and application of this line in the development of an *in vivo* infection model.

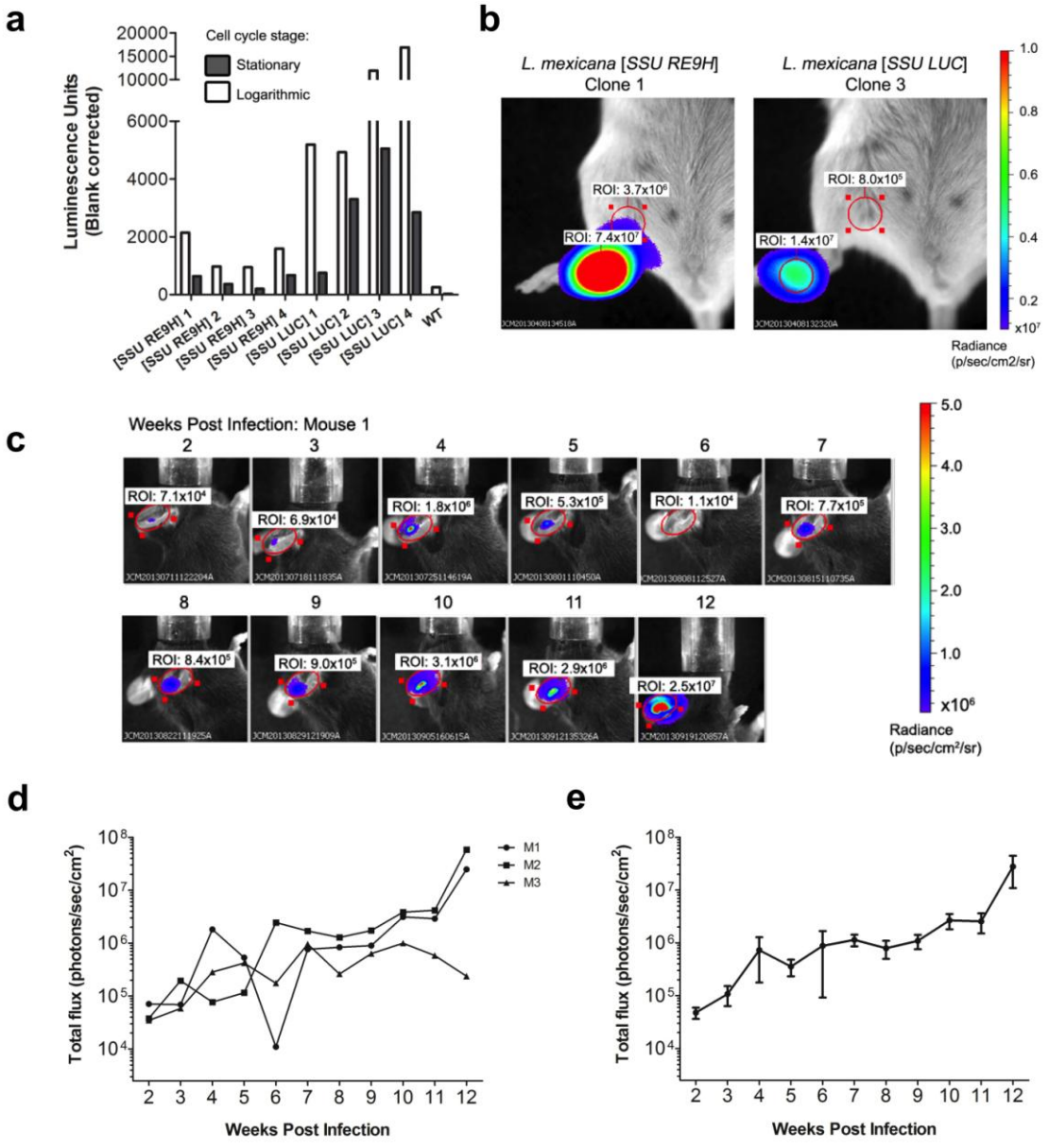


Figure 4:11- Generation of a bioluminescent *L. mexicana* line for determining parasite burden *in vivo*. a. Expression of *LUC* or *RE9H* determined by *in vitro* measures of light from 5x10^6 logarithmically growing or stationary promastigotes by a Pherestar plate reader. **b.** Region of

interest (ROI) measures from the footpads and draining lymph nodes of Balb/c mice infected with selected clones from each bioluminescent reporter line. **c.** Representative images of the bioluminescence signal detected using an IVIS from C57BL/6 mice infected in the ear pinna with the RE9H reporter line. Detection was weekly after week 2 post infection over the course of 12 weeks. **d.** The individual ROI values plotted over the course of 12 weeks post infection for 3 mice infected in the ear pinna with the RE9H reporter line and **e.** grouped analysis showing the average ROI value (error bars represent the standard error of the mean, N=3).

Amastigotes were extracted from the lesions caused by infection with both reporter lines, differentiated to promastigotes *in vitro* and stored as low passage stabilites. The [*SSU RE9H*] reporter line was used for subsequent infection of $5x10^4$ stationary phase promastigotes by intradermal injection into the right ear pinna of six C57BL/6 mice to monitor parasite burden in the ear over the course of 3 months infection (**Figure** 4:11c). Immune cell extraction was conducted by homogenising the ear and cervical lymph nodes and purifying the resident cells for phenotypic analysis by flow cytometry at the 3 month end time point. Left ears were inoculated with the vehicle 1xPBS as a 'sham' needle injury control, with ear and dLN resident populations quantified using naïve controls. Of the six mice inoculated, two (mouse 1 and 2) exhibited consistently increasing bioluminescent signal detection from the ear, whilst the signal from mouse 3 gradually increased until week 10 when the signal begins to drop in intensity (**Figure** 4:11d). The loss of signal from the remaining 3 mice which maintained low levels of flux detection was likely a result of the inoculation route, as infection into the ear pinna is a difficult procedure. As such, this model requires a higher sample size to control for the variability introduced from the infection route. Grouped analysis of the three mice shows an average increase in burden over time (**Figure** 4:11e), demonstrating the establishment of long term infection in these mice.

The infiltration of monocytes and in particular monocyte derived dendritic cells (mo-DC) into *Leishmania* infection sites has been implicated in the development of appropriate immunity against *L. major* in C57BL/6 mice (Petritus et al. 2012). In contrast, infection of these mice with *L. mexicana* results in reduced recruitment of mo-DC, impaired inducible nitric oxide synthase (iNOS) production in infected cells and their reduced migration to the draining lymph nodes for antigen presentation to T-cells relative to *L. major* infections (Petritus et al. 2012). The influence of these cells in driving an appropriate Th1 inflammatory immune response is an important aspect of host-mediated immunity to *Leishmania* infection. By inducible deletion of an essential gene or

virulence factor from *L. mexicana* during *in vivo* infection, the recruitment of these cell subsets to the infection site and draining lymph node represents a useful population to quantify in the context of infection resolution by an appropriate immune response.

Previous work (Goundry, 2015 Thesis) demonstrates the potential of studying monocyte subset as indicators of an appropriate immune response by utilisation of *in vivo* imaging to compare the parasite burden of C57BL/6 mice infected with LUC expressing *L. major* deficient in the genes encoding inhibitors of serine peptidases (*ISP2/3*). The loss of ISP2/3 virulence factor expression of these mutant reporter lines prevents parasite-mediated immunosuppression, resulting in an early recruitment of mo-DC to the infection site compared to wild-type and the accelerated resolution of lesions. These data implicate the efficacy of a similar approach by *in vivo* inducible gene deletion to establish the regulated gene as encoding an essential or immune modulating factor.

To establish the 'baseline' number of such cells at week 12 post infection, the red-shifted luciferase reporter expressing *L. mexicana* infected mice (**Figure** 4:11b-d) were culled and the immune cell populations at the ear infection site and the draining cervical lymph nodes extracted. Based on a published gating strategy (Ribeiro-Gomes et al. 2012), the extracted immune cells were phenotyped by staining with fluorescent antibodies against a variety cell surface markers and detect viable, innate immune leukocyte populations expressing CD45 and the leukocyte antigen CD11b (Hickstein et al. 1987) by flow cytometric analysis. This subset was further phenotyped by gating in respect to CD11c+ and MHCII surface receptor expression to detect Ly6G+ neutrophils, infiltrating Ly6C+ monocytes (Gordon & Taylor 2005), monocyte-derived dendritic cells and monocyte-derived macrophage (mo-MΦ) and Ly6C- skin or lymph node resident DC and MΦ (**Figure** 4:12). Both infected and sham ears and draining lymph nodes were extracted for analysis, with an equal number of ears and cervical lymph nodes used for immune cell extraction from uninfected, naïve mice as a non-inoculated control.

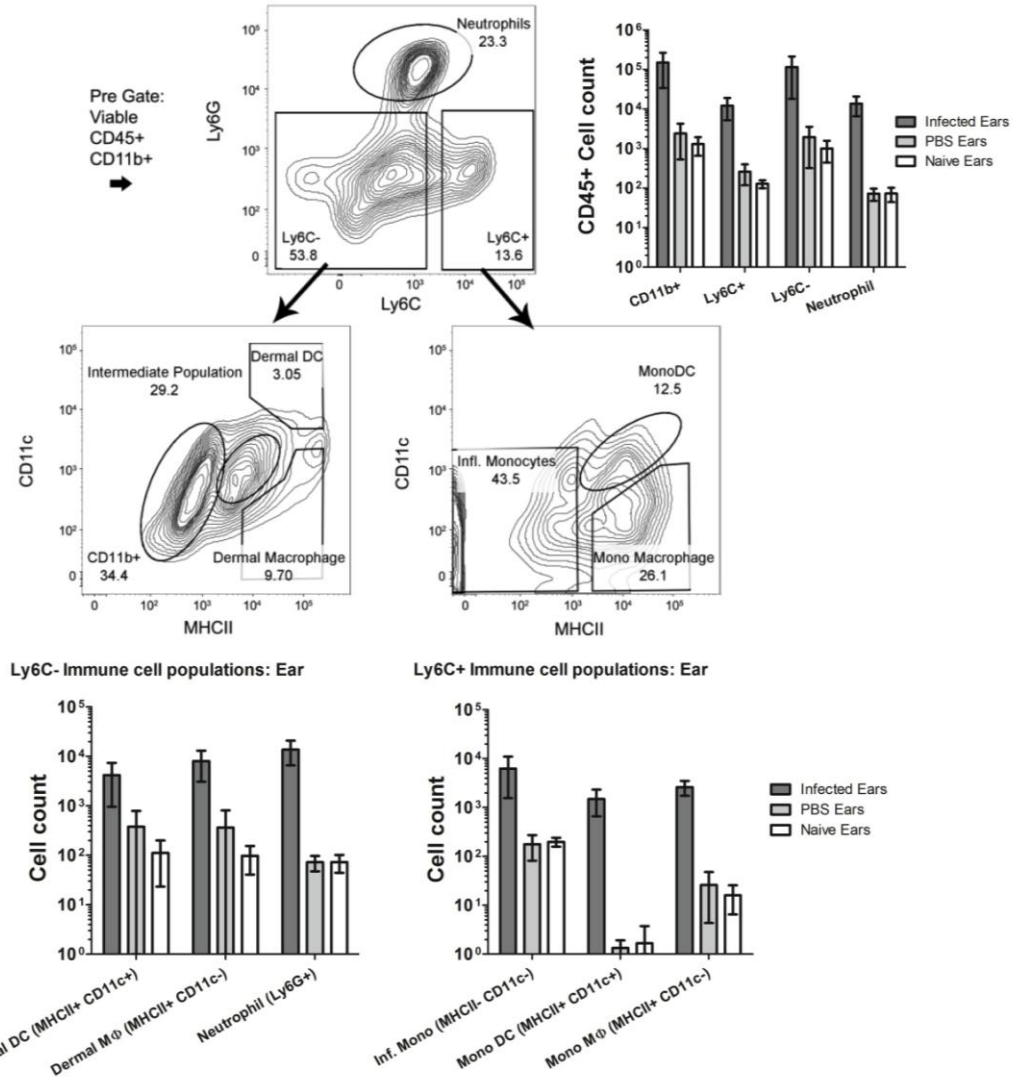


Figure 4:12- Immune cell populations at the infection site 3 months post infection. Ear derived dermal cells extracted from infected, PBS inoculated and naïve ears were analysed by flow cytometry. Representative gating on mouse 3 shown. Populations were pre-gated as single, viability stain negative, CD45+, CD11b+. Gating against Ly6C and Ly6G expression (top) allows quantification of a distinct neutrophil population as Ly6C+/Ly6G+, Ly6G-/Ly6C+ infiltrating monocytes (right) and Ly6G-/Ly6C- (left) skin resident populations. Quantification of dermal Dendritic cell (DC) and dermal macrophage (MΦ) populations by MHCII and CD11c expression (left, middle and lower). Quantification of inflammatory monocyte (Inf. Mono), monocyte derived DC (mono DC) and monocyte derived MΦ populations by MHCII and CD11c expression (right, middle and lower). N=3, error shown as standard deviation around the mean.

CD11b+ innate immune cell recruitment to ears infected with *L. mexicana* were around 100 fold higher relative to PBS and naïve treatments by three months post infection (**Figure** 4:12 upper panel), with a corresponding increase in the number of neutrophils and infiltrating Ly6C+ monocytes. The observable increase in ear vascularisation and lesion development at this time point was indicative of an increased recruitment of CD11b+ cells, such as neutrophils to the infection site. An equal number of neutrophils were recruited to ears of C57BL/6 infected as compared with a published study examining neutrophil recruitment to *L.*

major infected ears after only 14 days (Ribeiro-Gomes et al. 2012), suggesting an impaired level of recruitment for disease resolution.

A trend towards an increased Ly6C+/Ly6G- infiltrating monocyte population in infected ears was observed in this study, with the most pronounced increase in cell numbers relative to non-infected controls being the moDC population (**Figure** 4:12 bottom right panel). Previous experimentation identified the impaired recruitment of mo-DC at 14 days post infection C57BL/6 mice infected with *L. mexicana* compared to *L. major*, with around $7.5x10^3$ cells being identified (Petritus et al. 2012). The identification of an average $1.49x10^3$ moDC quantified at 3 months post infection demonstrated the low levels of recruitment at this chronic stage of infection. These data establish a basis for investigating the recruitment of monocytes and in particular moDC to the infection site. The use of parasites expressing RE9H allows a comparison between parasite burden at the infection site with immune cell recruitment following gene deletion of a potential drug target.

Migration of antigen presenting cells to the draining lymph node to induce the differentiation of naïve T Cells to Th1 effector cells is a crucial stage in the development of an appropriate immune response to *Leishmania* infection (Carvalho et al. 2012). Migration of moDC to the dLN is impaired in C57BL/6 infected with *L. mexicana* (Petritus et al. 2012; Hurrell et al. 2015a), therefore recovered migration of such cells following inducible deletion of an essential or virulence factor in *L. mexicana* represents a functional confirmation of the recovery of an appropriate immune response. The use of an 'innate' antibody strategy to investigate the recruitment of mo-DC in the draining lymph node was established for future use of C57BL/6 mice expressing the Kaede photo-switchable protein (Tomura et al. 2008). This method would enable cells from the infection site to be photo-switched, thereby inducing the expression of green fluorescence to red fluorescence in order to track dissemination from the infection site to the draining lymph node.

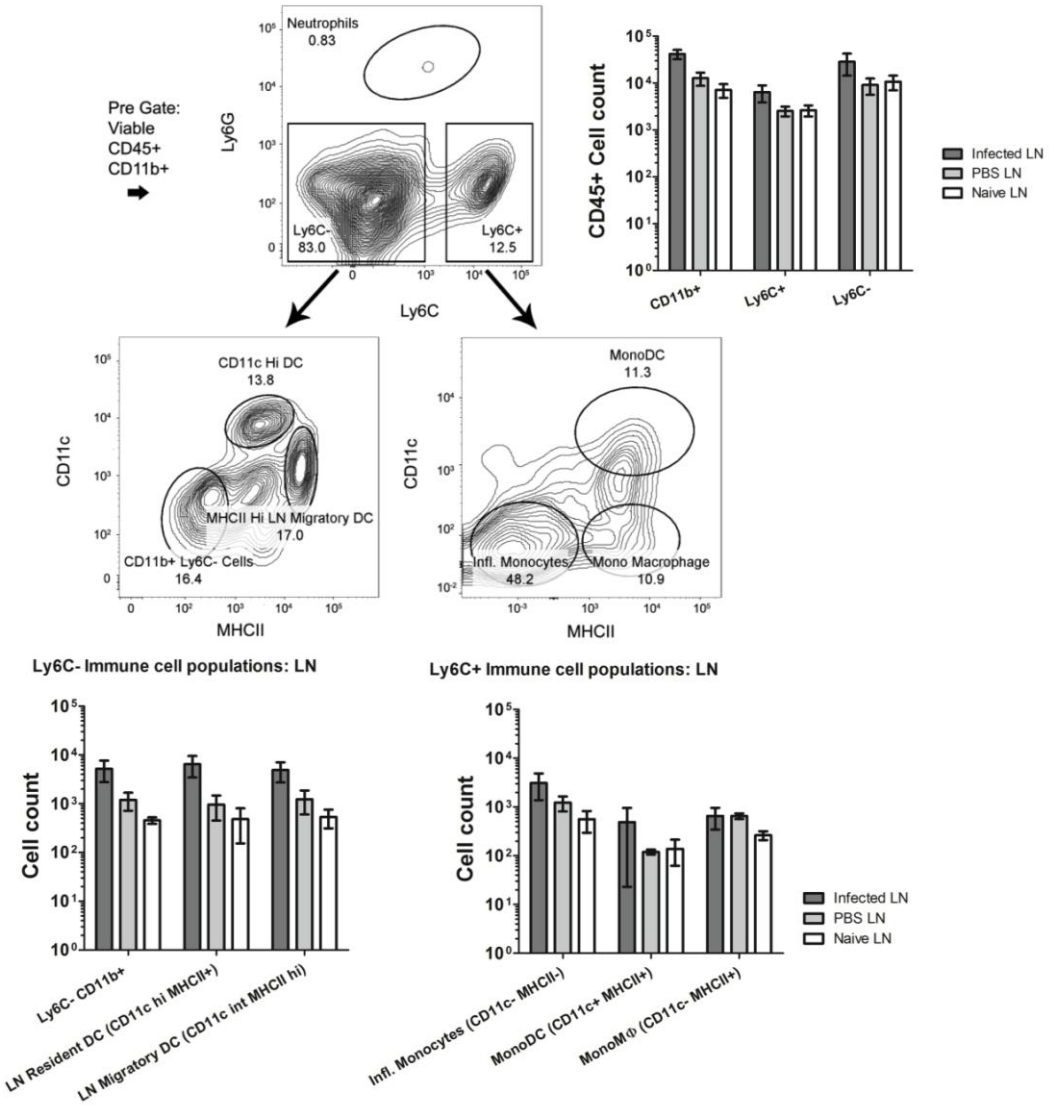


Figure 4:13- Immune cell populations at the cervical lymph node 3 months post infection. Cervical lymph node derived cells draining from the ear of infected, PBS inoculated and naïve mice were extracted and analysed by flow cytometry. Representative gating on mouse 3 shown. Populations were pre-gated as single, viability stain negative, CD45+, CD11b+. Gating against Ly6C and Ly6G expression (top) allows quantification of a distinct neutrophil population as Ly6C+/Ly6G+, Ly6G-/Ly6C+ infiltrating monocytes (right) and Ly6G-/Ly6C- (left) lymph node populations. Quantification of lymph node resident dendritic cell (DC) and lymph node migratory DC populations by MHCII and CD11c expression (left, middle and bottom). Quantification of inflammatory monocyte (Infl. Monocytes), monocyte derived DC (mono DC) and monocyte derived MΦ populations by MHCII and CD11c expression (right, middle and bottom). N=3, error shown as standard deviation around the mean.

To establish a basis for future application of Kaede expressing mouse models and *in vivo* inducible gene deletion in *L. mexicana,* immune cells from in the cervical, dLN were extracted, stained with an 'innate' antibody screen and phenotyped by flow cytometry (Figure 4:13). Quantification of the number of innate cell at this site identified an overall 5 fold increase in the number of CD11b+ cells relative to non-infected LNs, with an increase in both Ly6C+

myeloid cell numbers and Ly6C- resident populations (Figure 4:13, upper panel). Further phenotypic analysis of the populations showed a trend towards increased moDC recruitment in infected mice, however this was highly variable. A general trend towards increased numbers of Ly6C- DC and MO populations was observed, indicative of the expansion of the lymph nodes relative to uninfected controls however the recruitment of Ly6C+ infiltrating monocytes remained comparatively low throughout (Figure 4:13, middle and lower panels).

4.3 Discussion

4.3.1 Establishment of CRK3 as a validated drug target

The data presented in this chapter establishes that the inducible excision of essential genes in *L. mexicana* is a viable method for conditional null mutant generation by application to conditional deletion of the gene encoding the essential cdc2-like protein kinase (*CRK3*). CRK3 was previously proposed to be essential for promastigote growth by the inability to generate a *null* mutant line (P Hassan et al. 2001), thereby forming the basis for future study into the potential of this protein kinase as a drug target. Essentiality alone cannot be taken as a proof that a protein represents a validated drug target (as reviewed in section 4.1.2), however the production of active, recombinant CRK3/Cyclin complexes with which to conduct activity assays with CDK inhibitor screens (Walker et al. 2011) and probe active site residues (Gomes et al. 2010) demonstrate CRK3 activity can be inhibited with small molecules inhibitors and mutation of key residues. A limitation to such an approach is the lack of correlation between active protein kinase complex inhibition *in vitro* and *in situ* parasite cytotoxicity, as evidenced by previous screens of CRK3 inhibitors (Grant et al. 2004). A recent plasmid shuffle based methodology (Dacher et al. 2014; Morales et al. 2010) enables the generation of 'partial null mutants' with which activity can be probed directly in promastigotes (discussed in chapter 1.2), however this method not been applied to CRK3. Instead, the method of conditional complementation with a *CRK3*^{T178E} mutant has confirmed this as a key residue for mediating CRK3 function in *L. mexicana* promastigotes directly, implicating the inhibition of CRK3 activity *in situ* by chemical blocking of this active site. Our previous inability to regulate expression of CRK3 *in vivo* was a major limitation in establishing whether CRK3 activity was essential for

mediating the cell cycle of amastigotes. By deletion in stationary phase cells and subsequent infection, the attenuation of *CRK3* deficient *L. mexicana in vivo* indicates the protein kinase as essential for amastigote replication (see section 4.3.3 for further discussion).

In order to confirm that the cell cycle arrest phenotype demonstrated in promastigotes was specifically due to loss of *CRK3* rather than an artefact of rapamycin treatment or overexpression from the circularised fragment, a complementation was carried out through overexpression of $CRK3^{His}$ from the ribosomal locus. These cells no longer arrest once deletion is induced with rapamycin thereby confirming the specificity of gene deletion, however overexpression gives rise to cells with increased DNA content (**Figure** 4:4b) and a comparatively slow growth rate. In addition to cyclin binding and T178 phosphorylation, *S. pombe* cdc2 mutant functional rescue by *L. mexicana* CRK3 confirms dephosphroylation of T14/15A conserved residues is necessary for activity (Wang, 1998). Phosphorylation of T14/15A residues is thought to occur through negative regulation by wee1 kinase and positive regulation by a CDC25 phosphatase homologue (Gomes, 2010) therefore an overall abundance of CRK3 may impair the normal interactions with these regulators, resulting in uncoordinated mitosis thereby slowing growth. Previous study has shown that cells lacking endogenous CRK3 but complemented with CRK3 expression from a pTEX vector retain a normal cell cycle profile (Hassan, 2001) therefore our phenotype is likely an artefact of ribosomal overexpression. To address this we are currently developing vectors to facilitate integration into the beta-tubulin locus, thereby conferring lower expression of our complemented gene. This methodology represents an excellent method to identify and probe active sites within a protein.

Taken together, these findings establish CRK3 as a validated drug target in *L. mexicana* and demonstrates that the use of diCre will enable novel insights into essential gene function.

4.3.2 CRK3 is a mitotic regulator in promastigotes

Conditional gene deletion of floxed CRK3 results in growth arrest after 48 hours in *L. mexicana* promastigotes. Active CRK3 has been previously implicated in the

regulation of mitosis in *L. mexicana* promastigotes, with DNA content analysis following treatment with the CDK inhibitor flavopiridol indicative of cell cycle arrest in the G2 phase of the cell cycle (P Hassan et al. 2001). The conclusion that CRK3 mediates only the G2/M phase was deemed unsuitable, as absence of a block at a different cell cycle boundary such as G1/S could be explained by a differential inhibition of CRK3 in complex with a G1/S specific cyclin. Inducible deletion of floxed *CRK3* results in a block at the G2/M phase of the cell cycle (**Figure** 4:4b and c), thereby confirming the previous finding that active CRK3 in the promastigote stage controls the G2/M transition. Loss of *CRK3* results in the accumulation of enlarged, multi-flagellated cells (**Figure** 4:5 middle panels). This phenotype is indicative of an impaired mitotic regulation, whereby cells continue to synthesise organelles such as the flagellum and continue kinetoplast duplication in the absence of cytokinesis. In addition to continued protein synthesis, the increased nuclear DNA content of these cells is indicative of impaired mitosis, where re-initiation of G1 in the absence of cytokinesis results in the accumulation of nuclear DNA. It appears that these abnormal cells can eventually undergo cytokinesis by the observation that of one such daughter cells becoming 0N1K yet retaining two flagella (see bi-flagellated zoid in the lower panel of **Figure** 4:5). Such an interesting phenotype demonstrates the severe impairment in cell cycle regulation that loss of CRK3 induces, thereby confirming previous data that CRK3 was inhibited by flavopiridol treatment of promastigotes (P Hassan et al. 2001) leading to arrest at G2/M. RNAi of the syntenic orthologue of CYC6 in *T. brucei* results in growth arrest within 48 hours of induction and the accumulation of zoids and cells in G2/M in procyclic forms (Hammarton et al. 2003). This analogous phenotype is shown in our study through *CRK3* inducible excision, therefore it is likely that LmxCRK3 pairs with CYC6 to mediate mitosis (Walker et al. 2011).

The accumulation of cells with an increased DNA content and anucleated zoids by *CRK3* inducible deletion were previously observed following flavopiridol (Hassan et al. 2001) and indirubin treatment of *L. mexicana* promastigotes (Grant et al. 2004). The increased abundance of such aberrant cells likely results from the impairment of normal cell division, whereby loss of CRK activity results in an unequal sharing of nuclear DNA between daughter cells resulting in a single 2N1K cell and a 0N1K zoid. RNAi knockdown of the syntenic ortholog of *CRK3* in

Trypanosoma brucei procyclic forms also results in G2/M arrest and zoid formation (Tu & Wang 2004) with the accumulation of such aberrant cells rationalised by the lack of a checkpoint controlling mitosis to cytokinesis in the procyclic form (Ploubidou et al. 1999). The accumulation of zoids by CRK3 deletion and the same phenotype by indurubin treatment of promastigotes demonstrates the absence of a similar checkpoint for regulating premature cytokinesis in the absence of mitosis and implicates CRK3 as the target for indurubin inhibition. Yet the observation that indurubin treatment also results in the development of elongated cells (Grant et al. 2004) was not evidenced by *CRK3* deletion, thereby indicating that indurubin is also likely to inhibit other kinases as evidenced by its role in glycogen synthase kinase 3 (GSK3) inhibition (Leclerc et al. 2001).

4.3.3 CRK3 is essential for amastigote growth *in vivo*

In amastigotes, the effect of floxed *CRK3* deletion could not be dissected from the inhibitory effect that rapamycin has on the growth of *L. mexicana* amastigotes (**Figure** 4:8). CRK3 activity is essential for replication of amastigote *in vivo* as determined by their attenuation by stationary phase, floxed *CRK3* deletion and subsequent murine infection, but the utilisation of conditional gene deletion represents an important tool for dissecting the activity of a protein between life cycle stages. Previous RNAi of TbrCRK3 resulted in disparate phenotypes depending on transcript down-regulated in either bloodstream or procyclic forms (Tu & Wang 2004). A differential role for CRK3 in mediation of the cell cycle by the formation of an alternative protein kinase complex, such as CRK3:CYCA with an alternative regulatory function is therefore possible. Protein expression assays of the CYCA homologue *L. donovani* Cyc1 demonstrates an increased abundance during S-phase (Banerjee et al. 2006). In addition, histone phosphorylation by an active LdCYC1:CRK3 complex (Maity et al. 2011) are suggestive of S-phase kinase activity. Our initial observation of a G1/S cell cycle arrest in amastigotes by treatment with rapamycin (**Figure** 4:8) implicated the differential regulation by a CRK3:CYCA mediated protein kinase activity. However, this block in G1/S is likely due to rapamycin mediated inhibition instead. Despite the technical issues diCre conditional deletion represents an important method to dissect the function of an essential gene in amastigotes.

The process by which the conditional *null* phenotype can be assessed therefore requires optimisation to allow sufficient gene deletion in the absence of rapamycin mediated cell arrest. In place of a constant incubation of amastigotes in the presence of rapamycin, a possible improvement may be to treat the lesion derived cells for 24 hours with a sufficient dose to activate diCre mediated recombination. By removal of the rapamycin from the culture medium after this incubation period, the cells should recover their normal cell cycle progression. An additional improvement would be the use of rapalogs which may have reduced binding specificity to *L. mexicana* mTOR or FKBP12 homologues, thereby inducing diCre activity in the absence of inhibitory effects on cellular regulation. CRK3 protein kinase activity is dependent on its pairing with cognate, partner cyclins, therefore conditional deletion of CRK3 could potentially result in the accumulation of cells arrest in a stage other than G2/M if a different partner cyclin is necessary for activity. The dissection of such pathways has important implications for dissecting the role of other protein kinases in different life cycle stages.

4.3.4 Inducible mutant transgene complementation: a robust method for identifying active sites

Inducible complementation of an essential, but mutated transgene to recover activity and growth was developed to establish the essentiality of phosphorylation of the CRK3 T-loop residue. T178E mutagenesis of Lmx*CRK3* inhibits functional rescue in *S. pombe* (Wang et al. 1998) and ablates kinase activity with recombinant CRK3^{T178E}:CYCA (Gomes et al. 2010). The activity of CRK3^{T178E} has not been studied directly in *Leishmania* due to experimental constraints, therefore we utilised a conditional complementation approach to investigate the necessity of the phosphorylation of the T178 residue in *L. mexicana* promastigotes (**Figure** 4:6). The residue was identified as essential by excision of floxed *CRK3* in the *CRK3*^{T178E} complementation line, leading to cell cycle arrest in G2/M and zoid formation. Cell cycle arrest at 72 hours is later than the 48 hour arrest observed by conditional deletion of floxed CRK3 in the parental flox line, with a lower proportion of zoids when analysed by flow cytometry at 72 and 96 hours post induction. This result is likely attributed to the reduced growth rate of the complemented line relative to the parental flox

line, thereby leading to a delay in the onset of the arrest phenotype by slower cell division and protein turnover.

Interestingly with this cell line both induced and uninduced cells have dramatically reduced flagella length and are immotile (Figure 4:7f). This phenotype is likely due to a partial dominant negative effect due to overexpression of CRK3^{T178E}, resulting in impaired cell function, but maintenance of the cell cycle due to sufficient floxed *CRK3* expression and activity. The reduced flagellar size and growth are phenotypes mirrored by the generation of *L. major* deficient in *ATG5*, a gene encoding a key component of the autophagic pathway (Williams, 2012). The reduced size and growth of these cells is likely a result of their impaired ability to salvage material through the autophagic pathway, imparting selection on the parasites to reduce energy through flagellum regression. The partial dominant negative effect of CRK3^{T178E} may also result in metabolic stress in these cells, therefore inducing a compensatory response by flagellar loss. Another explanation may be the involvement of mitogen-activated protein (MAP) kinases, CDK-like kinases which respond to changes in environmental stress. Reduced cell size and flagellum length is a result of gene deletion of *LmxMPK3* and *LmxMKK* in promastigotes (Erdmann, 2006) (Wiese, 2003), however the involvement of this signalling cascade is unlikely. The G2/M arrest phenotype of this cell line resulting from floxed *CRK3* loss confirms the essentiality of T-loop residue phosphorylation to facilitate cyclin binding for the formation of an active complex to subsequently regulate progression through mitosis. These data establish this complementation assay as a rational approach for active site investigation.

4.3.5 Technical considerations for conditional deletion of essential genes by diCre mediated recombination

The generation of a Δ*crk3*::*DICRE*/Δ*crk3*::*CRK3*^{FLOX} cell line was aided by the production of gene replacement cassettes with an optimised Gateway cloning protocol (detailed in Figure 3:3). Gene replacement was also efficient, with the first round of gene replacement by the diCre construct yielded four blasticidin resistant clones out of six tested with diCre integrated into the *CRK3* locus. Subsequent replacement of the remaining *CRK3* allele by the floxed *CRK3* expression construct yielding four clones out of ten puromycin/blasticidin

resistant lines with both alleles replaced. Such amenable cell line generation holds promise for the utilisation of diCre as a method to regulate expression of a variety of essential genes, however the approach to delete the first gene copy by diCre replacement may not be advisable for the replacement of genes where replacement of a single copy results in altered ploidy (Martínez-Calvillo et al. 2005). In this circumstance, replacement of the first allele by loxP would be a preferable strategy to avoid such gene copy variation by initial 'add back' of the gene, followed by subsequent replacement with diCre. Even this approach may be limited for certain genes such as that encoding the *Leishmania* metacaspase (MCA), where gene expression is tightly regulated and deletion of a single allele leads to altered ploidy even in the presence of an integrated transgene (Ambit et al. 2008). Despite such potential complications, the efficacy of the diCre inducible deletion strategy for targeting essential genes is evidenced by the data presented in this chapter. In conjunction, the application of the system to induce deletion of a gene encoding an essential protein for cell cycle progression in *L. major* by our collaborator corroborates this (Damasceno, unpublished data).

Floxed *CRK3* is consistently excised by 24 hours post rapamycin treatment (**Figure** 4:3; **Figure** 4:8; **Figure** 4:10). Despite repeated attempts to detect loss of CRK3 by GFP tagging, protein extraction and SDS-page resolution and subsequent immunoblotting with an anti-GFP antibody, no detection of the CRK3-GFP fusion protein was obtained. Conditional complementation of *CRK3* as a transgene integrated at the 18S ribosomal locus yielded clones which grow at a reduced rate, but which were insensitive to loss of floxed *CRK3* (**Figure** 4:6). This experiment infers that growth arrest resulted from loss of CRK3 expression despite an absence of Western blot confirmation. In addition, diCre mediated inducible gene deletion of a protein essential for cell cycle progression by our collaborator (Damasceno, unpublished data) results in the loss of the target protein as detected by immunoblotting with an antibody specific to the target protein. We can therefore infer that loss of floxed *CRK3* results in loss of CRK3 protein and the inactivity of the cells.

4.3.6 Evaluation of the use of diCre inducible deletion as a tool for phenotypic screening of drug targets *in vivo*

By deletion of floxed *CRK3* in stationary phase promastigotes, the activity of the encoded protein kinase has been demonstrated as essential to the maintenance of infection in the susceptible murine BALB/c model. This finding demonstrates that diCre mediated conditional deletion will have important application to the study of other such proteins which have been implicated as essential by gene deletion in promastigotes. Prior to this study, the identification of essential genes *in vivo* was by the generation of mutant lacking both endogenous copies of the gene by complementation with the transgene expressed from an episome. The retention of the transgene in the absence of antibiotic selection following infection into a murine host (Wang et al. 2005) is the criteria for essentiality. There may be limitations to this approach, such as a more subtle selective advantage by expression of the protein as opposed to an essential function. To address this, expansion of this method to induce negative selection of such an episome by expressing the gene in array with a toxic thymidine kinase and *in vivo* ganciclocvir treatment (Paape, manuscript in preparation) further supports the requirement of the encoded protein for infectivity. Yet although this optimisation renders the method more robust, the inability to ablate the target gene and therefore dissect the resulting host immune response, or directly measure the parasite burden resulting from gene loss is a severe limitation.

In contrast, the application of this diCre technique can be applied to directly confirm essentiality in a more robust manner, with gene deletion directly resulting in impaired virulence and burden as measured by comparisons of *in vivo* bioluminescence relative to a non-induced control (**Figure** 4:10). A rapamycin treated, wild-type background [*SSU RE9H*] cell line established infection of mice following high dose rapamycin treatment, indicating that rapamycin treatment does not attenuate growth *in vivo*. The attenuated growth therefore results from deletion of the essential gene. More subtle side effects of *CRK3* deletion during the 24 hour treatment window which may alter aspects of cellular regulation influencing infection, such as metacyclogenesis were not explored. Such effects are unlikely however, as the proven insensitivity of promastigotes to rapamycin (Madeira da Silva et al. 2009), in conjunction with inducible deletion of *CRK3* inducing a growth arrest phenotype in replicative promastigotes after ~48 hours

of growth (**Figure** 4:2) substantiate this method as a practical strategy. The recovery of *in vivo* growth by week 10 post infection as measured by an increased bioluminescent signal from the footpads of mice infected with rapamycin treated, floxed *CRK3* line resulted from a subset of cells not undergoing conditional deletion. The necessity to amplify these populations by *in vitro* culture has the potential to enrich the population of cells retaining the gene, however the presence of these original cells in the cultures used for genomic DNA extraction would enable the detection of gene loss due to the sensitivity of PCR. In addition, the preferential amplification of the smaller, 'excised' fragment suggests that an absence of such an amplicon is an indication that none of the recovered cells retained floxed *CRK3*. In future, such experiments should utilise the axenic amastigotes media for enrichment of lesion-derived amastigotes to address this. Despite this consideration, this study demonstrates that attenuation of *L. mexicana in vivo* is a direct result of an absence of active CRK3, and by this method, the large body of evidence that this protein kinase represents a validated drug target has been confirmed by such experimentation.

4.3.7 A model for monitoring *L. mexicana* burden *in vivo* and phenotype the immune response to infection

The use of red-shifted luciferase expressing, bioluminescent reporter parasites represents a practical and powerful strategy to monitor parasite burden *in vivo* over the course in infection, and has already been applied to establish sensitive drug screening models for *T. brucei* (McLatchie et al. 2013) and *T. cruzi* (Lewis et al. 2014). The generation of LUC expressing *Leishmania* for measuring parasite burden by bioluminescent signal was first conducted in 2005 (Lang et al.), and has been applied for drug screening in murine models of cutaneous (Fortin et al. 2014) and visceral forms of the disease (Michel et al. 2011). However, the use of an RE9H based model for the study of *Leishmania* infection is preferable due to its enhanced tissue penetration and the subsequent increased detection sensitivity relative to LUC for the determination of parasite buden *in vivo* (**Figure** 4:11a). The establishment of such a model to measure parasite load by the proximate bioluminescent signal from the ears of C57BL/6 mice indicates that low dose infections are detected at early time points, before visible lesion development or vascularisation had occurred (**Figure** 4:11b).

However, the signal strength from infected ears generally increased throughout infection in only half of the infected mice, as the signal from half of the mice reduced to very low levels. This disparity may be due to variable doses of infectious parasites being inoculated into the ear pinna, as this infection route is technically difficult, however ear inoculation was chosen because it represents a practical site for downstream application of 2-photon microscopy.

By immune cell extraction and purification from ears (**Figure** 4:12) and draining, cervical lymph nodes (Figure 4:13) at three months post infection with the [*SSU RE9H*] reporter line, a methodology was established to quantify and analyse the recruitment of CD11b+ leukocytes at this chronic stage. The rationale for this model was the established experimental findings that CD11b+ cell recruitment to the infection site is higher in resolving *L. major* infections of C57BL/6 mice, with a subset of infiltrating, inflammatory monocyte derived cells being implicated in the development an appropriate Th1 CD4+ T cell response (Petritus et al. 2012). Phenotyping of a subset of monocytes which express the DC marker CD11c after *de novo* differentiation from inflammatory monocytes at the site of recruitment reveals an increased accumulation of monocyte-derived dendritic cells (mo-DC) (León et al. 2007) to the infection site of *L. major* mice relative to *L. mexicana* infected mice. These cells exhibit very low levels of iNOS production in the context of an *L. mexicana* infection, an antimicrobial response necessary for the clearance of *Leishmania* parasites (Alexander et al. 1998) and a further indication of the deficient immune response to *L. mexicana* relative to *L. major*. By quanitification of leukocyte populations in the ear during chronic infection, the levels of recruitment to a wild-type infection were established.

The migration of mo-DC to the draining lymph node for antigen presentation and CD4+ Th1 differentiation is also a key step in generating a Th1 response (Hurrell et al. 2015b). This migration is impaired however, as evidenced by adoptive transfer of monocytes into the infection site at 2 weeks post infection results in reduced dLN migration in *L. mexicana* infected mice relative to *L. major* (Petritus et al. 2012). The isolation and phenotyping of low levels of leukocytes in the draining lymph node (Figure 4:13) established a basis for the tracking of migration from infection site to dLN by Kaede (Tomura et al. 2008) reporter mouse infection and photo switching of the ear cells, or FITC painting. The

expansion of this model to include conditional gene deletion in *L. mexicana* will yield novel insights into the resolution of infection in the context of parasite burden and generation of an appropriate immune response.

4.3.8 *L. mexicana* conditional gene deletion for *In vivo* application: implications for multiple disease models

The initial strategy for the establishment of an *in vivo* model to measure parasite burden and immune cell recruitment was the application of *L. mexicana* conditional gene deletion by rapamycin treatment of chronically infected mice. This technique would allow the validation of the deleted gene as a 'druggable' target and phenotype the resulting immune response to identify the development of a resolving infection. By gene deletion of *CRK3* and monitoring of parasite burden *in vivo* we have established the protein kinase as essential for maintaining infection; however we are currently unaware of how deletion of *CRK3* influences the resulting immune response.

The persistence of a small population of parasites expressing CRK3 was able to drive subsequent re-infection in Balb/c mice after a period of 10 weeks; we may have hypothesised that the attenuation of the majority of cells would result in an appropriate Th1 response to prevent outgrowth of the remaining amastigotes retaining a copy of *CRK3*. The recovery of latent *L. major* infections in C57BL/6 infections has been demonstrated by the inhibition of appropriate iNOS mediated parasite killing (Stenger et al. 1996), therefore the recovery of parasite growth is likely a result of impaired immunity by the inherent disposition towards a Th2 mediated, humoral response in BALB/c mice (Rosas et al. 2005). BALB/c infection therefore represents an 'immuno-compromised' model of *Leishmania* infection. In the context of chronic infection and drug target validation, this would be an appropriate model for *in vivo* diCre activation and subsequent conditional gene deletion; loss of the gene would need to result in a pronounced effect on *in vivo* viability if the encoded factor is to be identified as a useful drug target. This is particularly important in the context of HIV co-infection, where immune compromised individuals would require an extremely efficient rate of drug mediated leishmania toxicity to prevent or treat the relapse of infection (Okwor & Uzonna 2013).

The treatment of infected mice with rapamycin or analogues of rapamycin (rapalogs) to induce gene deletion during *in vivo* infection would therefore be an excellent basis in the identification of genes encoding practical drug targets, particularly when coupled with the bioluminescent reporter parasites and the IVIS to monitor resulting parasite burden *in vivo*. The control of such experimentation would have to be stringent as a result of treatment with the immunoinhibitory compound rapamycin (Araki et al. 2011), as treatment may result in an altered immune response and influence the rate of attenuation. In addition, the bioavailability of rapamycin *in vivo* is poor (Rouf et al. 2009), therefore such a strategy may be experimentally challenging and is discussed further in chapter 6. The application of *in vivo* deletion to an essential *L. mexicana* gene during BALB/c infection is a powerful model to validate a drug target, but is not suitable for monitoring the outcome of appropriate immunity. With such considerations in mind, an appropriately controlled study represents a powerful tool to screen potential anti-leishmanial drug targets *in vivo*.

In contrast to BALB/c mice which are susceptible to infection with *L. major* and *L. mexicana*, C57BL/6 mice are only attenuated in a Th1 response by *L. mexicana* mediated immune modulation, therefore the attenuation of *L. mexicana* by gene deletion could potentially result in the induction of an appropriate immune response to target those remaining viable cells which retain the gene. In this respect, C57BL/6 infection represents an acute model to establish the outcome of gene deletion on infection resolution by an appropriate immune response. The application of the stationary phase conditional gene deletion and subsequent infection of C57BL/6 mice would enable the resulting immune response to be contextualised in regards to innate cell recruitment, and the subsequent development of adaptive and memory responses. The current antibody screen used to identify infiltrating moncoytes could be expanded to identify T helper cell differentiation and effector function. Additional components could be applied to the model depending on the biological outcome being tested, such as the use of photo-switchable Kaede mice (Tomura et al. 2008) to establish the effect of gene deletion on immune dissemination to the the draining lymph node. The application of this method would allow novel insights into the function of essential genes, the loss of which may induce an appropriate immune response early in infection. In addition, application to

encoded virulence factors would enable direct comparisons between the resulting immune response to cells expressing or lacking the target gene, thereby establishing the role of the encoded protein in mediating host-pathogen interactions.

The application of such multi-factorial studies would be important for establishing the resulting phenotype by inducible gene deletion in both the context of immune response, and by parasite burden to further validate the encoded parasite factors as essential for the maintenance of infection.

5 The role of repressor of differentiation protein kinase 1 in *Leishmania mexicana*

5.1 Introduction

5.1.1 *Leishmania* differentiation: from vector to host and back again

Differentiation between life cycle stages is an essential process for transmission and survival of *Leishmania*. To infect, survive and replicate between the contrasting environments of host and vector, *Leishmania* undergoes morphological and biochemical differentiation (Alexander et al. 1999). The transition to each of these stages must be tightly regulated in order for the parasite to complete its complex life cycle, with environmental changes such as pH variation and temperature shifts forming the basis for differentiation 'sensing' to occur. Such extracellular cues are received by specific receptors and transduced into an appropriate cell response by a signalling cascade to drive cellular differentiation, a chain of events which represent an essential process for maintenance of infection and life cycle transition. The mechanisms regulating this process are little understood, but signal transduction is likely mediated by post-transcriptional regulation by protein phosphatases and kinases, in particular the mitogen activated protein kinases (MAPK). These kinases are activated in response to environmental cues such as pH, temperature and nutritional stress in a signalling cascade which mediates transcriptional regulation and gene expression (Dacher et al. 2014). A body of evidence is establishing the role of MAPKs in regulating a number of cellular processes by phosphorylation of a range of substrates in the nucleus or cytosol (Kuhn & Wiese 2005). The relevance of these kinases in regulating differentiation is notable by their stage-specific activity; overexpression analysis of three such kinases (LmjPK4, 7 and 10) reveals an increase in kinase activity following exposure to 34°C and increased pH, mimicking the inoculation into the host and intracellular survival (Morales et al. 2007). A proteomics approach has further identified LmaMPK10 as an amastigote specific phosphoprotein (Morales et al. 2008). In addition, expression of the MAPK kinase homologue LmxPK4 is highest in promastigotes undergoing division and differentiation to amastigotes, with kinase activity highest at 40°C indicative of enhanced activity following

inoculation into the warm blooded host (Kuhn & Wiese 2005). The generation of an *LmaMPK4 null* mutant has been unobtainable (Wang et al. 2005), but a recent plasmid shuffle approach has identified key residues necessary for the protein kinase activity by a partial complementation approach and validated the essentiality of the protein in promastigotes (Dacher et al. 2014). The retention of the gene on an episome during murine infection also further demonstrates the requirement for active LmaPK4 in amastigotes, suggestive of a crucial role in regulation of differentiation at all life cycle stages. Stage specific activity has again been identified by gene knockout of *MPK1* in *L. mexicana* (Wiese 1998) to establish the necessity of LmxMPK1 protein kinase activity for amastigote proliferation and maintenance of infection.

Collectively, these studies are establishing MAP kinases as important transducers of extracellular cues in the context of disease maintenance, yet despite this expanding knowledge no studies have established a MAP kinase which directly regulates the differentiation of amastigotes to procyclic promastigotes. The premature entry of amastigote forms to avirulent procyclic promastigotes represents an important stage where stringent control must be exerted to prevent premature differentiation and maintain infection, but little is known about the chief regulators of this process. MAPK mediated signalling pathways are likely implicated in this, as exemplified by LmxMPK3 (Erdmann et al. 2006) and LmxMPK9 (Bengs et al. 2005). Both genes are expressed exclusively in dividing or differentiating promastigotes and can be efficiently deleted in promastigotes resulting in altered flagellar biosynthesis. The effect on flagellar development has implications regarding the regulation of amastigote to promastigote transition, yet this phenotype is not strong enough to exert a deleterious differentiation phenotype such as seen by *LmxMAPK1* deletion (Wiese 1998). This represents an important finding in preventing life cycle progression, with disruption of differentiation acting as a transmission blocker. An *LmxMAPK2 null* mutant has also been generated with amastigote attenuation and morphological disorders in the promastigote stage (Wiese 2007), however this phenotype is not as pronounced as the effect of TbMAPK2 depletion. Such powerful mediation of the cell cycle by a single protein is a desirable outcome in the study of regulatory factors, yet other than LmxMAPK1, no protein has yet been identified which can induce a more pronounced impairment on

differentiation between Leishmania life cycle stages. This may be in part the essentiality of many MAPKs (Morales et al. 2010) which prevents phenotypic analysis by the absence of a method to conditionally regulate essential gene expression in *Leishmania*. The diCre strategy discussed in Chapters 4 and 5 would lend itself to analysis of amastigote to promastigote differentiation, either *in vitro* with axenic amastigotes in culture or during *in vivo* infection. As discussed in Chapter 1.4, protein kinases are crucial regulators of cellular processes and represent important targets for drug inhibition, therefore the identification of protein kinases controlling differentiation during host infection would be of great interest both as drug targets.

5.1.2 A role for Repressor of differentiation kinase 1 (RDK1) in *L. mexicana* differentiation?

A body of knowledge is being generated on the role of signal transduction and regulation of differentiation in *T. brucei*. This is in part a result of the more amenable methods to manipulate this organism, particularly by RNA interference of target gene transcripts. Differentiation has been studied in depth in 'Stumpy' cells, the quiescent, transmissible stage of the parasite whereby differentiation is regulated by quorum sensing in a density dependent manner. TbMAPK5 has previously been identified as a regulator of stumpy differentiation, as BSF MAPK5 null mutants undergo stumpy generation prematurely both *in vitro* and *in vivo* resulting in impaired growth (Pfister et al. 2006). Interestingly, deletion of the *L. mexicana* homologue also impairs growth *in vivo* (Wiese 2007), yet the apparent lack of an established quiescent, 'stumpy-like' transmissible stage amastigote prevents direct comparison of such a phenotype in *Leishmania*. Application of RNAi to a genome wide screen has allowed RNAi target sequencing (Rit-seq) to induce protein knock down of *T. brucei* transfected with the genome fragment RNAi library (Mony et al. 2014). By this method, a number of protein kinases were identified and implicated in the quorum sensing pathway that results in stumpy differentiation. Of particular interest was the involvement of a never-in-mitosis A (NIMA) related kinase (NEK) in signal transduction. The role of NEK kinases in differentiation was expanded following a more concise, kinase-wide RNAi screen conducted by Jones et al. (2014). Knockdown of NEK 12.2 in bloodstream forms resulted in the induction of procyclic form differentiation, as identified by high levels of procyclin expression in the absence of heat shock and

the repositioning of the kinetoplast indicative of procyclic differentiation (Jones et al. 2014). In addition, this protein kinase was essential to the growth of the parasites, with RNAi resulting in a lethal phenotype. Due to its mediation of procyclic differentiation, this protein kinase was termed repressor of differentiation kinase 2 (RDK2). Interestingly, a related protein kinase may be expressed in *Leishmania* as evidenced by a gene with a high degree of DNA sequence identity (~63%) and genomic synteny to the LmxM.30.2960. Such a gene would represent an interesting target to conditionally regulate in *Leishmania*, as we may postulate that the loss of protein activity may induce a similar differentiation phenotype. To explore this, a *null* mutant could first be established by drug cassette replacement. RNAi of RDK2 results in a rapid lethal phenotype, therefore a shared function in *Leishmania* would be detrimental to the parasite. As such, conditional regulation by the diCre system represents a novel and robust approach to circumvent the current limitations on essential gene deletion (detailed in chapters 4 and 5). However the location of this gene on chromosome 30 obstructs knockout by either method, as this chromosome is associated with high copy number due to chromosomal duplication (Rogers et al. 2011). As such, at least four rounds of transfection and drug selection would be necessary to delete the gene. However, the Jones screen revealed another bloodstream to procyclic repressor of differentiation, termed RDK1. RNAi of this kinase induced procyclic differentiation in about 20% of cells with differentiation enriched to 38% by pretreatment of cells with 8-pCPT-cAMP and 27°C cold shock. An interesting property of this protein kinase is its sequence identity with STE11-like MAP kinase kinase kinases, which in conjunction with its localisation on the cell surface implicates RDK1 in initiating a MAPK signalling cascade following uptake by the Tsetse fly. As with RDK2, RDK1 has a syntenic orthologue in *L. mexicana* which represents an interesting target for gene deletion to investigate its role in differentiation. In conjunction, the gene encoding RDK1 (LmxM.31.0810) is likely diploid due to its location on chromosome 31 and was deemed amenable to deletion by replacement of drug selectable markers.

5.1.3 Utilising the diCre system to study LmxRDK1 function *in vivo*

We postulate that if LmxRDK1 is regulating differentiation with a similar mechanism to TbRDK1 that the active kinase would be involved in suppressing

the differentiation and early entry of mammalian infectious amastigotes to the sand fly infectious proliferative promastigotes. The induction of procyclic differentiation following RNAi of RDK1 in bloodstream form *T. brucei* at 37°C has important implications for the maintenance of infection, as this demonstrates the induction of inappropriate differentiation even in the absence of extracellular cues such as cold-shock or cis-aconitate treatment (Jones et al. 2014). In *Leishmania*, the procyclic promastigotes are non-infectious *in vivo* (Sacks & Perkins 1984) as they lack a thickened glycocalyx composed of elongated lipophosphoglycan (LPG) and the metalloprotease gp63 (Alexander et al. 1999), therefore RDK1 inhibition *in vivo* could result in down regulation of these virulence factors. The loss of virulence factor expression may enhance recognition and clearance of the parasites by the mammalian immune response. In conjunction, as metabolism is altered dramatically between amastigotes and promastigote life cycle stages (Kloehn et al. 2015) the induction of promastigote metabolism *in vivo* would be deleterious to parasite survival. TbRDK1 expression on the cell surface implicates it directly in the signalling of extracellular cues which are subsequently transduced by proteins such as the MAPKs in a signalling cascade. The increasing body of evidence for the regulation of life cycle differentiation by MAPK mediated signalling, in addition to the upstream signal transduction potential of RDK1 implicates a role as an important regulator of differentiation and therefore virulence in *L. mexicana*. These data suggest that RDK1 might play a potentially powerful role in regulating differentiation *in vivo*. By *in vivo* conditional deletion of *RDK1*, this hypothesis could be explored. The development of the diCre conditional gene deletion system represents a novel method to conduct gene deletion in axenic amasitogotes, or during the course of *in vitro* macrophage or murine infection. By *in vivo* application the infection dynamics of lesion size and immune cell recruitment following loss of RDK1 can be investigated.

5.1.4 Research aims

We aim to utilise the diCre system to conditionally regulate RDK1 expression during murine infection. Gene knockout by replacement of both *RDK1* copies with drug resistance cassettes will first be conducted to establish the essentiality of the gene in both life-cycle stages, followed by generation of a

conditional deletion line. The role of RDK1 in controlling the life cycle differentiation of *L. mexicana* will be investigated by:

1. Attempted generation of an *LmxRDK1 null* line.

2. Phenotype analysis of null mutants if generated, including murine infection with the *LmxRDK1 null* line to determine whether infection can be maintained *in vivo*.

3. If null mutants cannot be generated, the creation of a diCre mediated inducible knockout line with which to regulate *RDK1* expression.

4. Phenotype analysis of innate immune recruitment by ear infection of C57BL/6 mice following RDK1 inducible deletion to probe immune cell recruitment and disease maintenance *in vivo*.

5.2 Results

5.2.1 *In silico Trypanosome*/*Leishmania* RDK1 structural homology analysis

The coding sequences for TbRDK1 (Tb11.01.5650) and LmxRDK1 (LmxM.31.0810) were downloaded from TriTrypDB, translated to protein sequences and aligned to identify sequence conservation (Figure 5:1). Pfam and TMHMM prediction analysis revealed sequence identity between three transmembrane domains and a single protein kinase domain at the N and C-terminus of the protein respectively. These transmembrane domains in *T. brucei* RDK1 are functional as a myc-tagged RDK1 was detected in the membrane and cell surface by protein fractionation and IFA labelling (Jones et al. 2014). These conserved transmembrane domains suggest LmxRDK1 might be embedded in the cell membrane to transduce extracellular cues by a similar mechanism to TbRDK1.

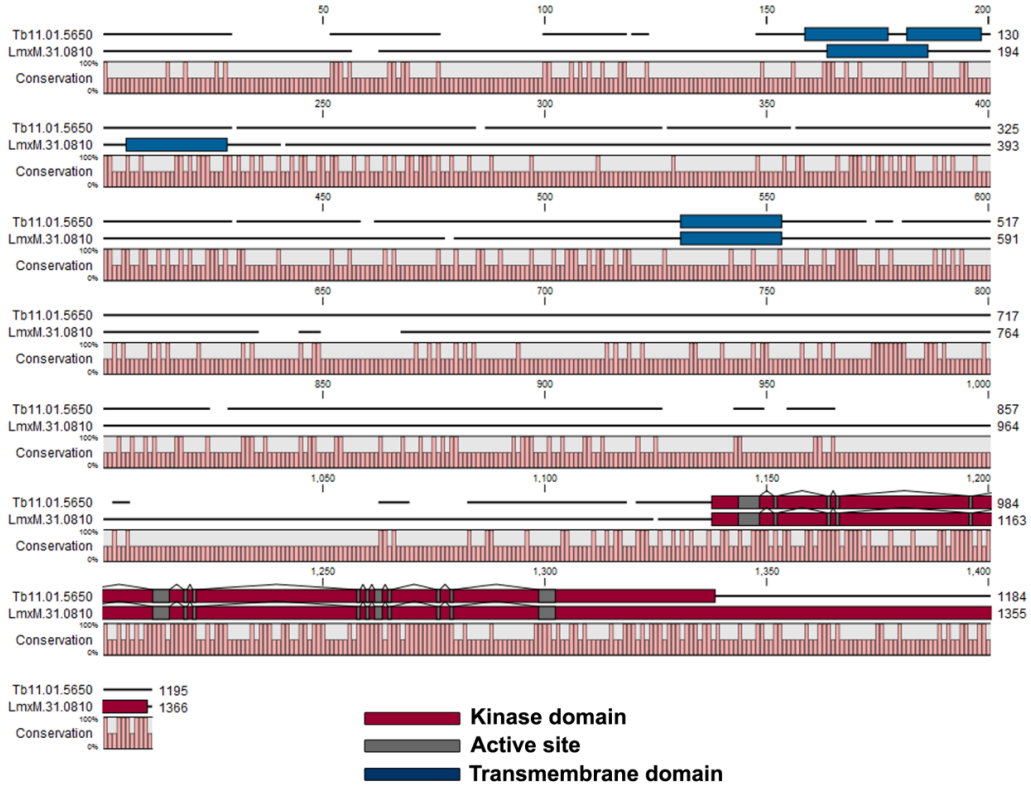


Figure 5:1- Schematic showing the alignment of *L. mexicana* and *T. brucei* RDK1 protein sequences. Black, horizontal lines indicate the region of the corresponding protein being mapped, with gene identification shown at left. Vertical, pink bars demonstrate sequence conservation between proteins. Highlighted domains were predicted by Pfam mapping and are annotated as protein kinase domains (red), active sites where substrate is either bound or phosphorylated (grey) and transmembrane domains (blue).

There are differences between the sequences, with RDK1 in *Leishmania* containing an additional 171 amino acids compared with *T. brucei* RDK1. The overall amino acid conservation between the sequences is around 28% and non-conserved extensions occur mainly at the N-terminal region and around 50 amino acids before the predicted kinase domain, thereby contributing to the low degree of conservation between sequences. However, analysis of the kinase domain reveals a relatively high 50% identity between the residues, suggestive of a conserved protein function at this region. In addition, the highly conserved mapping of active sites identified by the Pfam domain search implicates a shared protein kinase activity. Downstream cell signalling is likely to be a result of a RDK1 mediated phosphorylation of a MAP kinase signalling cascade, with conservation in this region suggestive of a homologous kinase activity towards MAPKs in *Leishmania*. Overall, the conservation of predicted transmembrane and catalytic domains implicates a role in differentiation of RDK1, therefore targeted replacement of the gene was conducted to establish the function of LmxRDK1.

5.2.2 Generation of an *RDK1 null* mutant

The 4.1kbp *RDK1* open reading frames were replaced by the integration of gene resistance cassettes. The strategy for attempted generation of an *L. mexicana RDK1* null mutant is shown in Figure 5:2. Constructs were created for the replacement of the *RDK1* gene with puromycin and hygromcyin drug resistance markers. PCR amplification of 500bp 5' and 3' flanks directly up and downstream of *RDK1* was conducted with primers containing appropriate attB sites to facilitate Gateway mediated insertion into pDONR vectors (explained in more detail in Chapter 3). Puromycin, blasticidin, nourseothricin and hygromycin drug resistance vectors were created by flanking the gene resistance cassettes with *RDK1* homologous arms by Gateway cloning. Only the puromycin and hygromcyin resistance constructs were used for subsequent transfection of wild-type *L. mexicana*.

Puromycin resistant clones were generated by transfection of the resistance cassette and replacement of *RDK1* confirmed by PCR amplification. Only amplification of clone 6 gDNA yielded a 1.13Kbp amplicon of expected size (Figure 5:2b upper), therefore this heterozygote for *RDK1* (Δ*rdk1*::*PACr*) was subsequently transfected with the hygromycin resistance cassette. A single clone (clone 4) was confirmed to have both *RDK1* alleles replaced by drug selection markers as evidenced by two 1.13Kbp amplicons by PCR amplification of genomic DNA with primers specific to the RDK1 locus and the resistance cassettes (Figure 5:2b lower). To confirm loss of *RDK1*, genomic DNA of clone 4 Δ*rdk1*::*PACr*/Δ*rdk1*::*HYGr* and clone 6 Δ*rdk1*::*PACr* gDNA was used for Southern blotting (Figure 5:2c & d.). By restriction digest and probing with a 3' *RDK1* flank, the lack of a 8.2Kbp fragment containing the *RDK1* coding sequence established clone 4 as an *RDK1 null* mutant (Δ*rdk1*).

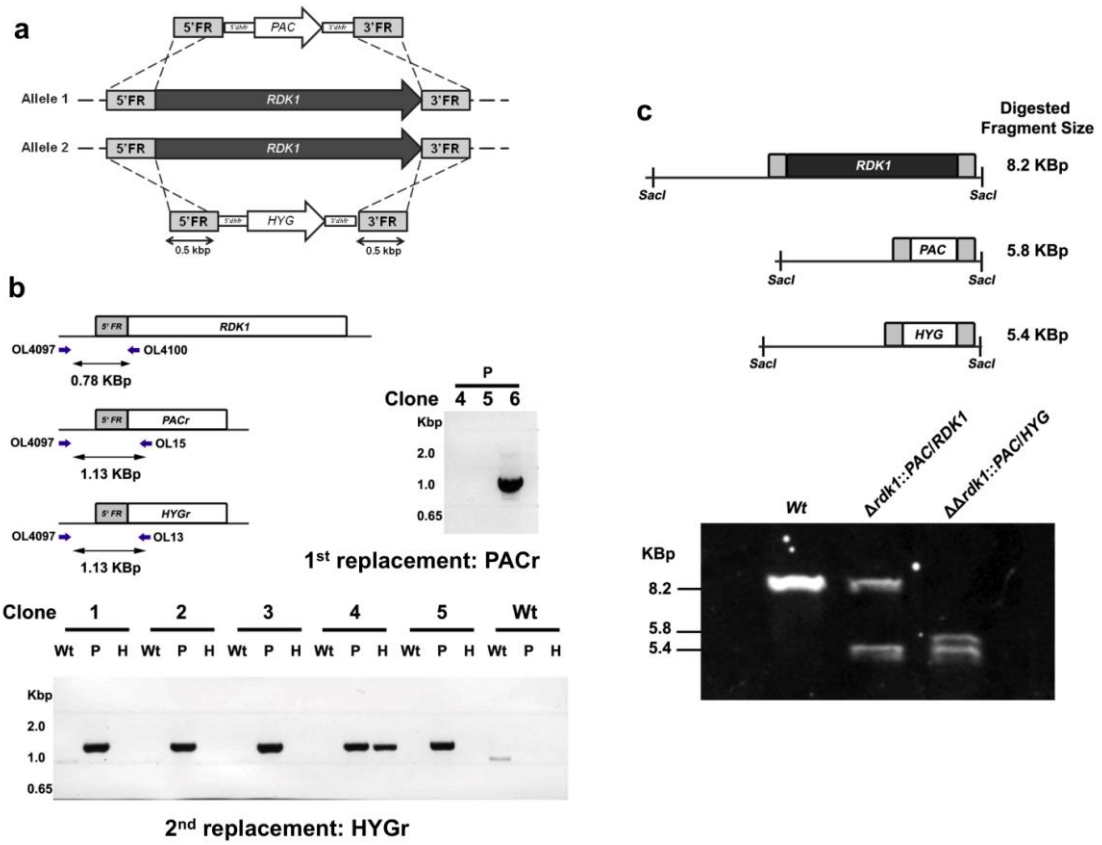


Figure 5:2- Replacement of *RDK1* with drug resistant cassettes. Panel **a.** The strategy for replacement of *RDK1* by homologous recombination of the puromycin (*PAC*) and hygromycin (*HYG*) resistance cassettes flanked 5' and 3' homologous flanking regions (FR). Panel **b.** Integration of drug resistance cassettes at the *RDK1* locus checked by PCR amplification. Genomic DNA was extracted from each of five puromycin and hygromycin resistant clones alongside a Wt control, followed by amplification with a forward primer binding upstream of the 5' flank and a primer binding within the *PAC* (P), *HYG* (H) or *RDK1* (Wt) coding sequences in the reverse orientation. A longer exposure of the gel reveals the presence of a Wt amplicon for all clones with the exception of clone 4. Panel **c.** (upper) depicts the strategy used for restriction enzyme digestion of genomic DNA by *SacI* to excise an 8.2KBp fragment if *RDK1* is retained. (lower) Genomic DNA was extracted from the Wt control, PAC integrated heterozygote and the subsequent PAC/HYG integrated clone 4. The DNA was digested by *SacI* and resolved on a 0.8% agarose gel, followed by transfer to a nitrocellulose membrane. The transferred, digested DNA was hybridised with a 500bp 3'FR chemiluminescent probe, allowing detection of each predicted DNA fragment (c.) by the development of X-Ray film following addition of a chemiluminescent substrate.

5.2.3 Murine infection by RDK1 deficient *L. mexicana*

□□□□□□□□□□□□□□□Δ*rdk1 in vitro,* was comparable to growth of wild-type *L. mexicana*. Neither Δ*rdk1*::*PACr* nor Δ*rdk1*::*PACr*/Δ*rdk1*::*HYGr* clones had aberrant morphology, with the only observable difference to wild-type being a slight elongation of the cells (data not shown). These observations were to be expected as the hypothesis was that RDK1 is a repressor of differentiation in the amastigote stage, therefore to test the hypothesis that RDK1 inactivity would result in the induction of differentiation and therefore attenuation *in vivo*, the

Δ*rdk1* line was inoculated into the footpads of Balb/c mice. Parasite burden was measured by footpad size over the course of 6 weeks, identifying the growth of footpad lesions at an equal rate to wild-type infection (**Figure 5:**3).

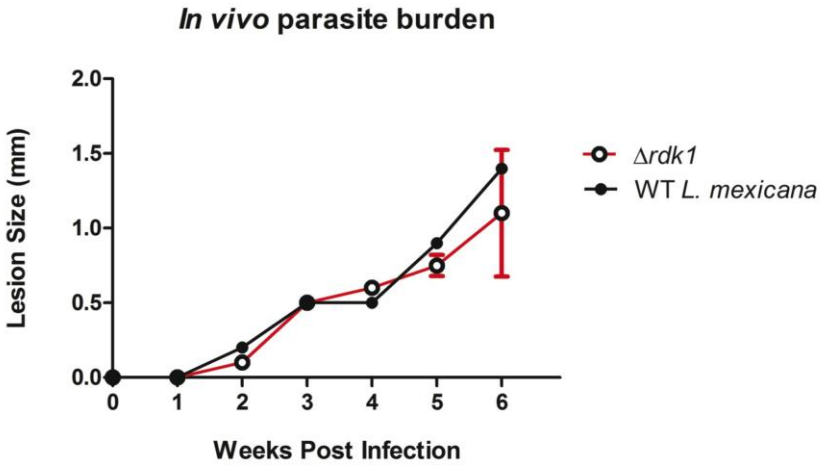


Figure 5:3- Infectivity of *Δrdk1* to mice. 2x10^6 stationary phase Δ*rdk1* stationary phase promastigotes were inoculated into the footpads of Balb/c mice and footpad size measured using a calliper (n=2, mean lesion size +/- S.E.). An *L. mexicana* wild-type control infection is included as a reference (n=1) and lesion size calculated by subtracting the measured footpad size from the initial size at week 1 post infection.

Viable Δ*rdk1* amastigotes were isolated from the footpad lesions. Purified amastigotes had a normal cell morphology and size as observed by light microscopy and were capable of differentiation to procyclic promastigotes within 24 hours of *in vitro* culture in supplemented HOMEM media, providing further evidence that cellular signalling and appropriate control of differentiation was in operation in this mutant.

5.3 Discussion

5.3.1 RDK1 is not essential for *L. mexicana* differentiation

We expected deletion of *LmxRDK1* to result in a phenotype akin to removal of an essential virulence factor, whereby growth and survival during murine infection would be attenuated. The generation of a Δ*rdk1 L. mexicana* cell line enabled the testing of this hypothesis, yet integration of puromycin and hygromycin resistance cassettes at this locus was infrequent as determined by PCR analysis (Figure 5:2b). Difficulties in gene replacement can indicate essentiality of a

gene (Ambit et al. 2008), however this was not the case as a single clone was isolated in which both copies of RDK1 were replaced (Figure 5:2b and c). The low frequency of gene replacement may therefore be a result of the large size of the 4Kbp gene. Despite the difficulties in generating this null line, RDK1 deletion yields viable promastigotes with an absence of altered ploidy to retain an extra copy of the gene, therefore it is not essential to growth and survival of promastigotes in culture, a trait shared by previously established virulence factor knockout lines (Wiese 1998, Buxbaum et al. 2003, Denise et al. 2003, Castanys-Muñoz et al. 2012). In contrast to the attenuated infectivity of these knockout mutants, RDK1 depletion yields *L. mexicana* which remain infective in Balb/c mice (**Figure 5**:3). There remains some minor experimental disparities between this study and those published examples; burden monitoring *in vivo* by lesion size was conducted for a longer time scale of between 8 and 70 weeks, and in the case of the Δ*cpb* trial (Buxbaum et al. 2003b) which ran for 18 weeks this longer time frame was crucial for identifying the subsequent lesion size reduction and reduced parasite burden manifested from 6 weeks post infection. Balb/c remain the most susceptible line to *L. mexicana* infection due to the polarised Th2 response, yet the susceptibility of this line may mask more subtle phenotypes such as impaired differentiation. The CPB study utilised C3H mice which are susceptible to *L. mexicana* infection as a result of an impaired Th1 response, therefore we could speculate that the infection of C3H or C57BL/6 lines with the Δ*rdk1* would result in an appropriate Th1 response as a result of a subtle impaired differentiation phenotype *in vivo*. Such a scenario is unlikely however, as the attenuation of growth would be expected in all cells due to the complete loss of RDK1, and in addition the growth of CPB deficient parasites in Balb/c mice is attenuated (Denise et al. 2003), further evidence for RDK1 not being necessary for virulence. A potential issue is the low number of mice inoculated in this study, however this was a preliminary experiment to establish whether further investigation by generation of a diCre conditional expression line was necessary. The equal rate of lesion development compared with the wild-type infected line in both infected mice, in conjunction with the extraction of viable amastigotes from footpad lesions leads to the conclusion that RDK1 does not function to block differentiation *in vivo*. These data lead to the conclusion that RDK1 does not regulate differentiation in *L. mexicana* in a similar manner to that of TbRDK1. The lack of a pronounced and discernible

differentiation phenotype following complete loss of RDK1, we did not generate an *L. mexicana* line where RDK1 expression could be conditionally regulated for further *in vivo* studies (see Research Aims 5.1.4).

5.3.2 Considerations for data mining of *T. brucei* RNAi screens

The use of genome-wide and more targeted RNAi approaches are generating a wealth of information about cell cycle control and differentiation in *T. brucei* (Subramaniam et al. 2006; Alsford et al. 2011; Jones et al. 2014; Mony et al. 2014). Aspects of *Leishmania* biology are less well understood due to the absence of broadly applicable strategies for regulating the expression of transcripts, yet the relatedness of kinetoplastids is such that 'hits' derived from *T. brucei* screens can be used to form testable hypotheses in *Leishmania*. We utilised such an approach in this study to investigate RDK1 as virulence factor with a role in repressing differentiation, based on the amino acid conservation and structural homology in both transmembrane and kinase domains (Figure 5:1). This study demonstrates that RDK1 does not function as a repressor of differentiation, yet there still holds promise for utilising such an approach for other kinases involved in differentiation such as the MAPKs.

A potential factor in explaining an absence of differentiation to promastigotes *in vivo* following RDK1 deletion are potential differences in the regulation of growth between *Leishmania* and *T. brucei*. The induction of stumpy arrested cells to facilitate transmission is crucial for life cycle progression in *T. brucei*, with the effect of RDK1 knock and procyclic differentiation taking effect in monomorphic 'stumpy-star' 2T1 cells (Jones et al. 2014), which are present at a population of around 20% in culture. Such growth arrest prepares the cells for procyclic differentiation once taken up by the Tsetse fly, however less is known about the development of such a quiescent stage of *Leishmania* amastigotes. Recent work (Kloehn et al. 2015) has established that the doubling rate of *L.mexicana* amastigotes in Balb/c mouse lesions takes around 12 days, with metabolism and protein synthesis during infection being dramatically reduced compared with axenic amastigotes or amastigotes infecting macrophages *in vitro*. The authors describe these lesion amastigotes as 'semi-quiescent', a necessary mechanism to cope with intracellular immune stresses such as iNOS and residence in an acidic phagolysosome. Amastigotes in a lesion may not

compose an entirely homogenous semi-quiescent population, as a small proportion of hyper-infected macrophage are present, suggestive of a more rapid growth rate and reduced quiescence. In contrast, we may speculate that a population of transmissible, stumpy-like amastigotes which are fully quiescent *in vivo* could be present, but these would likely be represented in very low numbers and are entirely hypothetical. TbRDK1 actively represses differentiation in quiescent stumpy cells, therefore an absence of an identified, fully quiescent stage in *L. mexicana* suggests an alternative mechanism to regulate amastigote to procyclic promastigote differentiation. The finding that RDK1 is not repressing differentiation in *L. mexicana* compared with *T. brucei* is analogous to the finding that MAPK5 induces procyclic arrest in *T. brucei* (Müller et al. 2002) but not promastigote arrest in *L. mexicana* (Wiese 2007). The underlying biology controlling differentiation between these kinetoplastids may be sufficiently divergent that conservation of protein sequence is not conducive to a homologous function in life cycle regulation.

5.3.3 RDK2 and the remaining limitations of *Leishmania mexicana* genetic manipulation

RDK2 may represent a better target to evaluate in the control of *Leishmania* differentiation because RNAi of the transcript results in a more rapid and higher number of cells undergoing procyclic differentiation relative to RDK1 knock down (Jones et al. 2014). Contrary to RDK1 ablation, this implies that differentiation is not as dependent on a prior differentiation step to a quiescent, cell cycle arrested stage such as the ‘stumpy*’ and could instead regulate differentiation in the semi-quiescent lesion amastigotes. *Leishmania* and *Trypanosoma* RDK2 share 63% amino acid sequence identity and have almost exactly the same number of residues (442 to 441 respectively), which may be indicative of a shared function in the cells. RDK2 regulation therefore represents a potentially fruitful target in the context of studying the control of differentiation, however as addressed above the location of the encoded gene on a supernumary chromosome is a major hindrance in conducting gene deletion studies. A time consuming drug cassette replacement and selection approach would have to be utilised to ablate the predicted four copies of the gene by replacement with four separate resistance cassettes. Despite the potential of such an approach, this method is further complication by the observation that

RDK2 RNAi is toxic to procyclic *T.brucei*; if such a phenotype occurs following targeted gene replacement of *RDK2* in *Leishmania* a *null* mutant is unobtainable by gene replacement. Chapters 3 and 4 deal with the development of a method to regulate expression of such essential genes in *Leishmania* which would permit conditional excision of *RDK2*, however the reliance on replacement of target genes through transfection and homologous recombination by this method remains a severe time limitation for targeting high copy number genes. Cas9-CRISPR technologies has real potential for deletion of multiple copies of RDK2 by targeting all gene alleles simultaneously (Sternberg & Doudna 2015; Sollelis et al. 2015) and in combination with diCre methodology could allow replacement of endogenous *RDK2* with floxed versions to enable inducible gene deletion of *RDK2* and previously unobtainable genes such as LmxMPK4, 5 and 10, thereby elucidating their role in differentiation. This study into the role of LmxRDK1 serves as an example as to the complications in translating 'hits' obtained from the study of the more amenable *T. brucei* to *Leishmania sp*.

6 General Discussion

The search for novel anti-leishmanial drugs is of crucial importance to address the currently sub-optimal repertoire of existing medicines. An elegant method to identify and assess the role of proteins that are not necessary for the survival of the parasite is by gene replacement and the generation of *null* mutant procyclic promastigotes. This approach has been used to a great extent since it was developed in 1990 (Cruz & Beverley) to generate *null* mutant clones to phenotypically assess parasite biology resulting from gene loss. As some phenotypes manifest once promastigotes differentiate to amastigotes, *null* mutants that remain viable as promastigotes enable further studies to be carried out *in vitro* or *in vivo*. Infection assays with such mutants can identify the gene as essential for survival in the mammalian host (Wiese 1998) or as virulence factors such as LPG and ISP where gene loss results in attenuation of infection (Späth et al. 2003; Eschenlauer et al. 2009). Gene replacement also enables the identification of genes that are essential to promastigote viability whereby replacement of the target alleles can only occur by expression of a transgene integrated elsewhere in the genome or present as an episome. Additionally, the retention of an episome during *in vivo* infection can be interpreted as selective pressure for gene expression and therefore an essential role during the amastigote life cycle stage (Wiese 1998). The loss of essential genes prevents growth during *in vitro* culture, therefore current techniques for genetic manipulation of *Leishmania* are limited to the confirmation of essentiality by the survival of clones which retain the gene. Recent expansions of this method by plasmid shuffle analysis is being utilized to explore the role of such essential genes through the generation of partial null mutants, and also by enabling a conditional complementation approach to identify active sites encoded within a gene sequence. Despite this improvement, reverse genetics of *Leishmania* and the assessment of essential genes encoding proteins which represent druggable targets remain limited. A conditional method of gene deletion is required to probe this, therefore this study sought to address this by applying the diCre system to conditionally delete cdc-related kinase CRK3 in *Leishmania mexicana*, which classic gene knockout approaches suggested is essential (Paul Hassan et al. 2001). By utilizing the existing approach of gene replacement by electroporation with transgenes flanked by gene homology, both copies of the

gene were replaced by the diCre sequence and a regulatable, loxP flanked *CRK3* open reading frame. Loss of the gene is rapid and stringently regulated, resulting in the cessation of procyclic promastigote growth due to a cell cycle arrest at the G2/M transition. The application of this method to the therapeutically significant amastigote stage was feasible by conditional deletion in infectious, stationary phase promastigotes and subsequent murine infection. The impaired growth of CRK3 deficient amastigotes establishes the essentiality of CRK3 *in vivo* and builds on previous research to confirm that the protein kinase is a validated drug target (Grant et al. 1998; Paul Hassan et al. 2001; Gomes et al. 2010; Walker et al. 2011). The application of this approach to other essential genes in *Leishmania* such as the MAP kinases (Wiese 1998; Wang et al. 2005; Dacher et al. 2014) or NMT (Price et al. 2003) will yield important information about the function of the target gene, in addition to the assessment of its potential as a suitable drug target. There are considerations that must be addressed to maximize the utility of diCre mediated recombination in *Leishmania*.

6.1 Considerations for *in vivo* application of the diCre system in *Leishmania*

The approach utilized in this study was conditional loss of *CRK3* in stationary phase promastigotes, however to better assess a gene as a drug target an approach to ablate the gene during murine or *in vitro* macrophage infection is desirable. A challenge in developing this system for *in vivo* use will be obtaining sufficient levels of diCre activity in intracellular amastigotes. An effective route of rapamycin administration is therefore a necessity. A chief consideration is that rapamycin is a potent immunosuppressant, and has been approved for oral use as Rapamune to prevent graft rejection in humans for over 15 years (Fogel et al. 2015). As such, the extensive and complex effect rapamycin exerts on the immune response through mTORC1 inhibition has been studied in particular depth; mTORC1 activity regulates immune cell proliferation, the generation of memory or effector cells and modulation can result in unexpected effects such as increasing longevity (reviewed by Araki et al. 2011). In this respect, *in vivo* administration of rapamycin to mice infected with diCre inducible deletion *Leishmania* would have to be stringently controlled to ensure rapamycin treatment alone is not influencing survival. There are a number of methods for

administration of rapamycin; orally, by intraperitoneal injection or topical application.

Oral treatment is an undesirable method due to the systemic side-effects that can come as a result of dissemination of rapamycin (Wheless & Almoazen 2013). With regards to species of *Leishmania* where pathology results in cutaneous lesions, topical application of rapamycin would be a rational administration route to induce deletion (Garnier & Croft 2002). Clinical studies into the use of topical rapamycin treatment have been conducted to treat symptoms caused by the autosomal-dominant genetic disorder tuberous sclerosis (TS) in humans which results in the formation of benign tumours in multiple organs. TS patients frequently present with facial angiofibromas in the skin as a result of impaired regulation of mTOR activation in dermal fibroblasts, causing the release of an epidermal growth factor (epiregulin) which results in proliferation of epidermal cells and the formation of skin papules (Koenig et al. 2012). To down regulate epiregulin production by mTOR inhibition, rapamycin has been administered topically in formulation with a cosmetic barrier cream (Koenig et al. 2012), a hydrophilic ointment (Wheless & Almoazen 2013) and by direct application of oral rapamycin (Mutizwa et al. 2011). Such studies hold promise for the topical application of rapamycin to a cutaneous lesion to induce diCre activity *in vivo*, however optimisation of the extensive dosing regimen of once or twice daily over the course of months used in these studies would have to be evaluated in terms of deletion efficacy. A limitation of this procedure is the application of creams to mice; personal communication from colleagues applying drug compounds in ointment report that the mice often groom the areas applied with cream, and that application can lead to variability in dosage.

An alternative to ointment could be the application to a lesion using rapamycin in formulation with a penetration enhancer such as DMSO or oleic acid (Garnier & Croft 2002), in a volatile carrier such as ethanol to facilitate rapid evaporation of the solution from the lesion and prevent loss through grooming. Again, the concentration of rapamycin used and the dosing regiment would need to be sufficiently optimised for *in vivo* deletion to be conducted. A pilot study using this method to dose the lesion of a mouse infected with the Δ*crk3::DICRE/CRK3* [*SSU GFP* ^{Flox}] line by direct application of 20µl 2µg/ml rapamycin in 10% DMSO,

90% ethanol over three days did not induce deletion, suggesting a higher dose and longer treatment is necessary. An issue with topical delivery is the multiple dermal layers which require penetration by the compound (Garnier & Croft 2002), in conjunction with the necessity to enter the infected host phagocytes, cross the parasitophorous vacuole membrane and enter the amastigotes. In addition, transmigration of immune cells harbouring *Leishmania* to the draining lymph node results in the establishment of infection (Baldwin et al. 2004) away from the infection site, therefore rapamycin treatment of the lesion would not induce deletion of these parasites, acting as a reservoir for subsequent re-infection.

Intraperitoneal (IP) administration of rapamycin has been established in many mouse and rat models due to the variety of cellular functions regulated by mTORC1. More relevant to this study is the induction of diCre activity in mice (Jullien et al. 2007) and studies which utilise the FRB and FKBP12 domains to regulate gene expression by reconstituting an active transcription factor in the presence of the rapamycin ligand *in vivo* (Wang et al. 2004; Koh et al. 2006). For *in vivo* induction of diCre activity and deletion of species causing visceral leishmaniasis, IP administration would allow systemic dosage and induction of deletion in the cells harboured by phagocytes in the viscera. However, there are a number of pharmacokinetic properties which render rapamycin a poor compound to administer by this route; 95% of rapamycin is sequestered by erythrocytes thereby hindering bioavailability, and the poor solubility of the drug makes preparation of injectable formulations difficult (Rouf et al. 2009). A liposomal formulation of rapamycin has been developed which the authors present as a more amenable delivery system, and one which will not be sequestered so highly by erythrocytes (Rouf et al. 2009). This represents a potentially valuable method for diCre induction *in vivo* because in contrast to free rapamycin, uptake of liposomes occurs highly in mononuclear phagocytic system (MPS) cells (Kelly et al. 2011) which represent the main immune cell niche of *Leishmania*. This would facilitate enhanced uptake in the cells infected with *Leishmania*, and this natural process can be enhanced by the addition of targeting proteins to the surface of liposomes, such as mannosylation to enhance uptake by macrophage and DC. The lipid formulation medicine AmBisome is the most efficacious anti-leishmanial drug available for treatment of visceral

leishmaniasis, showing that the uptake of liposomal formulations into intracellular parasites is a feasible method of delivery. In addition to intraperitoneal administration, this method could be utilised to directly inject rapamycin liposomes into cutaneous lesions to induce diCre activity, ablating the need for topical application. This may represent and an advantageous method for delivery of rapamycin directly to macrophage and dendritic cells harbouring the parasites, thereby inducing gene deletion in intracellular amastigotes *in vivo*.

The study of genes which encode virulence factors which influence the immune response requires strict control to dissect the effect of gene deletion from the influence of rapamycin treatment. Three treatment groups would be sufficient for *in vivo* analysis; the diCre conditional line with or without rapamycin administration, and a diCre or loxP heterozygote line with rapamycin administration. By this method, the background influence of rapamycin alone can be identified and the resulting effect on immune response and parasite burden compared between induced and uninduced infections. To further develop this methodology, the use of rapamycin analogues (rapalogs) represents an improved ligand for *in vivo* conditional deletion induction as these compounds have been developed by modification of the chemical structure to prevent inhibition of mTOR and differential binding to FRB binding domain mutants (Bayle et al. 2006). The rapalog C-16-(S)-7-methylindolerapamycin (AP21967) inhibits mTOR mediated phosphorylation of p70 S6K at an IC50 of 10nM compared to 0.1nM for rapamycin (Edwards & Wandless 2007) with no inhibitory effect on proliferation of rat fibroblasts *in vitro*, whilst still able to induce diCre activity with an FRB T2098L mutant (Jullien et al. 2007). The use of this raplog is also efficacious during *in vitro* studies requiring a rapamycin ligand (Wang et al. 2004; Koh et al. 2006) and represents a preferable compound for inducing diCre *in vivo* with a reduced immunosuppressive activity. However, these studies switched to rapamycin for *in vivo* dosage due to the high cost and volume necessary for efficacious gene expression so no reference can be made to the efficacy of AP21967 *in vivo*. DiCre induction by this rapalog was not effective *in vivo* (Jullien et al. 2007), however the successful *in vivo* administering of AP21967 by IP to induce protein dimerization of proteins regulating endothelial junctions during leukocyte extravasations does not entirely rule out the use of

this compound for diCre induction *in vivo* (Broermann et al. 2011). In addition, the low dose of rapamycin necessary to induce deletion of *GFP* (**Figure** 3:5) suggests a diCre efficiency that will facilitate sufficient rates of rapalog mediated diCre induction *in vivo*. The use of this rapalog will need to be validated and optimised in the context of *Leishmania* infection, however it represents a preferable ligand to prevent experimental caveats as a result of immune modulation by rapamycin treatment. With these considerations, a desirable method for diCre induction and conditional deletion of *Leishmania* encoded essential or virulence factors *in vivo* would be IP administration of rapalog in liposomal formulations to target the compound to phagocytes, whilst reducing mTOR inhibition in those cells.

6.2 Alternative inducible gene deletion: double floxing

This study has demonstrated efficient floxed *GFP* and *CRK3* excision in both promastigotes and amastigotes, whereby expression of each diCre subunit from the *CRK3* locus is driven by the presence of actin and beta-tubulin regulatory elements. Integration at this locus enables the generation of a heterozygote cell line with further complementation with a loxP flanked *CRK3* copy (Figure 6:1a), however the generation of a 'parental' diCre expression line has been applied in *T. gondii* (Andenmatten et al. 2012). The benefit of this strategy is consistent expression of diCre, as opposed to gene replacement and subsequent quantification of protein expression for each gene targeted (which is made problematic due to a limited repertoire of low affinity, anti-FRB and anti-FKBP12 antibodies). The generation of such a parental line in *Leishmania* is desirable to establish a quantified level of diCre protein expression prior to gene targeting. Altered ploidy in response to replacement of a single gene copy has been evidenced when replacing essential genes (Ambit et al. 2008), therefore by the strategy used in this study conditional regulation would be impractical. Instead, transfection of the diCre parental line to replace both gene alleles by loxP flanked 'add-back' gene copies would prevent any altered gene ploidy in response to gene replacement by diCre (**Figure** 6:1b). The cell line would then be amenable to diCre mediated gene loss of both copies. An issue may arise by the recombination between loxP sites, however a number of heterozygote loxP mutants exist which can be used to preferentially mediate recombination between specific sites and therefore prevent cross-over from gene alleles. To

prevent this, the loxP sites could contain mutations within the 8bp central spacer region where active diCre generates a 6bp staggered cut, and sequence homology to the second loxP is essential to enable recombination and therefore gene excision (Langer et al. 2002). These mutations generate heterospecific loxP sites which confer specificity to the corresponding loxP site mutant integrated on the same allele, thereby preventing chromosomal cross-over. The heterospecific lox511 and lox2272 sites have been applied to recombinase-mediated exchange (RMCE) of gene cassettes in mouse embryonic stem cells (ES) (Araki et al. 2002). Such mutant lox sites could be exploited to apply double allele deletion in *L. mexicana* as an alternative strategy to regulate essential gene expression.

Some aspects of diCre mediated gene deletion may be problematic when applied to essential genes. Gene expression levels may be altered by replacement of endogenous genes with floxed gene copies flanked by exogenous splice and poly-adenylation sites. This is an important consideration as inappropriate gene expression may result in a toxic phenotype, particularly if the gene is subsequently overexpressed or expressed at an insufficient level. A similar difficulty has been seen previously by the application of tet-operon based systems to regulate protein kinase expression in *T. brucei*. Insufficient T7 promoter repression can result in gene silencing and a subsequent impaired growth and survival, whilst poor regulation of inducible expression can result in a toxic phenotype being manifest prior to induction. If the replacement of endogenous *Leishmania* genes with floxed copies leads to altered expression, deleterious effects on cellular proliferation may manifest and render further phenotypic analysis by conditional gene deletion impractical. In this respect, the desired strategy of gene replacement can be decided upon.

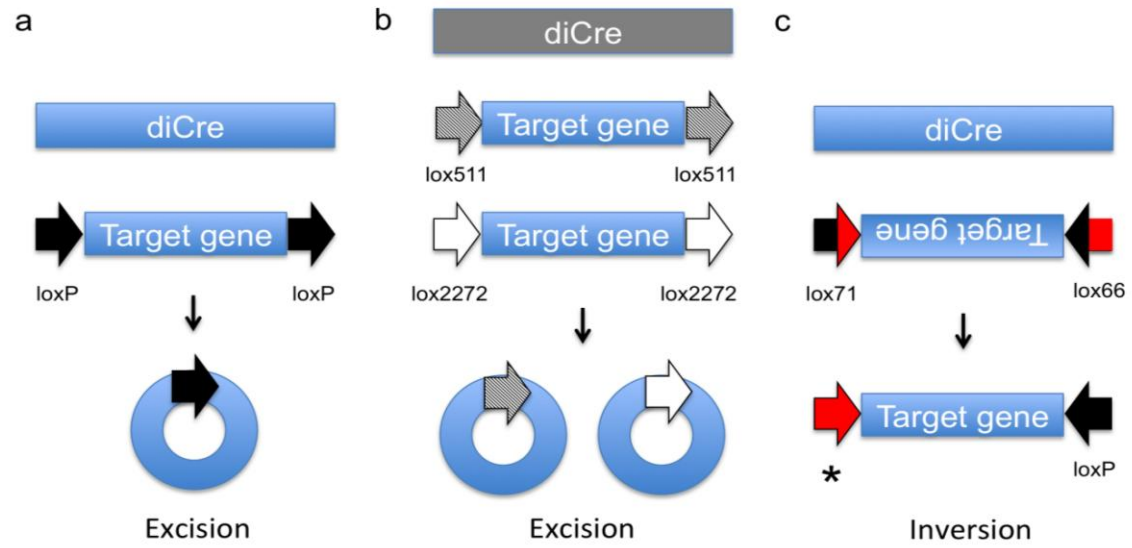


Figure 6:1- Strategies for diCre mediated gene regulation by mutant loxP orientation. A. The strategy used for inducible deletion requires replacement of a target gene by the diCre coding sequence (blue) and the target gene (blue) flanked by 'wild-type' loxP sequences (black arrows). Recombinase activity results in excision. B. Integration of the diCre coding sequence into an exogenous locus (grey) to confer expression and target gene replacement at each allele with distinct central spacer mutant lox sites (dashed or white arrows). Recombinase activity results in each gene being excised without cross over recombination. C. Inducible expression can be conferred by incorporating the diCre gene and an inverted target gene flanked by left and right element (LE/RE) mutated lox66 and 71 sequences (mixed black and red arrows). Recombinase activity results in the generation of a wild-type loxP site and a double mutant (*) with less affinity for Cre.

6.3 Expanding loxP site recombination: flip-flox

Inducible overexpression of a target gene would be a useful method to further explore the effects of the encoded protein on the physiology or virulence of *Leishmania*. The recent study by Kraeva et al. (2014) utilizes a T7-driven Tet-inducible system to accomplish this, however the flexibility of loxP mediated recombination opens up the possibility of achieving inducible gene expression by diCre mediation. Inducible expression requires an inverted gene of interest to be flanked with *cis* rather than *trans* orientated loxP sites (**Figure** 6:1c). In this orientation, there should be no read-through transcription of the gene and therefore no protein expression until diCre recombinase activity is induced by rapamycin treatment, whereupon the gene will 'flip' into a transcriptional 5'-3' orientation for expression. The application of method is complicated in *Leishmania* because of the evidence that transcription of both strands occurs (Belli et al. 2003). Therefore incorporation of this construct must be in a region where antisense transcription is silent to prevent protein expression in the absence of diCre activity. The identification of possible hairpin-loop forming transcription terminators around T1 and T2 sites in *L. infantum* (Abreu-Blanco et

al. 2010) suggests such sites could be utilized to prevent reverse read through transcription to silence the gene until 'flipped' at the ribosomal locus. In addition, integration of the gene into the ribosomal small subunit downstream of the PolI promoter would confer high levels of expression (Misslitz et al. 2000) to achieve inducible overexpression. Optimisation of a method to prevent reverse strand transcription by the addition of exogenous transcription terminators or by integration into a locus where the orientation of transcription is known would enable such gene invertion as a viable strategy in *Leishmania*.

The use of wild-type loxP sites would result in the gene constantly 'flipping' as Cre activity would continually catalyse recombination, however the use of left and right element (LE/RE) mutated lox sites such as lox66 and lox71 would prevent continual recombination; these have mutations in the inverted repeat regions flanking the loxP spacer where the Cre mediates a break in the DNA sequence (Albert et al. 1995). Despite not affecting Cre activity at the spacer, recombination between LE and RE mutants results in both a loxP site and a double mutant which has a dramatically reduced affinity for Cre. As such, the reaction can be skewed to undergo a single recombination event to prevent repeated inversion. This methodology opens up the possibility of inducible expression by diCre mediated recombination, and given the stringent regulation of diCre activity we may hypothesise that this would be more advantageous due to the 'leakiness' associated with tetracycline inducible systems in *T. brucei* (Barrett et al. 2004).

6.4 Applying diCre with existing molecular techinques

The flexibility of Cre:lox recombination potentiates its application to existing methods of *Leishmania* manipulation. A chief problem of such approaches is an absence of conditional regulation of activity, therefore the inversion strategy may be particularly desirable. The recent publication of tet-inducible gene expression in *L. mexicana* may also be applied in some cases, however the stringent control of activity by diCre may make it more desirable.

6.4.1 Active site analysis by conditional mutant complementation

The use of conditional complementation of floxed *CRK3* in this study has enabled the confirmation of the essentiality of T-loop phosphorylation for activity. This strategy is a useful method for probing active sites, but to enable a more high-throughput method to probe multiple coding sequence mutants by conditional complementation, a transposon based approach may be practical; this enables mutated coding sequences to be generated simultaneously by shuttle mutagenesis, enabling the cloning of multiple open reading frame (ORF) mutants for subsequent transfection into diCre:flox parental cells (Damasceno et al. 2010). Treatment with rapamycin will induce diCre activity to excise the floxed gene copy, and enable survival of clones expressing differentially mutated complementary ORFs (**Figure** 6:2 i). Dependent on the desired application, multiple cell lines could be generated and pooled to enable a high-throughput approach. In a simplistic example, survival or death within the population is the criteria for active or inactive complementation and therefore loss of cells bearing their respective mutant expresser could be identified by an approach similar to RIT-Seq. Such a conditional complementation (CC)-seq approach may be useful in determining multiple sites for chemical inhibition within a potential drug target. A similar approach to study multiple mutations in a gene sequence has been conducted by plasmid shuffle mediated conditional complementation (Dacher et al. 2014), however an advantage to the diCre approach is the potential to invert the mutant coding sequences and flank them with inverted LE/RE lox mutants to conduct inducible overexpression. This would enable dominant negative phenotypes to be assessed by transfection of plasmids into a wild-type expressing diCre cell line, and may represent a useful strategy to further probe the function of active sites.

6.4.2 RNAi

The current method for RNA interference in *Leishmania braziliensis* relies on the transfection of dsRNA or a stem-loop construct to initiate gene silencing. However, both approaches lack temporal regulation of the generation of dsRNA and therefore the siRNA generated by the RNAi pathway. A stem-loop inducible system is feasible with diCre, whereby one of the repeat sequences expressed to produce a hair-pin loop is flanked by inverted loxP sites and orientated in such a

way that no loop is produced (**Figure** 6:2 ii). Upon diCre activity, the sequence will invert to produce transcript that subsequently generates the stem loop, dsRNA and subsequently activates RNAi mediated degradation of transcript. The process of cloning such constructs may be made amenable by a PCR cloning based method such as Gibson Assembly, or by Gateway mediated cloning as used for high-throughput cloning of RNAi libraries for application to *T. brucei* (Jones et al. 2014). This system could potentially be applied for conditional RNAi in *L. braziliensis*, but first diCre expression and loxP mediated recombination would have to be developed in this species first.

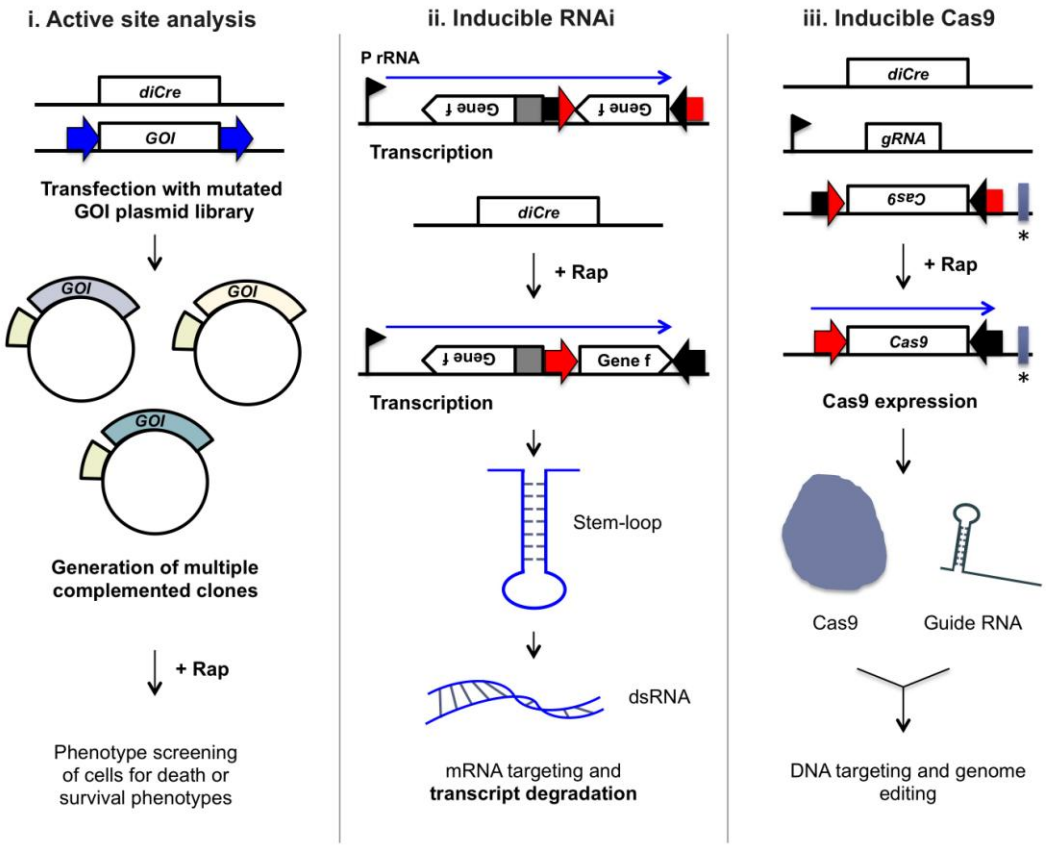


Figure 6:2- Application of diCre mediated recombination to other molecular methods for *Leishmania* manipulation. i. Multiple plasmids each containing an individual mutated gene of interest (GOI) cell lines can be generated to express mutant variants of the target. Rapamycin treatment to initiate diCre activity will result in floxed gene loss to induce a measurable phenotype resulting from expression of only the mutant sequence, and inactive or active mutants can be identified by sequencing. ii. Inducible RNAi by inversion of an open reading frame fragment (ORF f) by inverted lox recombination. Prior to rapamycin treatment no stem loop is formed by ribosomal promoter (P rRNA) driven transcription (blue arrow). After rapamycin treatment the gene fragment (Gene f) becomes orientated to enable read-through of both fragments and spacer (grey) to produce a stem-loop. Generation of dsRNA results in downstream silencing. iii. Generation of a cell line expressing guide RNA (gRNA), diCre and an inverted Cas9 ORF enables inducible Cas9 regulation. DiCre activity orientates the gene for expression, resulting in guide RNA binding and subsequent genome editing activity. An exogenous transcription termination site (*) at the 3' end would prevent reverse transcription.

6.4.3 CRISPR/Cas9

The use of CRISPR/Cas9 mediated genome tailoring has been applied in *Leishmania* to enhance homologous recombination for integration of exogenous genes (Sollelis et al. 2015) or to cause gene disruption and endogenous gene tagging (Zhang & Matlashewski 2015). However the application of such a method to target essential genes is still limited by a lack of conditional regulation of activity. There are regulated Cas9 enzymes available, such as destabilization domain (ddCas9) which may ameliorate this, however the use of diCre mediated Cas9 expression may also be a viable option. In this instance, the Cas9 coding sequence would be flanked with inverted loxP sites and integrated into the genome in the reverse orientation. Inducible diCre activity would therefore invert the gene and enable transcription and expression of the enzyme. A suitable method to retain the Cas9 guide DNA would have to be included in this plan, however expression downstream of the ribosomal promoter is a functional strategy. Incorporation of the diCre coding sequence elsewhere in the genome would confer the necessary expression, whilst the Cas9 coding sequence would be inserted in the reverse orientation and flanked by inverted LE/RE lox mutants (**Figure** 6:2 iii). In this reverse orientation there should be no transcription, however by diCre mediated recombination the Cas9 sequence will be flipped to enable read through transcription of the full sequence. Subsequent expression will drive the downstream CRISPR/Cas9 editing, depending on the guide sequence incorporated into the cell.

6.5 Concluding remarks

This study presents the efficacious utilisation of diCre inducible gene deletion in *L. mexicana* and its application to the cell cycle protein kinase CRK3. CRK3 has been shown to be essential for the progression of mitosis in promastigotes, whilst an active CRK3 is necessary to maintain murine infection. We can therefore conclude that CRK3 is a validated drug target, and that the application of this methodology to other essential genes will confirm further drug targets. Application of inducible deletion of essential genes, combined with the bioluminescent infection model will enable the development of amenable

murine assays of parasite burden and immune recruitment. Such studies will yield crucial information to accelerate drug development programs and help address the paucity of available drugs for leishmaniasis. Ideally, this work will form a basis for the establishment of a model of visceral infection to identify suitable targets in the disseminating *Leishmania* species which cause the deadly, visceral form of the disease.

List of References

Abdulla, M.-H. et al., 2008. RNA interference of Trypanosoma brucei cathepsin B and L affects disease progression in a mouse model. *PLoS neglected tropical diseases*, 2(9), p.e298.

Abremski, K., Hoess, R. & Sternberg, N., 1983. Studies on the properties of P1 site-specific recombination: evidence for topologically unlinked products following recombination. *Cell*, 32(4), pp.1301–1311.

Abreu-Blanco, M.T. et al., 2010. Analysis of ribosomal RNA transcription termination and 3' end processing in Leishmania amazonensis. *Gene*, 451(1-2), pp.15–22. Available at: http://dx.doi.org/10.1016/j.gene.2009.11.002.

Albert, H. et al., 1995. Site-specific integration of DNA into wild-type and mutant lox sites placed in the plant genome. *The Plant journal : for cell and molecular biology*, 7(4), pp.649–659.

Alexander, J., Coombs, G.H. & Mottram, J.C., 1998. Leishmania mexicana cysteine proteinase-deficient mutants have attenuated virulence for mice and potentiate a Th1 response. *Journal of immunology (Baltimore, Md. : 1950)*, 161(12), pp.6794–6801.

Alexander, J., Satoskar, a R. & Russell, D.G., 1999. Leishmania species: models of intracellular parasitism. *Journal of cell science*, 112 Pt 18, pp.2993–3002.

Alsford, S. et al., 2011. High-throughput phenotyping using parallel sequencing of RNA interference targets in the African trypanosome. , pp.915–924.

Alvar, J. et al., 2012. Leishmaniasis worldwide and global estimates of its incidence. *PLoS ONE*, 7(5).

Ambit, a et al., 2008. An essential role for the Leishmania major metacaspase in cell cycle progression. *Cell death and differentiation*, 15(1), pp.113–122.

Andenmatten, N., Egarter, S., Jackson, A.J., et al., 2012. Conditional genome engineering in Toxoplasma gondii uncovers alternative invasion mechanisms. *Nature methods*, (decembeR), pp.1–6. Available at: http://www.ncbi.nlm.nih.gov/pubmed/23263690 [Accessed December 25, 2012].

Araki, K., Araki, M. & Yamamura, K., 2002. Site-directed integration of the cre gene mediated by Cre recombinase using a combination of mutant lox sites. *Nucleic acids research*, 30(19), p.e103.

Araki, K., Ellebedy, A.H. & Ahmed, R., 2011. TOR in the immune system. *Current opinion in cell biology*, 23(6), pp.707–15. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3241972&tool=pmcentrez&rendertype=abstract [Accessed July 21, 2014].

Arrebola, R. et al., 1994. Isolation and characterization of a mutant dihydrofolate reductase-thymidylate synthase from methotrexate-resistant Leishmania cells. *Journal of Biological Chemistry*, 269(14), pp.10590–10596.

Baldwin, T. et al., 2004. Dendritic Cell Populations in Leishmania major-Infected Skin and Draining Lymph Nodes. *Infection and Immunity*, 72(4), pp.1991–2001.

Ballou, L.M. & Lin, R.Z., 2008. Rapamycin and mTOR kinase inhibitors. *Journal of chemical biology*, 1(1-4), pp.27–36.

Banaszynski, L. a. et al., 2006. A Rapid, Reversible, and Tunable Method to Regulate Protein Function in Living Cells Using Synthetic Small Molecules. *Cell*, 126(5), pp.995–1004.

Banerjee, S. et al., 2006. Leishmania donovani cyclin 1 (LdCyc1) forms a complex with cell cycle kinase subunit CRK3 (LdCRK3) and is possibly involved in S-phase-related activities. *FEMS Microbiology Letters*, 256, pp.75–82.

Barese, C.N. et al., 2012. Thymidine Kinase Suicide Gene-mediated Ganciclovir Ablation of Autologous Gene-modified Rhesus Hematopoiesis. *Molecular Therapy*, 20(10), pp.1932–1943.

Barrett, B., LaCount, D.J. & Donelson, J.E., 2004. Trypanosoma brucei: A first-generation CRE-loxP site-specific recombination system. *Experimental Parasitology*, 106(1-2), pp.37–44.

Barrett, M.P., Mottram, J.C. & Coombs, G.H., 1999. Recent advances in identifying and validating drug targets in trypanosomes and leishmanias. *Trends in microbiology*, 7(2), pp.82–8. Available at: http://www.ncbi.nlm.nih.gov/pubmed/10081086.

Bates, P. a, 1994. Complete developmental cycle of Leishmania mexicana in axenic culture. *Parasitology*, 108 (Pt 1(June 1993), pp.1–9.

Bayle, J.H. et al., 2006. Rapamycin analogs with differential binding specificity permit orthogonal control of protein activity. *Chemistry and Biology*, 13(1), pp.99–107.

Beattie, L. et al., 2008. Transgenic Leishmania and the immune response to infection. *Parasite immunology*, 30(4), pp.255–66. Available at: http://www.ncbi.nlm.nih.gov/pubmed/18266814 [Accessed January 31, 2012].

Belkaid, Y. et al., 2000. A natural model of Leishmania major infection reveals a prolonged “silent” phase of parasite amplification in the skin before the onset of lesion formation and immunity. *Journal of immunology (Baltimore, Md. : 1950)*, 165(2), pp.969–977.

Bell, A.S. et al., 2012. Selective inhibitors of protozoan protein N-myristoyltransferases as starting points for tropical disease medicinal chemistry programs. *PLoS Neglected Tropical Diseases*, 6(4).

Belli, S.I. et al., 2003. Sense and antisense transcripts in the histone H1 (HIS-1) locus of Leishmania major. *International Journal for Parasitology*, 33(9), pp.965–975.

Bengs, F. et al., 2005. LmxMPK9, a mitogen-activated protein kinase homologue affects flagellar length in Leishmania mexicana. *Molecular Microbiology*, 55(5), pp.1606–1615.

Berens, R.L., Brun, R. & Krassner, S.M., 1976. A simple monophasic medium for axenic culture of hemoflagellates. *The Journal of parasitology*, 62(3), pp.360–365.

Bhandari, V. et al., 2012. Drug susceptibility in Leishmania isolates following Miltefosine treatment in cases of Visceral Leishmaniasis and post Kala-Azar dermal Leishmaniasis. *PLoS Neglected Tropical Diseases*, 6(5), pp.1–6.

Brittingham, A. et al., 1995. Role of the Leishmania surface protease gp63 in complement fixation, cell adhesion, and resistance to complement-mediated lysis. *Journal of immunology (Baltimore, Md. : 1950)*, 155(6), pp.3102–3111.

Broermann, a. et al., 2011. Dissociation of VE-PTP from VE-cadherin is required for leukocyte extravasation and for VEGF-induced vascular permeability in vivo. *Journal of Experimental Medicine*, 208(12), pp.2393–2401.

Bryceson, a. D.M. et al., 1970. Immunity in cutaneous leishmaniasis of the guinea-pig. *Clinical and experimental immunology*, 7(3), pp.301–341.

Buchdunger, E. et al., 1996. Inhibition of the Abl protein-tyrosine kinase in vitro and in vivo by a 2-phenylaminopyrimidine derivative. *Cancer Research*, 56(1), pp.100–104.

Buxbaum, L.U. et al., 2003a. Cysteine protease B of Leishmania mexicana inhibits host Th1 responses and protective immunity. *Journal of immunology (Baltimore, Md. : 1950)*, 171(7), pp.3711–3717.

Buxbaum, L.U., 2015. Interleukin-10 from T Cells, but Not Macrophages and Granulocytes, Is Required for Chronic Disease in Leishmania mexicana Infection. *Infection and Immunity*, 83(4), pp.1366–1371. Available at: http://iai.asm.org/lookup/doi/10.1128/IAI.02909-14.

Buxbaum, L.U. & Scott, P., 2005. Interleukin 10- and Fc γ Receptor-Deficient Mice Resolve Leishmania mexicana Lesions Interleukin 10- and Fc □ Receptor-Deficient Mice Resolve Leishmania mexicana Lesions. , 73(4), pp.2101–2108.

Cameron, P. et al., 2004. Inhibition of lipopolysaccharide-induced macrophage IL-12 production by Leishmania mexicana amastigotes: the role of cysteine

peptidases and the NF-kappaB signaling pathway. *Journal of immunology (Baltimore, Md. : 1950)*, 173(5), pp.3297–304. Available at: http://www.ncbi.nlm.nih.gov/pubmed/15322192.

Carvalho, L.P. et al., 2012. Lymph node hypertrophy following Leishmania major infection is dependent on TLR9. *Journal of immunology (Baltimore, Md. : 1950)*, 188(3), pp.1394–401. Available at: http://www.ncbi.nlm.nih.gov/pubmed/22205030 [Accessed December 28, 2012].

Castanys-Muñoz, E. et al., 2012. Leishmania mexicana metacaspase is a negative regulator of amastigote proliferation in mammalian cells. Available at: http://eprints.gla.ac.uk/69550/.

Cayla, M. et al., 2014. Transgenic Analysis of the Leishmania MAP Kinase MPK10 Reveals an Auto-inhibitory Mechanism Crucial for Stage-Regulated Activity and Parasite Viability. *PLoS Pathogens*, 10(9), p.e1004347. Available at: http://dx.plos.org/10.1371/journal.ppat.1004347.

Charmoy, M. et al., 2010. Neutrophil-derived CCL3 is essential for the rapid recruitment of dendritic cells to the site of Leishmania major inoculation in resistant mice. *PLoS Pathogens*, 6(2).

Chen, J. et al., 1995. Identification of an 11-kDa FKBP12-rapamycin-binding domain within the 289-kDa FKBP12-rapamycin-associated protein and characterization of a critical serine residue. *Proceedings of the National Academy of Sciences of the United States of America*, 92(11), pp.4947–4951.

Cheng, H.-C. et al., 2011. Regulation and Function of Protein Kinases and Phosphatases. *Enzyme Research*, 2011, pp.1–3.

Choi, J. et al., 1996. Structure of the FKBP12-rapamycin complex interacting with the binding domain of human FRAP. *Science (New York, N.Y.)*, 273(5272), pp.239–242.

Cicenas, J. & Valius, M., 2011. The CDK inhibitors in cancer research and therapy. *Journal of Cancer Research and Clinical Oncology*, 137(10), pp.1409–1418.

Clayton, C. et al., 1998. Genetic nomenclature for Trypanosoma and Leishmania. *Molecular and Biochemical Parasitology*, 97(1-2), pp.221–224.

Cleghorn, L. a T. et al., 2011. Identification of Inhibitors of the Leishmania cdc2-Related Protein Kinase CRK3. *ChemMedChem*, 6(12), pp.2214–2224.

Collin, N. et al., 2009. Sand fly salivary proteins induce strong cellular immunity in a natural reservoir of visceral leishmaniasis with adverse consequences for Leishmania. *PLoS pathogens*, 5(5), p.e1000441. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2677456&tool=pmcentrez&rendertype=abstract [Accessed September 1, 2011].

Collins, C.R. et al., 2013. Robust inducible Cre recombinase activity in the human malaria parasite Plasmodium falciparum enables efficient gene deletion within a single asexual erythrocytic growth cycle. *Molecular Microbiology*, 88(4), pp.687–701.

Committee, W.H.O.E., 2010. Control of the leishmaniases. *World Health Organization technical report series*, (949), pp.xii–xiii, 1–186, back cover. Available at: http://www.ncbi.nlm.nih.gov/pubmed/21933303.

Constantinescu, C.S. et al., 1998. The role of IL-12 in the maintenance of an established Th1 immune response in experimental leishmaniasis. *European Journal of Immunology*, 28(7), pp.2227–2233.

Cox, F.E.G., 2002. History of Human Parasitology History of Human Parasitology. *Clinical microbiology reviews*, 15(4), pp.595–612.

Croft, S.L. & Olliaro, P., 2011. Leishmaniasis chemotherapy-challenges and opportunities. *Clinical Microbiology and Infection*, 17(10), pp.1478–1483.

Cruz, a, Coburn, C.M. & Beverley, S.M., 1991. Double targeted gene replacement for creating null mutants. *Proceedings of the National Academy of Sciences of the United States of America*, 88(August), pp.7170–7174.

Cruz, a K., Titus, R. & Beverley, S.M., 1993. Plasticity in chromosome number and testing of essential genes in Leishmania by targeting. *Proceedings of the National Academy of Sciences of the United States of America*, 90(4), pp.1599–603. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=45922&tool=pmcentrez&rendertype=abstract.

Cruz, A. & Beverley, S.M., 1990. Gene replacement in parasitic protozoa. *Nature*, 348(6297), pp.171–173.

Dacher, M. et al., 2014. Probing druggability and biological function of essential proteins in Leishmania combining facilitated null mutant and plasmid shuffle analyses. *Molecular microbiology*, 93(1), pp.146–66. Available at: http://www.ncbi.nlm.nih.gov/pubmed/24823804 [Accessed December 9, 2014].

Damasceno, J.D., Beverley, S.M. & Tosi, L.R.O., 2010. A transposon toolkit for gene transfer and mutagenesis in protozoan parasites. *Genetica*, 138(3), pp.301–11. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2813944&tool=pmcentrez&rendertype=abstract [Accessed July 20, 2014].

Denise, H. et al., 2003. Expression of multiple CPB genes encoding cysteine proteases is required for Leishmania mexicana virulence in vivo. *Infection and Immunity*, 71(6), pp.3190–3195.

Denny, P.W. et al., 2000. Acylation-dependent protein export in Leishmania. *Journal of Biological Chemistry*, 275(15), pp.11017–11025.

Druker, B.J., 2004. Imatinib as a paradigm of targeted therapies. *Advances in Cancer Research*, 91(1), pp.1–30.

Drummelsmith, J. et al., 2004. Differential protein expression analysis of Leishmania major reveals novel roles for methionine adenosyltransferase and S-adenosylmethionine in methotrexate resistance. *Journal of Biological Chemistry*, 279(32), pp.33273–33280.

Dunning, N., 2009. Leishmania vaccines : from leishmanization to the era of DNA technology The Immune Response to Leishmania. *Bioscience Horizons*, 2(1), pp.73–82. Available at: http://creativecommons.org/licenses/by-nc/2.0/uk/.

Edwards, S.R. & Wandless, T.J., 2007. The rapamycin-binding domain of the protein kinase mammalian target of rapamycin is a destabilizing domain. *Journal of Biological Chemistry*, 282(18), pp.13395–13401.

Ehrchen, J.M. et al., 2010. Keratinocytes determine Th1 immunity during early experimental leishmaniasis. *PLoS pathogens*, 6(4), p.e1000871. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2861693&tool=pmcentrez&rendertype=abstract [Accessed January 31, 2012].

Ehrchen, J.M. et al., 2008. The absence of cutaneous lymph nodes results in a Th2 response and increased susceptibility to Leishmania major infection in mice. *Infection and Immunity*, 76(9), pp.4241–4250.

Erdmann, M. et al., 2006. Interacting protein kinases involved in the regulation of flagellar length. *Molecular biology of the cell*, 17(4), pp.2035–2045.

Eschenlauer, S.C.P. et al., 2009. Influence of parasite encoded inhibitors of serine peptidases in early infection of macrophages with Leishmania major. *Cellular microbiology*, 11(1), pp.106–20. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2659362&tool=pmcentrez&rendertype=abstract [Accessed June 7, 2012].

Faria, M.S. et al., 2011. Leishmania inhibitor of serine peptidase 2 prevents TLR4 activation by neutrophil elastase promoting parasite survival in murine macrophages. *Journal of immunology (Baltimore, Md. : 1950)*, 186(1), pp.411–22. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3119636&tool=pmcentrez&rendertype=abstract [Accessed January 31, 2012].

Faurschou, M. & Borregaard, N., 2003. Neutrophil granules and secretory vesicles in inflammation. *Microbes and Infection*, 5(14), pp.1317–1327.

Filipe-Santos, O. et al., 2009. A dynamic map of antigen recognition by CD4 T cells at the site of Leishmania major infection. *Cell host & microbe*, 6(1), pp.23–33. Available at: http://www.ncbi.nlm.nih.gov/pubmed/19616763 [Accessed September 29, 2011].

Fogel, A.L., Hill, S. & Teng, J.M.C., 2015. Advances in the therapeutic use of mammalian target of rapamycin (mTOR) inhibitors in dermatology. *Journal*

of the American Academy of Dermatology, pp.1–11. Available at: http://linkinghub.elsevier.com/retrieve/pii/S0190962215000547.

Fortin, A. et al., 2014. Direct Comparison of the Efficacy and Safety of Oral Treatments with Oleylphosphocholine (OlPC) and Miltefosine in a Mouse Model of L. major Cutaneous Leishmaniasis. *PLoS Neglected Tropical Diseases*, 8(9), p.e3144. Available at: http://dx.plos.org/10.1371/journal.pntd.0003144.

Fraga, J. et al., 2013. Evolution and species discrimination according to the Leishmania heat-shock protein 20 gene. *Infection, Genetics and Evolution*, 18, pp.229–237. Available at: http://dx.doi.org/10.1016/j.meegid.2013.05.020.

Gade, D.W., 1979. Inca and colonial settlement, coca cultivation and endemic disease in the tropical forest. *Journal of historical geography*, 5, pp.263–280.

Garnier, T. & Croft, S.L., 2002. Topical treatment for cutaneous leishmaniasis. Available at: http://hdl.handle.net/2299/631.

Gibson, V.B. et al., 2012. A novel method to allow non-invasive, longitudinal imaging of the murine immune system in vivo. *Blood*, pp.2545–2551. Available at: http://www.ncbi.nlm.nih.gov/pubmed/22271449 [Accessed March 16, 2012].

Gomes, F.C. et al., 2010. Recombinant Leishmania mexicana CRK3:CYCA has protein kinase activity in the absence of phosphorylation on the T-loop residue Thr178. *Molecular and Biochemical Parasitology*, 171(2), pp.89–96. Available at: http://dx.doi.org/10.1016/j.molbiopara.2010.03.002.

Gomes, R. & Oliveira, F., 2012. The immune response to sand fly salivary proteins and its influence on Leishmania immunity. *Frontiers in Immunology*, 3(MAY), pp.1–8.

Gordon, S. & Taylor, P.R., 2005. Monocyte and macrophage heterogeneity. *Nature reviews. Immunology*, 5(12), pp.953–964.

Gossen, M. & Bujard, H., 1992. Tight control of gene expression in mammalian cells by tetracycline-responsive promoters. *Proceedings of the National Academy of Sciences of the United States of America*, 89(12), pp.5547–5551.

Gould, K.L. et al., 1991. Phosphorylation at Thr167 is required for Schizosaccharomyces pombe p34cdc2 function. *The EMBO journal*, 10(11), pp.3297–3309.

Goyard, S. & Beverley, S.M., 2000. Blasticidin resistance: A new independent marker for stable transfection of Leishmania. *Molecular and Biochemical Parasitology*, 108(2), pp.249–252.

Graeser, R., Franklin, R.M. & Kappes, B., 1996. Mechanisms of activation of the cdc2-related kinase PfPK5 from Plasmodium falciparum. *Molecular and Biochemical Parasitology*, 79(1), pp.125–127.

Grant, K.M. et al., 2004. Inhibitors of Leishmania mexicana CRK3 cyclin-dependent kinase: Chemical library screen and antileishmanial activity. *Antimicrobial Agents and Chemotherapy*.

Grant, K.M. et al., 1998. The crk3 Gene of Leishmania mexicanaEncodes a Stage-regulated cdc2-related Histone H1 Kinase That Associates with p12cks1. *Journal of Biological Chemistry*, 273(17), pp.10153–10159. Available at: http://www.jbc.org/cgi/doi/10.1074/jbc.273.17.10153 [Accessed December 9, 2014].

Gregory, D.J. et al., 2008. A novel form of NF-kappaB is induced by Leishmania infection: involvement in macrophage gene expression. *European journal of immunology*, 38(4), pp.1071–81. Available at: http://www.ncbi.nlm.nih.gov/pubmed/18383035 [Accessed September 3, 2011].

Gueirard, P. et al., 2008. Trafficking of Leishmania donovani promastigotes in non-lytic compartments in neutrophils enables the subsequent transfer of parasites to macrophages. *Cellular microbiology*, 10(1), pp.100–11. Available at: http://www.ncbi.nlm.nih.gov/pubmed/17651446 [Accessed August 13, 2011].

Guimarães-Costa, A.B. et al., 2009. Leishmania amazonensis promastigotes induce and are killed by neutrophil extracellular traps. *Proceedings of the National Academy of Sciences of the United States of America*, 106(16), pp.6748–6753.

Gutiérrez-Escribano, P. & Nurse, P., 2015. A single cyclin–CDK complex is sufficient for both mitotic and meiotic progression in fission yeast. *Nature Communications*, 6, p.6871. Available at: http://www.nature.com/doifinder/10.1038/ncomms7871.

Hamilton, D.L. & Abremski, K., 1984. Site-specific Recombination by the Bacteriophage system Pl. *Journal of molecular biology*, 178(2), pp.481–486.

Hammarton, T.C. et al., 2003. Stage-specific differences in cell cycle control in Trypanosoma brucei revealed by RNA interference of a mitotic cyclin. *The Journal of biological chemistry*, 278(25), pp.22877–86. Available at: http://www.ncbi.nlm.nih.gov/pubmed/12682070 [Accessed December 3, 2014].

Hanks, S. & Hunter, T., 1995. The eukaryotic protein kinase superfamily: kinase (catalytic) domain structure and classification. *FASEB journal*, 9, pp.576–596.

Hanks, S.K., 2003. Genomic analysis of the eukaryotic protein kinase superfamily: a perspective. *Genome biology*, 4(5), p.111.

Hassan, P. et al., 2001. The CRK3 protein kinase is essential for cell cycle progression of Leishmania mexicana. *Molecular and Biochemical Parasitology*, 113(2), pp.189–198. Available at: http://linkinghub.elsevier.com/retrieve/pii/S0166685101002201.

Hassan, P. et al., 2001. The CRK3 protein kinase is essential for cell cycle progression of Leishmania mexicana. *Molecular and biochemical parasitology*, 113(2), pp.189–98. Available at: http://www.ncbi.nlm.nih.gov/pubmed/11295173.

Heinzel, F.P. et al., 1991. Production of interferon gamma, interleukin 2, interleukin 4, and interleukin 10 by CD4+ lymphocytes in vivo during healing and progressive murine leishmaniasis. *Proceedings of the National Academy of Sciences of the United States of America*, 88(16), pp.7011–7015.

Herwaldt, B.L., 1999. Leishmaniasis. *Lancet*, 354(9185), pp.1191–1199.

Hickstein, D.D. et al., 1987. Isolation and characterization of the receptor on human neutrophils that mediates cellular adherence. *Journal of Biological Chemistry*, 262(12), pp.5576–5580.

Hlavacova, J., Votypka, J. & Volf, P., 2013. The effect of temperature on Leishmania (Kinetoplastida: Trypanosomatidae) development in sand flies. *Journal of medical entomology*, 50(5), pp.955–8. Available at: http://www.ncbi.nlm.nih.gov/pubmed/24180098.

Hoess, R.H., Wierzbicki, A. & Abremski, K., 1986. The role of the loxP spacer region in P1 site-specific recombination. *Nucleic acids research*, 14(5), pp.2287–2300.

Hsu, A.C. & Scott, P., 2007. Leishmania mexicana infection induces impaired lymph node expansion and Th1 cell differentiation despite normal T cell proliferation. *Journal of immunology (Baltimore, Md. : 1950)*.

Hurrell, B.P. et al., 2015. Rapid Sequestration of Leishmania mexicana by Neutrophils Contributes to the Development of Chronic Lesion. *PLOS Pathogens*, 11(5), p.e1004929.

Hutton, J.A. et al., 2014. Structure-Based Design of Potent and Selective Leishmania N -Myristoyltransferase Inhibitors. *Journal of Medicinal Chemistry*, 57(20), pp.8664–8670. Available at: http://pubs.acs.org/doi/abs/10.1021/jm5011397.

Ilgoutz, S.C. et al., 1999. Evidence that free GPI glycolipids are essential for growth of Leishmania mexicana. *The EMBO journal*, 18(10), pp.2746–55. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1171356&tool=pmcentrez&rendertype=abstract.

Inbar, E. et al., 2013. The Mating Competence of Geographically Diverse Leishmania major Strains in Their Natural and Unnatural Sand Fly Vectors. *PLoS Genetics*, 9(7).

Ivens, A.C. et al., 2005. The genome of the kinetoplastid parasite, Leishmania major. *Science (New York, N.Y.)*, 309(5733), pp.436–442.

Jänne, P. a, Gray, N. & Settleman, J., 2009. Factors underlying sensitivity of cancers to small-molecule kinase inhibitors. *Nature reviews. Drug discovery*, 8(9), pp.709–723. Available at: http://dx.doi.org/10.1038/nrd2871.

Jaramillo, M. et al., 2011. Leishmania repression of host translation through mTOR cleavage is required for parasite survival and infection. *Cell Host and Microbe*, 9(4), pp.331–341.

Jones, N.G. et al., 2014. Regulators of Trypanosoma brucei cell cycle progression and differentiation identified using a kinome-wide RNAi screen. *PLoS pathogens*, 10(1), p.e1003886. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3894213&tool=pmcentrez&rendertype=abstract [Accessed November 11, 2014].

Jullien, N. et al., 2007. Conditional transgenesis using Dimerizable Cre (DiCre). *PLoS ONE*, 2(12).

Jullien, N. et al., 2003. Regulation of Cre recombinase by ligand-induced complementation of inactive fragments. *Nucleic acids research*, 31(21), p.e131.

Kalderon, D. et al., 1984. A short amino acid sequence able to specify nuclear location. *Cell*, 39(3 Pt 2), pp.499–509.

Kamhawi, S. et al., 2004. A role for insect galectins in parasite survival. *Cell*, 119(3), pp.329–41. Available at: http://www.ncbi.nlm.nih.gov/pubmed/15543683.

Kamhawi, S., 2006. Phlebotomine sand flies and Leishmania parasites: friends or foes? *Trends in parasitology*, 22(9), pp.439–45. Available at: http://www.ncbi.nlm.nih.gov/pubmed/16843727 [Accessed August 25, 2011].

Kangussu-Marcolino, M.M. et al., 2014. Conditional removal of selectable markers in Trypanosoma cruzi using a site-specific recombination tool: Proof of concept. *Molecular and Biochemical Parasitology*, 198(2), pp.71–74. Available at: http://linkinghub.elsevier.com/retrieve/pii/S016668511500002X.

Kaye, P. & Scott, P., 2011. Leishmaniasis: complexity at the host-pathogen interface. *Nature reviews. Microbiology*, 9(8), pp.604–615. Available at: http://www.ncbi.nlm.nih.gov/pubmed/21747391 [Accessed July 16, 2011].

Kelly, C., Jefferies, C. & Cryan, S.-A., 2011. Targeted liposomal drug delivery to monocytes and macrophages. *Journal of drug delivery*, 2011, p.727241.

Kiely, J. et al., 2000. Functions of fission yeast Orp2 in DNA replication and checkpoint control. *Genetics*, 154(2), pp.599–607.

Kim, H.S. et al., 2013. Strategies to construct null and conditional null Trypanosoma brucei mutants using Cre-recombinase and loxP. *Molecular and Biochemical Parasitology*, 191(1), pp.16–19. Available at: http://dx.doi.org/10.1016/j.molbiopara.2013.08.001.

Kima, P.E. et al., 2000. Internalization of Leishmania mexicana complex amastigotes via the Fc receptor is required to sustain infection in murine cutaneous leishmaniasis. *The Journal of experimental medicine*, 191(6), pp.1063–1068.

Kimblin, N. et al., 2008. Quantification of the infectious dose of Leishmania major transmitted to the skin by single sand flies. *Proceedings of the National Academy of Sciences of the United States of America*, 105(29), pp.10125–10130.

Kloehn, J. et al., 2015. Characterization of Metabolically Quiescent Leishmania Parasites in Murine Lesions Using Heavy Water Labeling. *PLOS Pathogens*, 11(2), p.e1004683. Available at: http://dx.plos.org/10.1371/journal.ppat.1004683.

Knapp, S. & Sundström, M., 2014. Recently targeted kinases and their inhibitors—the path to clinical trials. *Current Opinion in Pharmacology*, 17, pp.58–63. Available at: http://linkinghub.elsevier.com/retrieve/pii/S1471489214000903.

Koenig, M.K. et al., 2012. Topical rapamycin therapy to alleviate the cutaneous manifestations of tuberous sclerosis complex: a double-blind, randomized, controlled trial to evaluate the safety and efficacy of topically applied rapamycin. *Drugs in R&D*, 12(3), pp.121–126. Available at: http://www.ncbi.nlm.nih.gov/pubmed/22934754.

Koh, J.T. et al., 2006. Use of a Stringent Dimerizer-Regulated Gene Expression System for Controlled BMP2 Delivery. *Molecular Therapy*, 14(5), pp.684–691.

Kolev, N.G., Tschudi, C. & Ullu, E., 2011. RNA interference in protozoan parasites: Achievements and challenges. *Eukaryotic Cell*, 10(9), pp.1156–1163.

Kraeva, N. et al., 2014. Tetracycline-inducible gene expression system in Leishmania mexicana. *Molecular and Biochemical Parasitology*, 198(1), pp.11–13. Available at: http://linkinghub.elsevier.com/retrieve/pii/S0166685114001662.

Kuhn, D. & Wiese, M., 2005. LmxPK4, a mitogen-activated protein kinase kinase homologue of Leishmania mexicana with a potential role in parasite differentiation. *Molecular Microbiology*, 56(5), pp.1169–1182.

Kumar, R. & Engwerda, C., 2014. Vaccines to prevent leishmaniasis. *Clinical & Translational Immunology*, 3(3), p.e13. Available at: http://www.readcube.com/articles/10.1038/cti.2014.4.

Lainson, R., 2010. The Neotropical Leishmania species: a brief historical review of their discovery, ecology and taxonomy. *Revista Pan-Amazônica de Saúde*, 1(2), pp.13–32.

Lainson, R., Ward, R.D. & Shaw, J.J., 1977. Leishmania in phlebotomid sandflies: VI. Importance of hindgut development in distinguishing between parasites of the Leishmania mexicana and L. braziliensis complexes. *Proceedings of the Royal Society of London. Series B, Containing papers of a Biological character. Royal Society (Great Britain)*, 199(1135), pp.309–320.

Lakso, M. et al., 1992. Targeted oncogene activation by site-specific recombination in transgenic mice. *Proceedings of the National Academy of Sciences of the United States of America*, 89(14), pp.6232–6236.

Lang, T. et al., 2005. Bioluminescent Leishmania expressing luciferase for rapid and high throughput screening of drugs acting on amastigote-harbouring macrophages and for quantitative real-time monitoring of parasitism features in living mice. *Cellular Microbiology*, 7(3), pp.383–392.

Langer, S.J. et al., 2002. A genetic screen identifies novel non-compatible loxP sites. *Nucleic acids research*, 30(14), pp.3067–3077.

Laufs, H. et al., 2002. Intracellular Survival of Leishmania major in Neutrophil Granulocytes after Uptake in the Absence of Heat-Labile Serum Factors Intracellular Survival of Leishmania major in Neutrophil Granulocytes after Uptake in the Absence of Heat-Labile Serum Factors. *Society*.

LeBowitz, J.H., Cruz, A. & Beverley, S.M., 1992. Thymidine kinase as a negative selectable marker in Leishmania major. *Molecular and Biochemical Parasitology*, 51(2), pp.321–326.

Leclerc, S. et al., 2001. Indirubins inhibit glycogen synthase kinase-3?? and CDK5/P25, two protein kinases involved in abnormal tau phosphorylation in Alzheimer's disease. A property common to most cyclin-dependent kinase inhibitors? *Journal of Biological Chemistry*, 276(1), pp.251–260.

León, B., López-Bravo, M. & Ardavín, C., 2007. Monocyte-Derived Dendritic Cells Formed at the Infection Site Control the Induction of Protective T Helper 1 Responses against Leishmania. *Immunity*, 26(4), pp.519–531.

Leprohon, P. et al., 2015. Drug resistance analysis by next generation sequencing in Leishmania. *International Journal for Parasitology: Drugs and Drug Resistance*, 5(1), pp.26–35. Available at: http://linkinghub.elsevier.com/retrieve/pii/S2211320714000281.

Lewis, M.D. et al., 2014. A New Experimental Model for Assessing Drug Efficacy against Trypanosoma cruzi Infection Based on Highly Sensitive In Vivo Imaging. *Journal of Biomolecular Screening*, 20(1), pp.36–43. Available at: http://jbx.sagepub.com/cgi/doi/10.1177/1087057114552623.

Liu, X. & Chang, K.P., 1992. The 63-kilobase circular amplicon of tunicamycin-resistant Leishmania amazonensis contains a functional N-

acetylglucosamine-1-phosphate transferase gene that can be used as a dominant selectable marker in transfection. *Molecular and cellular biology*, 12(9), pp.4112–4122.

Liu, Y. & Gray, N.S., 2006. Rational design of inhibitors that bind to inactive kinase conformations. *Nature chemical biology*, 2(7), pp.358–364.

Locksley, R.M. et al., 1987. Murine cutaneous leishmaniasis: susceptibility correlates with differential expansion of helper T-cell subsets. *Annales de l'Institut Pasteur. Immunology*, 138(5), pp.744–749. Available at: http://dx.doi.org/10.1016/S0769-2625(87)80030-2.

Lye, L.-F. et al., 2010. Retention and loss of RNA interference pathways in trypanosomatid protozoans. *PLoS pathogens*, 6(10), p.e1001161. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2965760&tool=pmcentrez&rendertype=abstract [Accessed March 9, 2012].

Lye, L.F., Cunningham, M.L. & Beverley, S.M., 2002. Characterization of quinonoid-dihydropteridine reductase (QDPR) from the lower eukaryote Leishmania major. *Journal of Biological Chemistry*, 277(41), pp.38245–38253.

Madeira da Silva, L. et al., 2009. Regulated expression of the Leishmania major surface virulence factor lipophosphoglycan using conditionally destabilized fusion proteins. *Proceedings of the National Academy of Sciences of the United States of America*, 106(18), pp.7583–8. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2678649&tool=pmcentrez&rendertype=abstract.

Madeira da Silva, L. & Beverley, S.M., 2010. Expansion of the target of rapamycin (TOR) kinase family and function in Leishmania shows that TOR3 is required for acidocalcisome biogenesis and animal infectivity. *Proceedings of the National Academy of Sciences of the United States of America*, 107(26), pp.11965–11970.

Maity, A.K., Goswami, A. & Saha, P., 2011. Identification of substrates of an S-phase cell cycle kinase from Leishmania donovani. *FEBS Letters*, 585(17), pp.2635–2639. Available at: http://dx.doi.org/10.1016/j.febslet.2011.06.017.

Martínez-Calvillo, S., Stuart, K. & Myler, P.J., 2005. Ploidy changes associated with disruption of two adjacent genes on Leishmania major chromosome 1. *International Journal for Parasitology*, 35(4), pp.419–429.

McCall, L.-I. et al., 2015. Targeting Ergosterol Biosynthesis in Leishmania donovani: Essentiality of Sterol 14alpha-demethylase. *PLOS Neglected Tropical Diseases*, 9(3), p.e0003588. Available at: http://dx.plos.org/10.1371/journal.pntd.0003588.

McLatchie, A.P. et al., 2013. Highly Sensitive In Vivo Imaging of Trypanosoma brucei Expressing “Red-Shifted” Luciferase. *PLoS Neglected Tropical Diseases*, 7(11), pp.1–12.

Mestas, J. & Hughes, C.C.W., 2004. Of mice and not men: differences between mouse and human immunology. *Journal of immunology (Baltimore, Md. : 1950)*, 172(5), pp.2731–8. Available at: http://www.ncbi.nlm.nih.gov/pubmed/14978070.

Metzger, D. et al., 1995. Conditional site-specific recombination in mammalian cells using a ligand-dependent chimeric Cre recombinase. *Proceedings of the National Academy of Sciences of the United States of America*, 92(15), pp.6991–6995.

Michel, G. et al., 2011. Luciferase-expressing Leishmania infantum allows the monitoring of amastigote population size, in vivo, ex vivo and in vitro. *PLoS Neglected Tropical Diseases*, 5(9), pp.1–7.

Miguel, D.C., Yokoyama-Yasunaka, J.K.U. & Uliana, S.R.B., 2008. Tamoxifen is effective in the treatment of Leishmania amazonensis infections in mice. *PLoS Neglected Tropical Diseases*, 2(6).

Millington, O.R. et al., 2010. Imaging of the host/parasite interplay in cutaneous leishmaniasis. *Experimental parasitology*, 126(3), pp.310–7. Available at: http://www.ncbi.nlm.nih.gov/pubmed/20501336 [Accessed August 4, 2011].

Millington, O.R. et al., 2007. Malaria impairs T cell clustering and immune priming despite normal signal 1 from dendritic cells. *PLoS Pathogens*, 3(10), pp.1380–1387.

Misslitz, a et al., 2000. Targeted integration into a rRNA locus results in uniform and high level expression of transgenes in Leishmania amastigotes. *Molecular and biochemical parasitology*, 107(2), pp.251–61. Available at: http://www.ncbi.nlm.nih.gov/pubmed/10779601.

Mollinedo, F. et al., 2010. Selective fusion of azurophilic granules with Leishmania-containing phagosomes in human neutrophils. *The Journal of biological chemistry*, 285(45), pp.34528–36. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2966068&tool=pmcentrez&rendertype=abstract [Accessed January 14, 2012].

Mony, B.M. et al., 2014. Genome-wide dissection of the quorum sensing signalling pathway in Trypanosoma brucei. *Nature*, 505(7485), pp.681–5. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3908871&tool=pmcentrez&rendertype=abstract.

Moradin, N. & Descoteaux, A., 2012. Leishmania promastigotes: building a safe niche within macrophages. *Frontiers in Cellular and Infection Microbiology*, 2(September), pp.1–7.

Morales, M. a et al., 2010. Phosphoproteome dynamics reveal heat-shock protein complexes specific to the Leishmania donovani infectious stage. *Proceedings of the National Academy of Sciences of the United States of America*, 107(18), pp.8381–8386.

Morales, M. a. et al., 2007. Over-expression of Leishmania major MAP kinases reveals stage-specific induction of phosphotransferase activity. *International Journal for Parasitology*, 37(11), pp.1187–1199.

Morales, M. a. et al., 2008. Phosphoproteomic analysis of Leishmania donovani pro- and amastigote stages. *Proteomics*, 8(2), pp.350–363.

Morales, M. a., Pescher, P. & Späth, G.F., 2010. Leishmania major MPK7 protein kinase activity inhibits intracellular growth of the pathogenic amastigote stage. *Eukaryotic Cell*, 9(1), pp.22–30.

Morrison, L.S. et al., 2012. Ecotin-like serine peptidase inhibitor ISP1 of Leishmania major plays a role in flagellar pocket dynamics and promastigote differentiation. *Cellular Microbiology*, 14(8), pp.1271–1286.

Mottram, J.C. et al., 1993. A novel CDC2-related protein kinase from Leishmania mexicana, LmmCRK1, is post-translationally regulated during the life cycle. *Journal of Biological Chemistry*, 268(28), pp.21044–21052.

Mottram, J.C. et al., 1996. Gene disruptions indicate an essential function for the LmmCRK1 cdc2-related kinase of Leishmania mexicana. *Molecular microbiology*, 22(3), pp.573–583.

Müller, I.B. et al., 2002. Stage-specific requirement of a mitogen-activated protein kinase by Trypanosoma brucei. *Molecular biology of the cell*, 13(11), pp.3787–3799.

Murta, S.M.F. et al., 2009. Methylene tetrahydrofolate dehydrogenase/cyclohydrolase and the synthesis of 10-CHO-THF are essential in Leishmania major. *Molecular microbiology*, 71(6), pp.1386–401. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2692627&tool=pmcentrez&rendertype=abstract [Accessed July 15, 2014].

Mutizwa, M.M., Berk, D.R. & Anadkat, M.J., 2011. Treatment of facial angiofibromas with topical application of oral rapamycin solution (1 mg mL - 1) in two patients with tuberous sclerosis. *British Journal of Dermatology*, 165(4), pp.922–923.

Nagy, a, 2000. Cre recombinase: the universal reagent for genome tailoring. *Genesis (New York, N.Y. : 2000)*, 26(2), pp.99–109. Available at: http://www.ncbi.nlm.nih.gov/pubmed/10686599.

Naula, C., Parsons, M. & Mottram, J.C., 2005. Protein kinases as drug targets in trypanosomes and Leishmania. *Biochimica et Biophysica Acta - Proteins and Proteomics*, 1754, pp.151–159.

Ng, L.G. et al., 2008. Migratory dermal dendritic cells act as rapid sensors of protozoan parasites. *PLoS pathogens*, 4(11), p.e1000222. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2583051&tool=pmcentrez&rendertype=abstract [Accessed June 17, 2011].

Ngô, H. et al., 1998. Double-stranded RNA induces mRNA degradation in Trypanosoma brucei. *Proceedings of the National Academy of Sciences of the United States of America*, 95(25), pp.14687–14692.

Okwor, I. & Uzonna, J.E., 2013. The immunology of Leishmania/HIV co-infection. *Immunologic Research*, 56(1), pp.163–171.

Orban, P.C., Chui, D. & Marth, J.D., 1992. Tissue- and site-specific DNA recombination in transgenic mice. *Proceedings of the National Academy of Sciences of the United States of America*, 89(15), pp.6861–6865.

Osorio, E. et al., 2013. Biochemical characterization of the bifunctional enzyme dihydrofolate reductase-thymidylate synthase from Leishmania (Viannia) and its evaluation as a drug target. *Biomédica : revista del Instituto Nacional de Salud*, 33(3), pp.393–401. Available at: http://www.ncbi.nlm.nih.gov/pubmed/24652175.

Paape, D. et al., 2014. Using a Non-Image-Based Medium-Throughput Assay for Screening Compounds Targeting N-myristoylation in Intracellular Leishmania Amastigotes. *PLoS Neglected Tropical Diseases*, 8(12), p.e3363. Available at: http://dx.plos.org/10.1371/journal.pntd.0003363.

Parkitna, J.R. et al., 2009. *Gene Knockout Protocols*, Available at: http://www.springerlink.com/index/10.1007/978-1-59745-471-1\nhttp://link.springer.com/10.1007/978-1-59745-471-1\nhttp://www.springerlink.com/content/978-1-934115-26-8/.

Parsons, M. et al., 2005. Comparative analysis of the kinomes of three pathogenic trypanosomatids: Leishmania major, Trypanosoma brucei and Trypanosoma cruzi. *BMC genomics*, 6, p.127.

Parsons, M., Nelson, R.G. & Agabian, N., 1986. The trypanosome spliced leader small RNA gene family: Stage-specific modification of one of several similar dispersed genes. *Nucleic Acids Research*, 14(4), pp.1703–1717.

Patterson, H. et al., 2014. Protein kinase inhibitors in the treatment of inflammatory and autoimmune diseases. *Clinical and Experimental Immunology*, 176(1), pp.1–10.

Peters, N.C., 2008. In vivo imaging reveals an essential role for neutrophils in leishmaniasis transmitted by sand flies. *Science*, 321(December), pp.970–975.

Peters, N.C. et al., 2008. In vivo imaging reveals an essential role for neutrophils in leishmaniasis transmitted by sand flies. *Science*, 321(5891), pp.970–974. Available at: http://www.ncbi.nlm.nih.gov/pubmed/18703742.

Peters, N.C. & Sacks, D.L., 2009. The impact of vector-mediated neutrophil recruitment on cutaneous leishmaniasis. *Cellular microbiology*, 11(9), pp.1290–6. Available at: http://www.ncbi.nlm.nih.gov/pubmed/19545276 [Accessed January 31, 2012].

Petritus, P.M. et al., 2012. Leishmania mexicana Induces Limited Recruitment and Activation of Monocytes and Monocyte-Derived Dendritic Cells Early during Infection. *PLoS neglected tropical diseases*, 6(10), p.e1858. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3475671&tool=pmcentrez&rendertype=abstract [Accessed December 5, 2012].

Pfister, D.D. et al., 2006. A mitogen-activated protein kinase controls differentiation of bloodstream forms of Trypanosoma brucei. *Eukaryotic Cell*, 5(7), pp.1126–1135.

Pflicke, H. & Sixt, M., 2009. Preformed portals facilitate dendritic cell entry into afferent lymphatic vessels. *The Journal of experimental medicine*, 206(13), pp.2925–35. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2806476&tool=pmcentrez&rendertype=abstract [Accessed August 17, 2011].

Pinheiro, R.O. et al., 2009. Induction of autophagy correlates with increased parasite load of Leishmania amazonensis in BALB/c but not C57BL/6 macrophages. *Microbes and Infection*, 11(2), pp.181–190.

Ploubidou, a et al., 1999. Evidence for novel cell cycle checkpoints in trypanosomes: kinetoplast segregation and cytokinesis in the absence of mitosis. *Journal of cell science*, 112 (Pt 2, pp.4641–4650.

Price, H.P. et al., 2003. Myristoyl-CoA:Protein N-myristoyltransferase, an essential enzyme and potential drug target in kinetoplastid parasites. *Journal of Biological Chemistry*, 278(9), pp.7206–7214.

Rachidi, N. et al., 2014. Pharmacological assessment defines Leishmania donovani casein kinase 1 as a drug target and reveals important functions in parasite viability and intracellular infection. *Antimicrobial Agents and Chemotherapy*, 58(3), pp.1501–1515.

Racoosin, E.L. & Beverley, S.M., 1997. Leishmania major: promastigotes induce expression of a subset of chemokine genes in murine macrophages. *Experimental parasitology*, 85(3), pp.283–295.

Ramdas, S., 2012. Social Science & Medicine Cruel disease , cruel medicine : Self-treatment of cutaneous leishmaniasis with harmful chemical substances in Suriname. *Social Science & Medicine*, 75(6), pp.1097–1105. Available at: http://dx.doi.org/10.1016/j.socscimed.2012.04.038.

Ribeiro-Gomes, F.L. et al., 2012. Efficient Capture of Infected Neutrophils by Dendritic Cells in the Skin Inhibits the Early Anti-Leishmania Response I. Müller, ed. *PLoS Pathogens*, 8(2), p.e1002536. Available at:

http://dx.plos.org/10.1371/journal.ppat.1002536 [Accessed February 18, 2012].

Ritter, U., Frischknecht, F. & van Zandbergen, G., 2009. Are neutrophils important host cells for Leishmania parasites? *Trends in parasitology*, 25(11), pp.505–10. Available at: http://www.ncbi.nlm.nih.gov/pubmed/19762280 [Accessed August 31, 2011].

Robertson, C.D., 1999. The Leishmania mexicana proteasome. *Molecular and Biochemical Parasitology*, 103(1), pp.49–60.

Robinson, K. a. & Beverley, S.M., 2003. Improvements in transfection efficiency and tests of RNA interference (RNAi) approaches in the protozoan parasite Leishmania. *Molecular and Biochemical Parasitology*, 128(2), pp.217–228.

Rogers, M. et al., 2009. Proteophosophoglycans regurgitated by Leishmania-infected sand flies target the L-arginine metabolism of host macrophages to promote parasite survival. *PLoS pathogens*, 5(8), p.e1000555. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2722086&tool=pmcentrez&rendertype=abstract [Accessed September 21, 2011].

Rogers, M.B. et al., 2011. Chromosome and gene copy number variation allow major structural change between species and strains of *Leishmania*. *Genome Res.*, 21, pp.2129–2142.

Rogers, M.E., 2012. The role of Leishmania proteophosphoglycans in sand fly transmission and infection of the mammalian host. *Frontiers in Microbiology*, 3(JUN), pp.1–13.

Romano, A. et al., 2014. Cross-species genetic exchange between visceral and cutaneous strains of Leishmania in the sand fly vector. *Proceedings of the National Academy of Sciences*, 111(47), pp.16808–16813. Available at: http://www.pnas.org/lookup/doi/10.1073/pnas.1415109111.

Rosas, L.E. et al., 2005. Genetic background influences immune responses and disease outcome of cutaneous L. mexicana infection in mice. *International immunology*, 17(10), pp.1347–57. Available at: http://www.ncbi.nlm.nih.gov/pubmed/16141242 [Accessed June 27, 2013].

Rouf, M.A. et al., 2009. Development and characterization of liposomal formulations for rapamycin delivery and investigation of their antiproliferative effect on MCF7 cells. *Journal of liposome research*, 19(4), pp.322–331.

Russell, S.H., Hoopes, J.L. & Odell, J.T., 1992. Directed excision of a transgene from the plant genome. *Molecular & general genetics : MGG*, 234(1), pp.49–59.

Sacks, D.L. et al., 1995. Stage-specific binding of Leishmania donovani to the sand fly vector midgut is regulated by conformational changes in the

abundant surface lipophosphoglycan. *The Journal of experimental medicine*, 181(2), pp.685–697.

Sacks, D.L. & Perkins, P. V, 1984. Identification of an infective stage of Leishmania promastigotes. *Science (New York, N.Y.)*, 223(4643), pp.1417–1419.

Saha, a K., Mukherjee, T. & Bhaduri, a, 1986. Mechanism of action of amphotericin B on Leishmania donovani promastigotes. *Molecular and biochemical parasitology*, 19(3), pp.195–200.

Sando, R. et al., 2013. Inducible control of gene expression with destabilized Cre. *Nature methods*, 10(11), pp.1085–8. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3947879&tool=pmcentrez&rendertype=abstract.

Sauer, B., 1987. Functional expression of the cre-lox site-specific recombination system in the yeast Saccharomyces cerevisiae. *Molecular and cellular biology*, 7(6), pp.2087–2096.

Sauer, B. & Henderson, N., 1988. Site-specific DNA recombination in mammalian cells by the Cre recombinase of bacteriophage P1. *Proceedings of the National Academy of Sciences of the United States of America*, 85(14), pp.5166–5170.

Scahill, M.D., Pastar, I. & Cross, G. a M., 2008. CRE recombinase-based positive-negative selection systems for genetic manipulation in Trypanosoma brucei. *Molecular and Biochemical Parasitology*, 157(1), pp.73–82.

Schwarz, T. et al., 2013. T Cell-Derived IL-10 Determines Leishmaniasis Disease Outcome and Is Suppressed by a Dendritic Cell Based Vaccine. *PLoS Pathogens*, 9(6).

Secundino, N. et al., 2010. Proteophosphoglycan confers resistance of Leishmania major to midgut digestive enzymes induced by blood feeding in vector sand flies. *Cellular Microbiology*, 12(7), pp.906–918.

Shi, H. et al., 2000. Genetic interference in Trypanosoma brucei by heritable and inducible double-stranded RNA. *RNA (New York, N.Y.)*, 6(7), pp.1069–1076.

Da Silva, R. & Sacks, D.L., 1987. Metacyclogenesis is a major determinant of Leishmania promastigote virulence and attenuation. *Infection and Immunity*, 55(11), pp.2802–2806.

Da Silva, R.P. et al., 1989. CR1, the C3b receptor, mediates binding of infective Leishmania major metacyclic promastigotes to human macrophages. *Journal of immunology (Baltimore, Md. : 1950)*, 143(2), pp.617–622.

Sollelis, L. et al., 2015. First efficient CRISPR-Cas9-mediated genome editing in Leishmania parasites. *Cellular Microbiology*, p.n/a–n/a. Available at: http://doi.wiley.com/10.1111/cmi.12456.

Späth, G.F. et al., 2003. The role(s) of lipophosphoglycan (LPG) in the establishment of Leishmania major infections in mammalian hosts. *Proceedings of the National Academy of Sciences of the United States of America*, 100(16), pp.9536–9541.

Stenger, S. et al., 1996. Reactivation of latent leishmaniasis by inhibition of inducible nitric oxide synthase. *The Journal of experimental medicine*, 183(4), pp.1501–1514.

Sternberg, N. & Hamilton, D., 1981. Bacteriophage P1 site-specific recombination. I. Recombination between loxP sites. *Journal of molecular biology*, 150(4), pp.467–486.

Sternberg, N., Hamilton, D. & Hoess, R., 1981. Bacteriophage P1 site-specific recombination. II. Recombination between loxP and the bacterial chromosome. *Journal of molecular biology*, 150(4), pp.487–507.

Sternberg, S.H. & Doudna, J.A., 2015. Expanding the Biologist's Toolkit with CRISPR-Cas9. *Molecular Cell*, 58(4), pp.568–574. Available at: http://linkinghub.elsevier.com/retrieve/pii/S1097276515001641.

Stierhof, Y.D. et al., 1999. Filamentous proteophosphoglycan secreted by Leishmania promastigotes forms gel-like three-dimensional networks that obstruct the digestive tract of infected sandfly vectors. *European journal of cell biology*, 78(10), pp.675–689.

Subramaniam, C. et al., 2006. Chromosome-wide analysis of gene function by RNA interference in the African trypanosome. *Eukaryotic Cell*, 5(9), pp.1539–1549.

Sundar, S. et al., 2000. Failure of pentavalent antimony in visceral leishmaniasis in India: report from the center of the Indian epidemic. *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America*, 31(4), pp.1104–1107.

Sundar, S. et al., 2007. *Injectable paromomycin for Visceral leishmaniasis in India.*,

Tal, O. et al., 2011. DC mobilization from the skin requires docking to immobilized CCL21 on lymphatic endothelium and intralymphatic crawling. *The Journal of experimental medicine*, 208(10), pp.2141–53. Available at: http://www.ncbi.nlm.nih.gov/pubmed/21930767 [Accessed September 29, 2011].

Tomura, M. et al., 2008. Monitoring cellular movement in vivo with photoconvertible fluorescence protein "Kaede" transgenic mice. *Proceedings of the National Academy of Sciences of the United States of America*, 105(31), pp.10871–6. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2504797&tool=pmcentrez&rendertype=abstract.

Tu, X. & Wang, C.C., 2004. The Involvement of Two cdc2-related Kinases (CRKs) in Trypanosoma brucei Cell Cycle Regulation and the Distinctive Stage-specific Phenotypes Caused by CRK3 Depletion. *Journal of Biological Chemistry*, 279(19), pp.20519–20528.

Walker, R.G. et al., 2011. High throughput screens yield small molecule inhibitors of Leishmania CRK3:CYC6 cyclin-dependent kinase. *PLoS neglected tropical diseases*, 5(4), p.e1033. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3071374&tool=pmcentrez&rendertype=abstract [Accessed December 9, 2014].

Walters, L.L., Chaplin, G.L. & Modi, G.B., 1989. Biology of Bra Ziliensis Pa Na Mensls) in Lutzomyia Gomezi (Diptera : Psychodidae): a Natural Host-Parasite Association. *Society*, 40(1), pp.19–39.

Wang, J. et al., 2004. Rapamycin control of exocrine protein levels in saliva after adenoviral vector-mediated gene transfer. *Gene therapy*, 11(8), pp.729–733.

Wang, Q. et al., 2005. LmxMPK4, a mitogen-activated protein (MAP) kinase homologue essential for promastigotes and amastigotes of Leishmania mexicana. *Kinetoplastid biology and disease*, 4, p.6.

Wang, Y. et al., 1998. Stage-specific activity of the Leishmania major CRK3 kinase and functional rescue of a Schizosaccharomyces pombe cdc2 mutant. *Molecular and Biochemical Parasitology*, 96(1-2), pp.139–150.

Wasan, K.M. et al., 1994. Decreased Toxicity of Liposomal Amphotericin-B Due To Association of Amphotericin-B With High-Density-Lipoproteins - Role of Lipid Transfer Protein. *Journal of Pharmaceutical Sciences*, 83(7), pp.1006–1010. Available at: <Go to ISI>://WOS:A1994NX39000015.

Webster, B. et al., 2006. Regulation of lymph node vascular growth by dendritic cells. *The Journal of experimental medicine*, 203(8), pp.1903–13. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2118366&tool=pmcentrez&rendertype=abstract [Accessed November 14, 2012].

Wheless, J.W. & Almoazen, H., 2013. A Novel Topical Rapamycin Cream for the Treatment of Facial Angiofibromas in Tuberous Sclerosis Complex. *Journal of child neurology*, 28(7), pp.933–936. Available at: http://www.ncbi.nlm.nih.gov/pubmed/23680945.

WHO, 2007. *Control of leishmaniasis.*,

Wierzbicki, a et al., 1987. A mutational analysis of the bacteriophage P1 recombinase Cre. *Journal of molecular biology*, 195(4), pp.785–794.

Wiese, M., 1998. A mitogen-activated protein (MAP) kinase homologue of Leishmania mexicana is essential for parasite survival in the infected host. *EMBO Journal*, 17(9), pp.2619–2628.

Wiese, M., 2007. Leishmania MAP kinases - Familiar proteins in an unusual context. *International Journal for Parasitology*, 37, pp.1053–1062.

Wirtz, E. & Clayton, C., 1995. Inducible gene expression in trypanosomes mediated by a prokaryotic repressor. *Science (New York, N.Y.)*, 268(5214), pp.1179–1183.

Wright, J.H., 1903. Protozoa in a Case of Tropical Ulcer ("Delhi Sore"). *The Journal of Medical Research*, 10(3), pp.472–482.7.

Wu, J. & Filutowicz, M., 1999. Hexahistidine (His6)-tag dependent protein dimerization: A cautionary tale. *Acta Biochimica Polonica*, 46(3), pp.591–599.

Yan, S., Myler, P.J. & Stuart, K., 2001. Tetracycline regulated gene expression in Leishmania donovani. *Molecular and biochemical parasitology*, 112(1), pp.61–69.

Yap, A. et al., 2014. Conditional expression of apical membrane antigen 1 in Plasmodium falciparum shows it is required for erythrocyte invasion by merozoites. *Cellular Microbiology*, 16(5), pp.642–656.

Yip, C.K. et al., 2010. Structure of the Human mTOR Complex I and Its Implications for Rapamycin Inhibition. *Molecular Cell*, 38(5), pp.768–774. Available at: http://dx.doi.org/10.1016/j.molcel.2010.05.017.

Van Zandbergen, G. et al., 2004. Cutting edge: neutrophil granulocyte serves as a vector for Leishmania entry into macrophages. *Journal of immunology (Baltimore, Md. : 1950)*, 173(11), pp.6521–6525.

Zhang, W. & Matlashewski, G., 2015. CRISPR-Cas9-Mediated Genome Editing in Leishmania donovani. *mBio*, 6(4), pp.e00861–15. Available at: http://mbio.asm.org/lookup/doi/10.1128/mBio.00861-15.