This thesis has been submitted in fulfilment of the requirements for a postgraduate degree (e.g. PhD, MPhil, DClinPsychol) at the University of Edinburgh. Please note the following terms and conditions of use:

This work is protected by copyright and other intellectual property rights, which are retained by the thesis author, unless otherwise stated.
A copy can be downloaded for personal non-commercial research or study, without prior permission or charge.
This thesis cannot be reproduced or quoted extensively from without first obtaining permission in writing from the author.
The content must not be changed in any way or sold commercially in any format or medium without the formal permission of the author.
When referring to this work, full bibliographic details including the author, title, awarding institution and date of the thesis must be given.
What do kinetoplastids need a kinetoplast for?

Life cycle progression of *Trypanosoma brucei* in the presence and absence of mitochondrial DNA

Caroline E. Dewar

University of Edinburgh
2016
Abstract
The parasitic protist *Trypanosoma brucei* is the causative agent of human African trypanosomiasis. The parasite undergoes a complex life cycle involving stages within the mammalian bloodstream and its tsetse fly vector. The fundamental differences between energy metabolism in the procyclic insect form (PCF) and long slender bloodstream form (BSF) *T. brucei* involve a switch in the directionality of the F₁F₀-ATPase. In PCF, the need for oxidative phosphorylation in low glucose conditions requires the enzyme to generate ATP. In the slender BSF, the enzyme uses ATP from glycolysis to drive proton pumping to maintain the essential mitochondrial membrane potential. F₀-ATPase subunit 6 (A6) is critical for proton translocation in either direction and is encoded in the mitochondrial DNA (kDNA). The parasite’s kDNA is therefore essential in the slender BSF, and also in PCF where it encodes multiple subunits of the respiratory chain complexes that constitute the oxidative phosphorylation pathway.

Specific point mutations in the nuclearly encoded γ subunit of the mitochondrial F₁F₀-ATPase allow survival in the absence of kDNA in the slender BSF *T. brucei* (Dean et al., 2013). These mutations, even in the heterozygous genotype, cause an increase in resistance to multiple drugs *in vitro* (Gould and Schnaufer, 2014).

This thesis investigates two questions:

1. What is the molecular mechanism of compensation for kDNA loss?
2. Are kDNA and a functional F₀F₁-ATPase required for life cycle progression?
Slender BSF *T. brucei* were generated expressing ATPase L262Pγ. The effects of this γ mutation and kDNA loss, respectively, on structure/function of the $F_1F_\text{o}$-ATPase were probed. Cells expressing L262Pγ show decreased sensitivity to $F_\text{o}$ inhibitor oligomycin compared to WT cells, suggesting that the L262Pγ mutation functionally uncouples the enzyme. The impact of the L262Pγ mutation on the structure of the enzyme was probed by high resolution clear native electrophoresis. This shows there are dramatic consequences to $F_1F_\text{o}$ structure in the presence of the L262Pγ mutation. The apparent selection for cells that no longer express intact $F_1F_\text{o}$ suggests that L262Pγ uncouples the enzyme, resulting in a lethal proton leak.

Pleomorphic *T. brucei* with and without kDNA were also generated by expressing mutant γ in strain AnTat1.1 90:13. Differentiation studies demonstrate kDNA0 cells can differentiate to insect-transmissible stumpy forms. These cells show a decreased lifespan, suggesting a critical role for a kDNA-encoded product in the stumpy form. Tsetse fly infections show kDNA is indispensable for progression to the PCF. Unexpectedly, parasites homozygous for L262Pγ can establish a midgut infection, while they do not infect the salivary glands. Heterozygous parasites, on the other hand, can form animal-transmissible metacyclics in the salivary glands, providing a potential mechanism for spreading decreased sensitivity to multiple drugs.
Lay summary

A long long time ago, a bacterial-like creature gobbled up another bacterial-like creature. This seemingly innocuous occurrence actually ended up kick-starting life on this planet as we know it. The now-internal bacterium benefitted the consumer so much that the consumer ended up retaining it, and so the little bacterium continued to survive within its consumer. So, after millions of years of evolution, the descendant of that little bacterium still exists within our cells. Now, it has become the primary energy producer in our cells, the so-called ‘powerhouse’ of the cell: the mitochondrion. Without it, we, along with other creatures that now enjoy a multi-cellular existence, with many different tissues specialized in their function like eyes, and heart and teeth, would simply not exist.

This little bacterium contained DNA essential for its survival. DNA encodes within it the building blocks of life, proteins. Although much of this bacterium’s DNA has eroded away over the years as it was no longer needed for life within another cell, some of this DNA does still remain. It is this mitochondrial DNA that is now essential to forensic technique. In fact it was his mitochondrial DNA that allowed that skeleton in a Leicester car park to be identified as being Richard III.

I currently work on mitochondrial DNA. This thesis details research undertaken on an African parasite called *Trypanosoma brucei*, a single-celled organism that contains a single mitochondrion per cell. This parasite causes Sleeping Sickness, a human and animal disease spread by the tsetse fly. The fly lands on its victim, starts to suck blood whilst the trypanosomes within the fly saliva swim out into the
victim’s bloodstream, prepared to proliferate and survive within that new host. When that victim is bitten again, some of those trypanosomes can be taken up by the fly, which will bite another victim once it is hungry again. In this way the disease is spread. New drugs are needed to cure the disease and stop the trypanosomes surviving in the mammalian bloodstream.

The mitochondrial DNA in trypanosomes has a very unusual structure, as it is made up of many circular strands of DNA that are interlinked like chainmail. Because this ‘kinetoplast’ structure requires much regulation to be built and maintained, and our human mitochondria have a less complex DNA structure, the kinetoplast represents a perfect target for new drugs: the drug will kill the parasite by preventing this essential structure from staying together, whereas it will not affect us and our mitochondrial DNA at all. Sounds great!

Unfortunately, this is more difficult than it sounds. Trypanosomes, being ‘tricksy’ little creatures that have caused disease for centuries, have an answer to everything. Some mutated trypanosomes are able to survive without their mitochondrial DNA-and as not having mitochondrial DNA means that there is no kinetoplast structure, these drugs will not work.

My research centres around trying to understand how these mutated trypanosomes survive in the mammalian bloodstream without their mitochondrial DNA, a normally essential piece of survival toolkit. It turns out they have a mutation in their $F_1F_0$-ATP synthase, the protein that basically generates energy currency for the cell.
Spectacularly, this one tiny mutation makes the proteins encoded in the mitochondrial DNA completely redundant, allowing the DNA encoding these proteins to be deleted with no effect to the cell.

Understanding the process of how these parasites survive without their mitochondrial DNA is important because (1) if we know what a creature needs to stay alive, we also know how to kill them. This will enable us to design drugs to eradicate these mutated parasites and the disease they cause. (2) As many other parasites and disease-causing creatures also can survive without their mitochondrial DNA, this work can be used to further understand how they survive.
Declaration

I declare that the work within this thesis is my own, unless otherwise stated. The work has not been submitted for any other degree previously. No work in this thesis has yet been published.

Caroline Dewar

Experimental contribution

Dr Paula MacGregor performed half of the morphology scoring, contributed to optimisation of the IFA protocol and contributed to the generation of Figures 3.5, 3.6, 3.7 and 3.8 in Chapter 3.

Dr Nick Savill designed the mathematical models, fit the data to the models, analysed model fit and generated Figs 3.10-3.17 in Chapter 3.

Aitor Casas performed half of the dissections in Chapter 4.
Acknowledgements

At the risk of this becoming like an Oscars speech, there are many people I would like to thank for their help and support during the last 4 years.

I would like to thank my supervisor Achim Schnaufer for enabling me to work on this fascinating area of biology. I want to express my gratitude for the constant challenges and opportunities provided. I really appreciate the amount of time and effort you have spent in mentoring me, and for the support, guidance and encouragement I have received during my PhD.

I have been extremely fortunate to have had a great lab group around me during my PhD. To previous members Laura and Matt, thank you for taking me under your wing, for creating a great environment to work in and for the still constant support and guidance. To Stephan, it is still such a shock that you are no longer with us. Your presence is sorely missed. And to the current members of the lab, Sinclair, thank you in particular for the laughs and for your bioinformatic wizardry. To Claudia, Migla, Julie, Gloria and Karolina, I look forward to properly getting to know you all now this is done!

I would like to thank my second supervisor Keith Matthews for his advice on the experiments in Chapter 3 and for his interest in my project. I would like to express my gratitude to Paula MacGregor for the morphology scoring she performed, staring at blue dots down a microscope for hours on end in a dark room. I learnt a lot about experimental planning and process from our discussions. Thank you to Nick Savill for the mathematical modeling performed in Chapter 3, and for being patient with my constant questioning.

I would like to express my appreciation to our collaborator Alvaro Acosta Serrano and his lab group for making me feel extremely welcome upon my visits to LSTM in Liverpool. I would like to thank Aitor, Lee and Naomi for teaching me the intricacies of tsetse fly dissections, and to also thank Aitor for all the hours of dissections he carried out with me. I would like to thank Dan for keeping my flies 'fed and watered' and for the training I received in tsetse fly infections. To Alvaro, thank you for supporting our project, and for the time spent chatting about potential hypotheses and also about career plans. It was very much appreciated.
I would also like to thank past and present members of the Matthews lab group and Robbie. I’ve really benefitted from being able to work alongside you all and hearing about the progress in your various projects. Thank you for the advice, the support and friendships. In particular thank you to Julie for the support and help with PIL work. And to Rachel- we got there in the end!

I would also like to thank a long list of extremely important people in my life:

Firstly, to the people that came into my life when I moved to Edinburgh. To Angela and Georgina, and Miguel and James, it is so hard to know that after 4 years we are all going our separate ways. Your friendships and this time we spent together have meant the world to me. Thank you for the lunches, the sports chat (well two of you anyway), the gym sessions, the karaoke screeching, the evenings spent watching the Notebook…and for always cooking for me.

And to the other people I have met in Edinburgh- Dawn, Bex, Marta, Jana, Gillian, Bridget, Alison to name just a few. I am incredibly fortunate to be surrounded by such a supportive, fun and brilliant group of people. In particular, thank you to Simon, who unwittingly entered this finishing PhD student’s life, and didn’t run a mile.

To the Circus crew- Dan, Charlie, honorary member Cecilia, and in particular to Hazel. I am so grateful that our friendships are just as strong as they were, despite the differences in geography. It is incredibly difficult for me to put into words how important our memories are to me, they never fail to make me smile. Thank you for being a constant source of support, love and laughter in my life, and I thoroughly look forward to our next chapter.

To my oldest friends Charlie, Katie, Nikki, Emma and Alice, despite all of our lives now being very different, thank you for still always being there, for listening and for visiting. Long may our November trips continue.

And, finally, to my parents and brother. Thank you for the belief and confidence you have in me that seemingly never dims, and for always knowing exactly what to say.

This is dedicated to my grans, Vera and Betty, whose unconditional love and support will never leave me.
Abbreviations

1,3-BPG 1,3-bisphosphoglycerate
3C three carbon-molecule containing compound
8pCPT-cAMP 8-(4-chlorophenylthio)adenosine 3’,5’-cyclic monophosphate
A273P Alanine to proline substitution at amino acid position 273
A281- alanine deletion at amino acid position 281
A6 subunit a of T. brucei F₁Fₒ ATPase
AAC ATP/ADP carrier
AIC Akaike information criterion
AK akinetoplastic
ApoL1 apolipoprotein L1
ASCT acetyl:succinate CoA-transferase
ATOM archaic translocase of the outer mitochondrial membrane
BARP bloodstream alanine-rich proteins (misnomer)
BSA bovine serum albumin
BSF bloodstream form
CA cisaconitate
cAMP cyclic adenosine monophosphate
CBP calmodulin binding protein
COX cytochrome oxidase subunit
CR cytosine-rich template
CyB cytochrome b
DAPI 4’, 6-diamidino-2-phenylindole
DDM n-Dodecyl β-D-maltoside
DFMO D,L-alpha-difluoromethylornithine, eflornithine
DHAP dihydroxyacetone phosphate
DK dyskinetoplastic
dTMP thymidine monophosphate
E. coli Escherichia coli
EC₅₀ half maximal effective concentration
ED₅₀ median effective dose
EP glutamate-proline repeat containing protein
ER endoplasmic reticulum
EtBr  ethidium bromide
FAD  flavin adenine dinucleotide
FAZ  flagellar attachment zone
FCCP  carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone
FCS  fetal calf serum
Fe/S  iron sulfur centre
FMN  flavin mononucleotide
GCC  glycine cleavage complex
G-3-P  glyceraldehyde-3-phosphate
Gly-3-P  glycerol-3-phosphate
G3PDH  glycerol-3-phosphate dehydrogenase
GPEET  glycine-proline-glutamate-glutamate threonine
pentapeptide repeat-containing protein
GPI  glycosylphosphatidylinositol
gRNA  guide RNA
HAT  Human African Trypanosomiasis
HpHbR  haptoglobin-haemoglobin receptor
HPR  haptoglobin-related protein
hrCNE  high resolution clear native electrophoresis
IC_{50}  half minimal inhibitory concentration
IFA  immunofluorescence assay
IMS  intermembrane space
IP  intraperitoneal
ISG  invariant surface glycoprotein
KCN  potassium cyanide
kDNA  kinetoplast DNA
kDNA^+  cells retaining their kDNA
K. lactis  Kluyveromyces lactis
L262P  lysine to proline substitution at amino acid position 262
M282L  methionine to lysine substitution at amino acid position 282
MoA  minicircle type A
mtDNA  mitochondrial DNA
MURF  maxicircle unidentified reading frame
NAD+  nicotinamide adenine dinucleotide (oxidised)
NADH  nicotinamide adenine dinucleotide (reduced)
<table>
<thead>
<tr>
<th>Term</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>ND</td>
<td>NADH dehydrogenase subunit</td>
</tr>
<tr>
<td>NDH2</td>
<td>alternative NADH dehydrogenase</td>
</tr>
<tr>
<td>OSCP</td>
<td>oligomycin sensitivity-conferring protein (misnomer)</td>
</tr>
<tr>
<td>P. serpens</td>
<td><em>Phytomonas serpens</em></td>
</tr>
<tr>
<td>PAD1</td>
<td>proteins associated with differentiation</td>
</tr>
<tr>
<td>PAGE</td>
<td>polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PCF</td>
<td>procyclic form, first insect stage</td>
</tr>
<tr>
<td>PDH</td>
<td>pyruvate dehydrogenase</td>
</tr>
<tr>
<td>PFA</td>
<td>paraformaldehyde</td>
</tr>
<tr>
<td>PM</td>
<td>peritrophic matrix</td>
</tr>
<tr>
<td>PI</td>
<td>propidium iodide</td>
</tr>
<tr>
<td>PV</td>
<td>proventriculus</td>
</tr>
<tr>
<td>PVDF</td>
<td>polyvinylidene fluoride</td>
</tr>
<tr>
<td>q+</td>
<td>positive charge</td>
</tr>
<tr>
<td>qPCR</td>
<td>quantitative polymerase chain reaction</td>
</tr>
<tr>
<td>qRT-PCR</td>
<td>quantitative reverse transcriptase-polymerase chain reaction</td>
</tr>
<tr>
<td>REL1</td>
<td>RNA editing ligase 1</td>
</tr>
<tr>
<td>RNAi</td>
<td>RNA interference</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>RPS</td>
<td>40S ribosomal protein S12</td>
</tr>
<tr>
<td>rRNA</td>
<td>ribosomal RNA</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
</tr>
<tr>
<td>S. cerevisiae</td>
<td><em>Saccharomyces cerevisiae</em></td>
</tr>
<tr>
<td>SHAM</td>
<td>Salicylhydroxamic acid</td>
</tr>
<tr>
<td>SILAC</td>
<td>stable isotope labeling by amino acids in cell culture</td>
</tr>
<tr>
<td>SIF</td>
<td>Stumpy Induction Factor</td>
</tr>
<tr>
<td>SNP</td>
<td>single nucleotide polymorphism</td>
</tr>
<tr>
<td>SRA</td>
<td>serum resistance-associated</td>
</tr>
<tr>
<td>T. b. gambiense</td>
<td><em>Trypanosoma brucei gambiense</em></td>
</tr>
<tr>
<td>T. b. brucei</td>
<td><em>Trypanosoma brucei brucei</em></td>
</tr>
<tr>
<td>T. b. equiperdum</td>
<td><em>Trypanosoma brucei equiperdum</em></td>
</tr>
<tr>
<td>T. b. evansi</td>
<td><em>Trypanosoma brucei evansi</em></td>
</tr>
<tr>
<td>T. b. rhodesiense</td>
<td><em>Trypanosoma brucei rhodesiense</em></td>
</tr>
<tr>
<td>T. vivax</td>
<td><em>Trypanosoma vivax</em></td>
</tr>
<tr>
<td>TAC</td>
<td>tripartite attachment complex</td>
</tr>
<tr>
<td>Acronym</td>
<td>Definition</td>
</tr>
<tr>
<td>---------</td>
<td>------------------------------------------------</td>
</tr>
<tr>
<td>TAP</td>
<td>tandem affinity purification</td>
</tr>
<tr>
<td>TAO</td>
<td>trypanosome alternative oxidase</td>
</tr>
<tr>
<td>TgsGP</td>
<td><em>T. b. gambiense</em>-specific glycoprotein</td>
</tr>
<tr>
<td>TLF</td>
<td>trypanosome lytic factor</td>
</tr>
<tr>
<td>TMRE</td>
<td>tetramethylrhodamine</td>
</tr>
<tr>
<td>UTR</td>
<td>Untranslated region</td>
</tr>
<tr>
<td>VSG</td>
<td>variable surface glycoprotein</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organisation</td>
</tr>
<tr>
<td>WT</td>
<td>wild type</td>
</tr>
<tr>
<td>Δψm</td>
<td>mitochondrial membrane potential</td>
</tr>
</tbody>
</table>
# Table of Contents

Abstract .........................................................................................................................i

Lay summary .................................................................................................................. iii

Declaration ......................................................................................................................... vi

Experimental contribution ............................................................................................... vi

Acknowledgements ............................................................................................................ vii

Abbreviations .................................................................................................................... ix

Table of Contents ............................................................................................................. xiii

1. Introduction ................................................................................................................. 1
   1.1 Trypanosomes as a pathogen ...................................................................................... 2
      1.1.1 The human disease .......................................................................................... 2
      1.1.2 Nagana .......................................................................................................... 5
      1.1.3 Vector control .................................................................................................. 5
      1.1.4 Vaccination prospects ...................................................................................... 6
      1.1.5 The current therapies against HAT ............................................................... 7
      1.1.6 Drug resistance in T. brucei ........................................................................... 9
      1.1.7 New therapies ............................................................................................... 10
   1.2 A summary of the cell biology of the parasite ....................................................... 11
   1.3 Trypanosoma brucei as a model organism .............................................................. 12
      1.3.1 Unusual aspects of cell biology .................................................................... 13
      1.3.1.1 VSG ....................................................................................................... 13
      1.3.1.2 Glycosomes ............................................................................................ 14
      1.3.1.3 Gene expression ...................................................................................... 14
      1.3.2 Unusual aspects of mitochondrial biology .................................................. 15
      1.3.2.1 kDNA ................................................................................................... 15
      1.3.2.2 RNA Editing ......................................................................................... 16
   1.4 Cell cycle of trypomastigote stages ......................................................................... 17
   1.5 The T. brucei life cycle ............................................................................................ 18
      1.5.1 Bloodstream stages ....................................................................................... 19
      1.5.2 Insect stages ................................................................................................ 21
      1.5.3 Differentiation within a fly ............................................................................ 24
      1.5.4 Difficulty studying certain stages of the trypanosome life cycle .............. 26
      1.5.5 Mitochondrial function through the life cycle ............................................ 27
   1.6 Mitochondrion ......................................................................................................... 29
      1.6.1 The mitochondrial respiratory chain ............................................................. 29
      1.6.2 Supercomplexes of the inner mitochondrial membrane ................................... 32
      1.6.3 The T. b. brucei respiratory chain ................................................................. 33
      1.6.3.1 TAO ...................................................................................................... 33
      1.6.3.2 G3PDH ................................................................................................. 34
      1.6.3.3 Complex II ............................................................................................. 35
      1.6.3.4 AAC ..................................................................................................... 35
      1.6.4 The respiratory complexes containing maxicircle gene products .............. 36
      1.6.4.1 Complex I ............................................................................................... 36
      1.6.4.2 Complex III ........................................................................................... 38
2. Investigation into the mechanism for survival of BSF *T. brucei* in the absence of kDNA..........................83

2.1 Introduction.................................................................................................................84
   2.1.1 Hypothesis.................................................................................................................85
   2.1.2 Study design.................................................................................................................86

2.2 Materials and Methods .............................................................................................87
   2.2.1 Generation of cell lines with TAP-tagged ATPase subunits ..............................87
   2.2.2 Generation of heterozygous γL262P cell lines.....................................................88
   2.2.2.1 Confirmation of γ genotype in transfected clones.................................88
   2.2.3 Generation of homozygous γL262P/L262P cell lines...........................................89
   2.2.4 Generation of AK cell lines ......................................................................................90
   Table 2.1 Primer combinations used to amplify kDNA and nuclear components from gDNA ........................................................................................................90
   2.2.5 Growth analysis *in vitro* .......................................................................................91
2.2.6 Pyrosequencing................................................................. 91
2.2.6.1 Design of pyrosequencing primers and PCR of pyrosequencing templates..... 91
2.2.6.2 Pyrosequencing reaction.................................................... 91
2.2.7 Pulldown analysis of mitochondrial F_{1}F_{o}-ATPase ....................................... 92
2.2.8 Western blot................................................................. 93
Table 2.2 Antibodies used for western blotting in this chapter ......................... 94
2.2.9 Mitochondrial extraction .................................................... 94
2.2.9.1 Preparation of crude mitochondrial extracts........................................ 94
2.2.9.2 hrCNE................................................................. 95
2.2.10 cDNA preparation ....................................................... 96
2.2.10.1 RNA extraction....................................................... 96
2.2.10.2 DNase treatment of extracted RNA........................................... 97
2.2.10.3 Nano Agilent analysis of RNA............................................. 97
2.2.10.4 Reverse transcription of T. b. brucei RNA......................................... 97
2.2.11 Quantitative analysis of expression levels of maxicircle gene transcripts ...... 98
2.2.12 Amplification of A6 transcripts............................................... 99
2.2.13 In vitro analysis of sensitivity to F_{1}F_{o}-ATPase inhibitors ......................... 99
2.2.14 Δψm measurement ......................................................... 100
2.3 Results .............................................................................. 101
2.3.1 Generation of TAP-tagged cell lines ............................................. 101
2.3.2 Generation of WT/L262Pγ and L262P/L262Pγ cell lines .............................. 102
2.3.3 Generation of AK cell lines.................................................. 103
Table 2.3 Cell lines produced in Chapter 2 ............................................... 104
2.3.4 Assessment of the effect of L262Pγ alleles and kDNA loss on in vitro growth 105
2.3.5 Expression levels of the L262Pγ allele in heterozygous cell lines .............. 105
2.3.6 Probing the effect of L262Pγ mutation on F_{1}F_{o}-ATPase complex subunit composition .............................................................................. 106
2.3.7 Optimisation of crude mitochondrial extractions ....................................... 106
2.3.8 Putative F_{o} and stator subunits are not detectable in L262P/L262Pγ cell line clones A, B and 3 ................................................................. 111
2.3.9 The L262Pγ mutation causes structural disruption of the F_{1}F_{o}-ATPase complex in most, but not all L262P/L262Pγ clones ................................................................. 112
2.3.10 L262P/L262Pγ clones C, 1 and 2 have structurally coupled F_{1}F_{o}-ATPases ... 115
2.3.11 The impact of L262Pγ on RNA editing of subunit A6 ................................ 116
2.3.12 The effect of L262Pγ on oligomycin sensitivity ..................................... 118
2.3.13 The effect of L262Pγ and kDNA loss on azide sensitivity ....................... 121
2.3.14 The effect of L262Pγ and kDNA loss on Δψm .................................... 124
2.4 Discussion ......................................................................... 126
Table 2.4 Summary of data from Chapter 2 ............................................... 128
2.4.1 kDNA loss causes disruption of the F_{o} moiety ..................................... 129
2.4.2 The L262Pγ mutation causes functional uncoupling of the F_{1}F_{o} complex .... 130
2.4.3 The L262Pγ mutation also causes a proton leak .................................... 131
2.4.4 How does L262Pγ impact Δψm generation? ....................................... 133
2.4.5 Do the same conclusions apply to pleomorphic cell lines? ..................... 135
2.4.6 The effect of prolonged culture on the compensatory mechanism ............ 137
2.4.7 What else affects Δψm generation? ............................................. 138
2.4.8 Does Δψm affect the growth of cells? ........................................... 140
2.4.9 Suggestions for follow up studies ............................................... 141
2.4.9.1 Kinetic analysis on F_{1}F_{o}-ATPase containing L262Pγ ....................... 142
2.4.9.2 Additional bioenergetic parameters ............................................ 143
2.4.9.3 Further subunit analysis of F_{1}-containing complexes ..................... 143
3. The requirement for kDNA in stumpy forms

3.1 Introduction

3.2 Materials and methods

3.2.1 Growth analysis of T. brucei cell lines in vitro
3.2.2 Animal handling .................................................................................................................. 183
3.2.3 In vivo mouse infection with T. brucei .................................................................................. 184
3.2.4 In vitro slender to stumpy differentiation of T. brucei AnTat1.1 90:13 WT/WTγ, WT/L262Pγ, WT/L262Pγ (AK) cell lines .................................................................................. 187
3.2.5 Anti-PAD1 IFA protocol using cells from in vivo mouse infections ................................. 187
3.2.6 Assessing life cycle stage markers PAD1 and COX IV via western blot ................. 188
Table 3.1 Antibodies used for western blotting in this chapter .............................................. 190
3.2.7 Mathematical model for T. brucei infection dynamics ...................................................... 190
3.2.8 Cell cycle analysis via flow cytometry .............................................................................. 190
3.2.9 Measurement of relative ΔΨm using flow cytometry ........................................................ 191

3.3 Results .................................................................................................................................. 192
3.3.1 Pleomorphic slender cells independent of kDNA are viable in in vitro culture. 192
3.3.2 Pleomorphic cells independent of kDNA are able to differentiate to morphologically stumpy forms in vivo...................................................................................... 192
3.3.3 AK cells that are morphologically stumpy express PAD1 .............................................. 193
3.3.4 AK pleomorphic slender cells are able to differentiate to a stumpy-like form in in vitro culture ................................................................................................. 194
3.3.5 A lack of kDNA does not affect the dynamics of slender to stumpy differentiation in vivo .............................................................................................................. 195
3.3.6 AK T. brucei have an altered first peak of parasitaemia ................................................... 197
3.3.7 Normalisation of morphology scoring by PAD1 staining .............................................. 199
3.3.8 Mathematical modelling of an in vivo infection with kDNA+ and AK T. brucei200
3.3.8.1 The mathematical model ............................................................................................... 202
3.3.8.2 A model including a term for SIF-independent differentiation better fits our infection dynamics data ................................................................................................. 203
Table 3.2 AIC analysis for comparison of models presented in this thesis ............................... 205
Table 3.3 AIC analysis for comparison of models used in MacGregor et al., 2011 207
3.3.8.3 Fitting alternative models to the data .......................................................................... 207
3.3.9 Infection dynamics as predicted from models with and without SIF independent differentiation term ............................................................................................................. 210
3.3.10 Parameter estimation using models with and without SIF independent differentiation term .................................................................................................................. 211
Table 3.4 A comparison between the average parameter estimates for all cell lines ......... 212
3.3.10.1 Differentiation ............................................................................................................. 213
3.3.10.2 Cell cycle duration ..................................................................................................... 213
3.3.10.3 Differentiated AK cells are predicted to have a shorter lifespan than kDNA+ cells ................................................................................................................................. 215
3.3.10.4 AK cells are predicted to activate the mouse immune system at an earlier time point ......................................................................................................................... 217
3.3.10.5 AK stumpy cells clearance is predicted to be independent of the immune system .............................................................................................................................. 218
3.3.11 AK cells do not have a second slender peak at day 7................................................. 219
3.3.12 Absence of kDNA can be associated with loss of ΔΨm in stumpy forms ....... 219

3.4 Discussion ............................................................................................................................ 222
3.4.1 The characteristics of in vivo mouse infections with AK trypanosomes ......................... 224
3.4.2 The generation of ΔΨm in stumpy forms ........................................................................ 227
3.4.3 Complex V direction in stumpy forms ............................................................................ 231
3.4.4 T. b. evansi is monomorphic not due to a loss of kDNA ............................................. 233
3.4.5 The mathematical model supports SIF concentration dependent differentiation 236
3.4.6 The model also supports that there is a constant level of background slender to stumpy differentiation in vivo .................................................................................. 237

xvii
3.4.7 Limitations of this study ................................................................. 238
3.4.7.1 Does ethidium bromide or acriflavine treatment alter infection dynamics? ... 238
3.4.7.2 Stumpy longevity in the absence of an immune system ............................. 239
3.4.7.3 Improvements to the mathematical model ........................................... 241
3.4.8 Outlook ....................................................................................... 242
3.4.8.1 Pleomorphic AK trypanosomes as a tool ............................................. 242
3.4.8.2 Is SIF independent differentiation an actual phenomenon? ...................... 244

4. The role of F1F0 ATP synthase and kDNA in differentiation and survival of T. brucei ................................................................. 265
4.1 Introduction ..................................................................................... 266
4.1.1 Hypothesis .................................................................................. 268
4.1.2 Study design ............................................................................... 268
4.2 Materials and Methods .................................................................. 269
4.2.1 Generation of stumpy forms ......................................................... 269
4.2.2 In vitro differentiation and PCF culture ............................................ 270
4.2.3 Assessing life cycle stage markers via western blot ............................ 271

3.5 Figures ......................................................................................... 245
Figure 3.1 Cumulative in vitro growth analysis of WTγ and kDNA-independent AnTat1.1 90:13 cell lines .......................................................... 245
Figure 3.2 AnTat1.1 90:13 cells with and without kDNA are able to differentiate to the stumpy form ............................................................... 246
Figure 3.3 Investigation into differentiation of kDNA-independent and AK cell lines to the stumpy-like form in the presence of 100 µM 8pCPT-cAMP ........................................ 247
Figure 3.4 Representative phase contrast images showing the morphology of cells through induction to the stumpy-like form using 8pCPT-cAMP ........................................ 248
Figure 3.5 The morphological changes occurring during in vivo mouse infection over time ........................................................................... 249
Figure 3.6 Representative phase contrast images showing the morphology of cells at days 4 to 8 of mouse infection ................................................. 250
Figure 3.7 A comparison between the morphology scoring of individual 1 and 2 .... 251
Figure 3.8 Parasitaemia within in vivo mouse infections over time ................. 252
Figure 3.9 Schematic of the mathematical model ......................................... 253
Figure 3.10 A comparison between models with and without SIF independent differentiation term ................................................................. 254
Figure 3.11 Measuring the goodness of fit of three alternative mathematical models to the data ........................................................................ 255
Figure 3.12 The fit of the mathematical model to the data for infections with T. brucei of genotype WT/WTγ ............................................................ 256
Figure 3.13 The fit of the mathematical model to the data for infections with T. brucei of genotype WT/L262Pγ ......................................................... 257
Figure 3.14 The fit of the mathematical model to the data for infections with T. brucei of genotype WT/L262Pγ (AK) clone 1 ........................................ 258
Figure 3.15 The fit of the mathematical model to the data for infections with T. brucei of genotype WT/L262Pγ (AK) clone 2 ........................................ 259
Figure 3.16 Parameter estimation for each cell line by a mathematical model incorporating both SIF independent and SIF dependent differentiation ........... 260
Figure 3.17 Parameter estimation for each cell line by a mathematical model incorporating only SIF dependent differentiation .................................. 261
Figure 3.18 A second slender peak does not appear at day 7 post infection for akinetoplast cell lines ..................................................................... 262
Figure 3.19 Absence of kDNA can be associated with loss of ΔΨm in stumpy forms. ......................................................................................... 263

4. The role of F1F0 ATP synthase and kDNA in differentiation and survival of T. brucei ................................................................. 265
Table 4.1 Antibodies used for western blotting in this chapter ........................................ 272
4.2.4 Quantitative measurement of EP expression ......................................................... 272
4.2.5 Tsetse fly handling and infections ................................................................. 273
4.2.6 Tsetse fly dissections .................................................................................... 274
4.2.7 Confirmation of γ genotype in AnTat1.1 90:13 clones ........................................ 275

4.3 Results .............................................................................................................. 276
4.3.1 AK clones express EP procyclin in response to CA ......................................... 276
4.3.2 L262P/L262Pγ PCF clones do not enter exponential growth in vitro .......... 278
4.3.3 SDM79 does not support exponential growth of freshly differentiated AnTat1.1
90:13 cell lines .................................................................................................. 279
4.3.4 AK stumpy form trypanosomes do not establish a tsetse fly midgut infection.. 280
4.3.5 L262Pγ-expressing stumpy forms can establish a tsetse fly PCF midgut infection ......................................................................................................................... 281
4.3.6 kDNA-independent cell line WT/L262Pγ clone 1 can establish a metacyclic
salivary gland infection ......................................................................................... 283
4.3.7 Metacyclic form WT/L262Pγ cells can infect mice ............................................. 284

4.4 Discussion ...................................................................................................... 285
4.4.1 kDNA is required for a midgut PCF infection .................................................... 286
4.4.2 Cell lines with the WT/L262Pγ genotype can be transmitted by tsetse flies .... 289
4.4.3 In vitro and in vivo conditions affect homozygous L262P/L262Pγ cells differently
.............................................................................................................................. 293
4.4.4 Why do freshly differentiated cells not grow in SDM79? ............................... 297
4.4.5 Limitations of this study .................................................................................. 299
4.4.5.1 Does L262P cause F1F0 ATP synthase to be less efficient? ......................... 299
4.4.5.2 Is completion of the life cycle by WT/L262Pγ cells dependent on
downregulation of the L262P allele? .................................................................. 300
4.4.6 Outlook .......................................................................................................... 302
4.4.6.1 Why do cell lines WT/WTγ and WT/L262Pγ clone 2 not differentiate to the
metacyclic form? ............................................................................................ 302
4.4.6.2 Are L262P/L262Pγ cell lines able to differentiate into the other fly stages in the
life cycle? ..................................................................................................... 303
4.4.6.3 How can L262P/L262Pγ cells generate ATP? .............................................. 304

4.5 Figures ......................................................................................................... 306
Figure 4.1 After treating stumpy cells for 24 hrs with CA, cells look morphologically
PCF .................................................................................................................... 306
Figure 4.2 EP procyclin is expressed by CA-induced AK cells ................................. 307
Figure 4.3 Newly differentiated L262P/L262Pγ cells are unable to proliferate in
vitro; glucose is toxic to freshly differentiated cells .............................................. 308
Figure 4.4 PCF specific markers can be detected in L262Pγ-expressing PCF cells .... 309
Figure 4.5 Experimental infection of tsetse fly midguts with kDNA independent and
AK trypanosomes .............................................................................................. 311
Figure 4.6 The morphology of PCF cells within the midgut ....................................... 312
Figure 4.7 Generation of kDNA-independent metacyclic form trypanosomes ...... 313

5. General discussion ........................................................................................ 315
5.1 So why do kinetoplastids need a kinetoplast? .................................................... 321
5.2 Outlook .......................................................................................................... 322
5.3 Figures .......................................................................................................... 327
Figure 5.1 The impact of kDNA loss and L262Pγ mutation on life cycle progression 327

6. Appendices .................................................................................................... 329
Appendix A: A quantification of the expression levels of ATPase α alleles in T. b. evansi strains

Introduction .................................................................................................................. 330
Materials and Methods ............................................................................................ 331
Table 6.1 Primers used in this chapter ...................................................................... 331
Results ......................................................................................................................... 338
Discussion .................................................................................................................... 342
Figures ......................................................................................................................... 345
Figure 6.1 Pyrosequencing analysis of ATPase α from DK subspecies of T. brucei .... 346

Table 6.2 Pyrosequencing results quantifying the % of α alleles in the amplicon mixture that are WT from the SNP position ......................................................... 347
Table 6.3 Pyrosequencing results quantifying the % of α alleles in the amplicon mixture that are WT from the deletion region ......................................................... 347
Table 6.4 Pyrosequencing results for the control PCR reactions ............................... 348

Appendix B: For Chapter 2 ........................................................................................ 349
Table 6.5 Genotypes of cell lines generated in this study ......................................... 349
Table 6.6 Primers used in Chapter 2 ........................................................................ 350
Figure 6.2 Gating strategy for flow cytometry ......................................................... 351

Appendix C: For Chapter 3 ....................................................................................... 352
Assessing the error in scoring morphology by PAD1 IFA ........................................ 352
Figure 6.3 Assessment of the accuracy of the morphology scoring of two individuals ......................................................................................................................... 354

Appendix D: The mathematical model for trypanosome within-host dynamics ...... 356
Construction of the model ......................................................................................... 356
Table 6.7 Variables and parameters used in the model ............................................ 359
Model fitting and parameter estimation .................................................................. 360
Likelihood .................................................................................................................... 360

7. References ............................................................................................................. 363
1. Introduction
1.1 Trypanosomes as a pathogen

Sleeping sickness, or Human African Trypanosomiasis (HAT), is a parasitic disease that could potentially occur in 36 sub-Saharan African countries (http://www.dndi.org/diseases-projects/diseases/hat.html). The disease is caused by the African trypanosome, Trypanosoma brucei. The parasite lives extracellularly in the mammalian bloodstream and is spread by the tsetse fly (species within the genus Glossina). As such, the parasite is able to survive within both hosts.

Disease transmission occurs in foci within the distribution of its tsetse fly vector (Franco et al., 2014). The people most at risk to exposure to the tsetse fly live in rural areas (Fevre et al., 2008). There is limited access to adequate health services in these areas. Surveillance for the disease is difficult, so diagnosis can be slow. Many of these regions are affected by political and economic unrest. War, mass migration of the population and poverty all aid disease transmission by disrupting surveillance, diagnosis, health care provision and education (Franco et al., 2014).

1.1.1 The human disease

The human disease is split into two stages, the first stage occurring immediately after initial infection. Here trypanosomes proliferate in blood, lymph and subcutaneous tissue. Symptoms include fever, headaches, joint pains and itching (Franco et al., 2014). In the second stage the parasites are able to cross the blood-brain barrier, infecting the central nervous system. Obvious symptoms of the disease appear here, with disturbance of circadian cycle, changes of behaviour, confusion, sensory disturbances and poor coordination (Franco
et al., 2014). In general, if left untreated the disease is considered to be fatal (Brun et al., 2010).

There are two subspecies of human infective trypanosomes:

- **T. brucei gambiense** accounts for more than 98% of reported cases (World Health Organisation (http://www.who.int/mediacentre/factsheets/fs259/en/)). Affecting countries in west and central Africa, the disease caused is chronic, with symptoms not apparent for months or even years after infection. By the time symptoms emerge the patient is already in an advanced disease stage, and the central nervous system is already affected.

- **T. brucei rhodesiense** affects countries in eastern and southern Africa (http://www.who.int/mediacentre/factsheets/fs259/en/). This parasite causes an acute infection with the first symptoms observed a few weeks after infection. The disease develops rapidly and invades the central nervous system. The difference in the disease progression for these two subspecies is not well understood (Morrison, 2011).

In 2009 the number of infections reported dropped below 10 000 for the first time in 50 years, and in 2013 there were 6314 cases reported (http://www.who.int/mediacentre/factsheets/fs259/en/). The estimated number of actual cases is around 20 000, most of which go untreated. Despite the fact that the number of tsetse flies containing human-infective forms of the parasite is estimated to be low (Brun et al., 2010) (section 1.5.3), 65 million people live in the area infested by the tsetse fly, and are thus deemed at risk (Franco et al., 2014). Screening of this population is continuously required for early detection of **T. b. gambiense** infection. The disease has been targeted for elimination by the WHO by 2020 (http://www.who.int/mediacentre/factsheets/fs259/en/).
*T. brucei brucei* is a subspecies of trypanosome that is animal infective, but not human infective. Humans and other primates have lipid-rich particles, containing trypanosome lytic factor (TLF) 1 and 2, a lipid-binding protein, apolipoprotein L1 (ApoL1), and haptoglobin-related protein (HPR) which binds haemoglobin, in their blood serum (Rifkin, 1978, Hajduk et al., 1989). TLF-1 is taken up into *T. b. brucei* via the parasite haptoglobin-haemoglobin receptor (HpHbR) (Vanhollebeke et al., 2008, Vanhollebeke and Pays, 2010), as the parasite is a haem and lipid scavenger (Koreny et al., 2010, Green et al., 2003). Once endocytosed, ApoL1 is released in the lysosome, embeds in the membrane and causes lysis of the parasite (Hager et al., 1994, Vanhollebeke and Pays, 2010, Vanhollebeke and Pays, 2006).

The two human-infective subspecies of *T. brucei* are able to resist the actions of ApoL1. *T. b. rhodesiense* express a single protein named serum resistance-associated (SRA) which confers human serum resistance on the parasite (Xong et al., 1998, Gibson, 2005); SRA is a marker for this subspecies (Welburn et al., 2001). This protein is homologous to the variable surface glycoprotein (VSG) that covers the cell surface of the parasite in the mammalian bloodstream. It is present on the cell surface and in the endosomes and lysosomes during protein turnover (Milner and Hajduk, 1999, Vanhamme et al., 2003). SRA interacts with ApoL1 in the lysosome and neutralises its ability to puncture the lysosomal membrane (Vanhamme et al., 2003).

*T. b. gambiense* has a mutated HpHbR that reduces affinity for TLF-1 (Kieft et al., 2010, Higgins et al., 2013). *T. b. gambiense*-specific glycoprotein (TgsGP) is a gambiense-specific VSG-like protein (Berberof et al., 2001) that confers sensitivity to TLF-2 (Capewell et al., 2013, Uzureau et al., 2013). The mechanism of action is not fully understood.
1.1.2 Nagana

50 million head of cattle are at risk of trypanosome infection (Fevre et al., 2008). The symptoms of this trypanosomosis are fever, listlessness, emaciation, hair loss, discharge from the eyes, oedema, anaemia, and paralysis (Steverding, 2008). The disease prevents the establishment of sustainable farming in many parts of sub Saharan Africa, reducing the number of animals that can be used for transport, milk production, ploughing and for food. 3 million cattle deaths occur per year (http://www.fao.org), despite constant dosing of trypanocidal drugs. The disease causes the area US$4 billion in loss of crop and livestock production (http://www.fao.org). The drugs used to treat cattle include isometamidium chloride and homidium, an ethidium bromide (EtBr)-based drug (Dolan et al., 1990, Stevenson et al., 1995). Drug resistance encourages the spread of the disease. Prophylactic treatment increases the risk of resistance, with the parasites experiencing sub-lethal concentrations of the drug for an extended time (Matovu et al., 2001).

1.1.3 Vector control

Control of the tsetse fly vector would help to control the spread of human and animal disease, as transmission of trypanosomes is proportional to vector density. Humans are the main reservoir host for *T. b. gambiense*, unlike *T. b. rhodesiense* where cattle are the main reservoir host (Franco et al., 2014). Hence animal-human, animal-animal, and human-human transmission all occur with *T. b. rhodesiense*. In order to reduce vector density, several techniques are being used: aerial insecticide spraying that targets adult flies during the first spray and pupal stages and resting flies on the ground during subsequent sprays, odour or colour-baited insecticide treated traps, radiation-sterilised insect release and insecticide treatment of cattle (Welburn et al., 2009).
1.1.4 Vaccination prospects

A vaccine for human or animal trypanosomatid diseases has never been produced. The eradication of the trypanosome reservoir would be extremely difficult, and so priming the immune system to mount an anti-trypanosome response would be ideal. There have been cases of trypanotolerance seen in animals (D’ieteren et al., 1998, Orenge et al., 2012, Naessens et al., 2003) and humans (Jamonneau et al., 2012, Jamonneau et al., 2004), hence under certain circumstances the mammalian immune systems can control the disease. The development of a vaccine is limited by the use of the mouse as a model organism in which to study trypanosomiasis (La Greca and Magez, 2011). Notably, disease progression in mice is faster which alters the ratio between parasites and immune cells to a level not experienced in human or cattle infection.

Vaccine candidates have included the surface coat of the trypanosome form found in the mammalian bloodstream, VSG (see section 1.3.1.1 of this thesis) (La Greca and Magez, 2011). However, the antigenic variation in VSG allowing trypanosomes to evade an immune response makes a vaccine to these proteins impossible (Cornelissen et al., 1985). Trypanosomes have an enormous potential to generate novel VSG through gene rearrangement (Horn, 2014, Mugnier et al., 2015), and VSG only elicits a short-lived IgM response (Reinitz and Mansfield, 1990). Invariant antigens from the flagellar pocket (Mkunza et al., 1995) and surface glycoproteins (ISG) (Lanca et al., 2011), and even tubulin (Lubega et al., 2002) have been explored as potential vaccine targets, with no success. The use of non-pathogenic trypanosome species is also being explored as a possible vaccine. These are being manipulated into becoming a vehicle for establishing an immune response to particular antigen, or as delivery vehicles for therapeutics (Mott et al., 2011).
1.1.5 The current therapies against HAT

HAT affects a small number of poor African populations, limiting the attractiveness of developing a new drug for pharmaceutical companies. There is a heavy reliance on therapies developed during the first half of the 20\textsuperscript{th} century, and these can be by themselves highly toxic.

- Pentamidine, a diamidine, was first discovered in 1940 and is used for the treatment of the first stage of \textit{T. \textit{b. gambiense}} infection. Despite undesirable side effects including hyper/hypoglycaemia, hypotension and heart problems, it is in general well tolerated by patients. It is highly potent, with a half minimal inhibitory concentration (IC\textsubscript{50}) and half effective dose (ED\textsubscript{50}) in the nanomolar range (Brun et al., 2011, Merschjohann et al., 2001). The method of action is not well understood (Barrett et al., 2007).

- Suramin was discovered in 1920, and used for the treatment of the first stage of \textit{T. \textit{b. rhodesiense}}. It is highly potent, with IC\textsubscript{50} and ED\textsubscript{50} also in the nM range (Otoguro et al., 2008, Merschjohann et al., 2001). It provokes undesirable effects, including urinary tract infections, skin lesions, renal failure and allergic reactions. The mode of action is not well understood (Barrett et al., 2007).

- Melarsoprol was introduced in 1949, and has efficacy against the second stage of both \textit{gambiense} and \textit{rhodesiense} infections. Derived from arsenic, it has many undesirable side effects, including encephalopathy which is fatal in 5\% of cases. It is highly potent, with activity in nM range. The way this drug kills trypanosomes is not well understood (Barrett et al., 2007). It does cross the blood-brain barrier, but to levels 1-2\% of the concentration found in blood, meaning that parasites with less sensitivity to the drug can easily become non susceptible (Brun et al., 2011).
• Eflornithine (DFMO) is less toxic than melarsoprol but still can cause symptoms such as alopecia, seizures and bone marrow toxicity. It was registered in 1990 as an analog of ornithine, thus is a specific inhibitor of ornithine decarboxylase, preventing polyamine biosynthesis (Bacchi et al., 1983, Phillips et al., 1987). It only effective against second stage *T. b. gambiense* (Iten et al., 1997), with activity in the μM range (Otoguro et al., 2008), requiring a large amount of drug to be administered by intravenous injection.

• Nifurtimox is a nitrofuran derivative, developed in the 1960s, and is registered for the treatment of American trypanosomiasis but not for HAT. The drug causes oxidative stress (Docampo and Moreno, 1986, Enanga et al., 2003). The drug can accumulate in the brain at levels half of those in plasma (Brun et al., 2011). The side effects include toxicity to the nervous systems (Barrett et al., 2007).

A combination treatment of nifurtimox and eflornithine was introduced in 2009. This is currently recommended as first-line treatment for the second stage of the *gambiense* form. This reduces the time needed for treatment from 14 to 10 days and the number of intravenous doses required is reduced by 75%, with an overall halving of the therapeutic cost (Priotto et al., 2009). Side effects include tremors and weight loss.

Treatment failure can occur for multiple reasons.

• Problems in drug distribution and administration: many countries with trypanosomiasis incidence have economic and political unrest, making distribution of the drugs complicated. Treatment programs are often complicated and long, requiring hospitalisation. For example, for stage 1, pentamidine treatment involves a once-daily 4 mg/kg dosage for 7 days. For stage 2, the nifurtimox-eflornithine combination therapy involves eflornithine doses of 200 mg/kg intravenously twice
per day for 7 days and nifurtimox doses of 5 mg/kg orally three times a day for 10 days (Priotto et al., 2009).

• Drug efficacy can vary between people; for example, around 10% of patients who receive eflornithine do not respond to treatment (Barrett et al., 2011).

• Diagnosis difficulty: correct diagnosis of stage 1 vs. 2 is important as different treatments are used for each, and as drugs have differing abilities to persist in different compartments. For example, suramin and pentamidine are able to bind serum proteins and have long half-lives, but only cross the blood-brain barrier at extremely low levels (Sanderson et al., 2009). This differential diagnosis can be challenging, however (Franco et al., 2014).

### 1.1.6 Drug resistance in *T. brucei*

*T. brucei* is able to develop drug resistance through genetic mutations. A molecular understanding of drug resistance is essential, and can be instrumental in switching between therapies across entire areas of countries. Resistance has been seen against all drugs used, but only certain mechanisms of resistance have been understood. For example:

• Melarsoprol and pentamidine resistance can be caused by point mutations in TbAT1, a purine nucleoside transporter, and TbAQP2, an aquaporin. Loss of TbAT1 does not solely account for high level resistance of the drugs (Stewart et al., 2010, Matovu et al., 2003). AQP2 mediates high affinity uptake; deletion or rearrangements of TbAQP2 was found to be present in strains resistant to both drugs (Munday et al., 2014, Baker et al., 2012).

• TbAAT6, an amino acid transporter, controls the uptake of eflornithine. Gene deletion causes drug resistance, with reexpression of the gene causing restoration of sensitivity (Vincent et al., 2010). The gene is non-essential, hence in the presence of
the drug the selective pressure to maintain the wild type (WT) gene is overcome, allowing resistance to be obtained relatively easily.

1.1.7 New therapies

There is a need for new therapies that are safer and easier to administer. DB289 is an oral diamidine prodrug recently developed as a replacement for pentamidine in the first stage of sleeping sickness. However during a stage III clinical trial, severe liver toxicity was detected in a small number of patients (Wenzler et al., 2009). This led to the program being discontinued. Fexinidazole is currently in phase III trials for development as the first oral therapy for stage one and two disease (www.dndi.org), eliminating the need to differentiate between stages of the disease (Kaiser et al., 2011). The compound is metabolised to a sulfone via the action of cytochrome P450 enzymes (Brun et al., 2011). The target of the sulfone is not known. A high blood brain barrier permeation has been predicted, with a good safety profile. SCYX-7158 and derivatives are oxaboroles that have been shown to have efficacy against stage 2 of the disease in mice. Testing is currently at phase II/III trial stage (www.dndi.org). The target of this drug is not known. Both drugs require large amounts of the drug to be administered. There have been attempts to alter the delivery system of drugs: for example melarsoprol-cyclodextran complexes to allow melarsoprol to be given orally (Rodgers et al., 2011), negating the need for long hospital stays.
1.2 A summary of the cell biology of the parasite

*T. brucei* are protozoan parasites. They are classified in the phylum Euglenozoa and the class Kinetoplastea due to their mitochondrial DNA (mtDNA) being present in a disc-like structure called a kinetoplast (kDNA).

The parasite has many features allowing survival in the multitude of environmental conditions that its lifestyle dictates. The trypanosome must be able to survive in the glucose-rich environment of the mammalian bloodstream in the presence of the host immune system. The parasite must also be able to survive and migrate between several compartments within the tsetse fly vector, all differing environments with respect to nutrition availability, pH and O₂ levels. The need to adapt to the conditions found within each niche environment is reflected by morphological and metabolic changes during the parasite’s life cycle. Differentiation between the stages of the life cycle is ordered and programmed within the cell, and occurs as a consequence of signals received from the environment. The two forms of the parasite that have been most studied are those found within the mammalian bloodstream (BSF) and those found within the insect midgut, the procyclic form (PCF). Growth conditions for both of these forms can be mimicked in *in vitro* culture (Hirumi and Hirumi, 1989, Brun and Schonenberger, 1979).

A trypanosome cell is elongated with a defined posterior and anterior end (Figure 1.1). The cytoskeleton of microtubules defines the cell shape. The single mitochondrion runs the entire length of the cell. Like all mitochondria, it is encased in two membranes, the outer and the inner. The intermembrane space (IMS) lies between these two, and the compartment enclosed in the inner membrane is named the matrix. Complexes of the respiratory chain are located in the inner membrane (section 1.6.3, Figure 1.3B) and generate the essential mitochondrial membrane potential (Δψm) across this boundary.
The trypanosome mitochondrion is present in a single copy, and must segregate with precision during cell division. There is regulation of mitochondrial activity depending on the external environment (section 1.5.5). The mitochondrion contains a genome, termed kDNA (section 1.3.2.1). This is a disk of interlinked DNA comprising of maxicircles and minicircles. The maxicircles encode protein-encoding genes and ribosomal RNAs corresponding to the mitochondrial DNA of other organisms (section 1.6.4). Some of these genes are encrypted. Minicircles contain the guide RNAs (gRNA) necessary for their decryption in a process called RNA editing (section 1.3.2.2). The kDNA is linked to the base of the flagellum by a tripartite attachment complex (TAC), containing the differentiated mitochondrial membranes, and the basal body (Figure 1.1). The TAC provides the structural link allowing kDNA segregation to be linked to the flagellum and basal body replication and segregation during the cell cycle (Ogbadoyi et al., 2003, Robinson and Gull, 1991)(section 1.4).

The flagellar pocket is where the flagellum protrudes from the cell body (Figure 1.1). This invagination is the site of endo- and exocytosis (Overath and Engstler, 2004), and thus the single Golgi apparatus and endoplasmic reticulum (ER) are positioned close. The flagellum allows the cell to swim in the direction the flagellum grows in, the anterior pole of the cell, and also has a central role in the cell cycle (section 1.4). This has a 9+2 microtubule configuration typical of motile flagella. The paraflagellar rod spans the flagellum and is semi-rigid, contributing to motility (Bastin et al., 1998). The flagellum is attached to the cytoskeleton of the cell along its length by the flagella attachment zone (FAZ).

1.3 Trypanosoma brucei as a model organism

Trypanosomes make useful model organisms as certain life stages can be grown in vitro and due to their genetic tractability: established methods include gene knock-in and -out via
homologous recombination (Clayton, 1999), RNA interference (RNAi)-based knock-down (Ngo et al., 1998), and overexpression of genes via tetracycline-inducible promoters. The nuclear genome has been sequenced (Berriman et al., 2005) and annotation is available for free online (TriTrypDB). Thought to have diverged early in evolution from other eukaryotic organisms, it has been controversially argued that the eukaryotic root lies between the Euglenozoa and other eukaryotes (Cavalier-Smith, 2010, He et al., 2014, Adl et al., 2012). As single-celled eukaryotes with a unique place in the eukaryotic tree, they are useful organisms for investigating many aspects of fundamental eukaryotic and kinetoplastid-specific cell biology.

Many features of cell biology have been discovered in trypanosomes, including RNA editing (Benne et al., 1986), glycosylphosphatidylinositol (GPI) anchors (Ferguson et al., 1988), glycosomes (Opperdoes and Borst, 1977), acidocalcisomes (Philosoph and Zilberstein, 1989, Docampo et al., 1995) and trans-splicing (Boothroyd and Cross, 1982, Agabian, 1990).

1.3.1 Unusual aspects of cell biology

There are many examples of kinetoplastid-specific cell biology, a few of which are detailed below.

1.3.1.1 VSG

Slender BSF cells continuously switch the VSG surface coat they are expressing in order to prevent recognition by the host immune system. The repertoire of potential VSG genes and pseudogenes is extremely large (Marcello and Barry, 2007), with only one being expressed per cell at a time and the rest staying silent (Hertz-Fowler et al., 2008, Callejas et al., 2006). Switches can be mediated by gene conversion, telomere exchange or active site switching (Taylor and Rudenko, 2006, Horn and Cross, 1997). The huge repertoire available for
potential genetic recombination to generate novel mosaic VSGs is responsible for establishing chronic infection (Roth et al., 1989, Berriman et al., 2005, Barry and Mcculloch, 2001) (section 1.5.1).

1.3.1.2 Glycosomes

Belonging to the peroxisome family, glycosomes contain many glycolytic enzymes (Figure 1.2). This sequestration of part of the glycolytic pathway away from the cytosol is unique to kinetoplastids. The compartmentalisation is essential, and modelling has suggested this allows the prevention of toxic hexose phosphate accumulation, compensating for a loss in ability to regulate some of the enzymes encased (Bakker et al., 2000). Enzymes involved in other metabolic pathways are also present in the glycosome (Michels et al., 2006).

The content of glycosomes fluctuates during the life cycle of T. brucei (Colasante et al., 2006, Bauer et al., 2013). Peroxisome and glycosome populations can be turned over by autophagy when the nutritional status of the cell renders the current metabolic situation redundant (Erdmann et al., 1997, Herman et al., 2008). New organelles can thus be generated with an adapted enzymatic mixture present. In the context of a parasitic lifestyle where multiple environmental changes are experienced, the ability to reroute metabolism rapidly would allow flexibility (Szoor et al., 2014).

1.3.1.3 Gene expression

Unlike other eukaryotes, most protein-encoding genes in kinetoplastids are encoded in tandem arrays that are polycistronically transcribed (Martinez-Calvillo et al., 2003). Mature mRNA is generated via post transcriptional processing, including trans-splicing (Liang et al., 2003). Most genes are under post-transcriptional control, for example translational regulation and mRNA destabilisation (Clayton, 2002). In other eukaryotes, RNA polymerase I
exclusively transcribes ribosomal RNA (rRNA). In *T. brucei*, this enzyme also transcribes the genes encoding the surface proteins of the BSF and insect forms (Gunzl et al., 2003).

### 1.3.2 Unusual aspects of mitochondrial biology

The single mitochondrion of *T. brucei* has many unique features, some of which are detailed below, making this complex organelle a fascinating research topic. The *T. brucei* life cycle requires regulation of mitochondrial activity making this organism an ideal candidate for studying the processes of mitochondrial biogenesis and regulation. The mitochondrial genome cannot be manipulated as readily as the nuclear genome, with no technique for mitochondrial transfection or genome editing yet developed in trypanosomes.

#### 1.3.2.1 kDNA

30-50 copies of the maxicircle are present per *T. brucei* cell, and thousands of 1 kb minicircles are present, making kDNA replication an extremely complex process (Verner et al., 2015). The minicircle population is heterogeneous, each one encoding between two to five gRNAs (Pollard et al., 1990). The gRNAs encoded on one minicircle can edit the same maxicircle gene, or different maxicircle genes (section 1.3.2.2).

kDNA is essential in PCF and BSF (Schnaufer et al., 2001, Schnaufer et al., 2002). Despite this, akinetoplastic (AK) and dyskinetoplastic (DK) subspecies of *T. b. brucei* do exist. These can survive in the BSF without kDNA (AK) or with losses of functional parts of kDNA (DK). They do not survive within the insect (Schnaufer et al., 2002, Stuart, 1971); the genes retained within the kDNA maxicircles mostly encode hydrophobic membrane-integral components of the mitochondrial respiratory chain required for oxidative phosphorylation (section 1.6.4, Figure 1.3A, B). Six of these genes are never-edited and are in a form that can
be translated straightaway. The other 12 are subjected to RNA editing. Alternative editing also can occur, increasing the diversity of the proteins encoded (Ochsenreiter et al., 2008b).

The kinetoplast is replicated once per cell cycle, synchronised with the replication of the nucleus (section 1.4). Segregation of kDNA minicircles into daughter cells is thought to be random (Savill and Higgs, 1999), but must maintain gRNA diversity to obtain the full complement of minicircles and maintain viability within the cell population. Transcription, RNA processing, including RNA editing, and translation of maxicircle gene products all occur within the mitochondria (Verner et al., 2015).

1.3.2.2 RNA Editing

The process of RNA editing is essential for T. brucei (Babbarwal et al., 2007, Schnaufer et al., 2001), and this process is an attractive drug target (Durrant et al., 2010). This particular type of editing, which inserts or removes uridylyl nucleotides from transcripts of maxicircle genes, is a kinetoplastid-specific process (Benne et al., 1986, Feagin et al., 1988b, Feagin et al., 1988a, Alfonzo et al., 1997). This generates open reading frames that encode a translatable protein. Some transcripts are differentially edited between the BSF and PCF, and it has been proposed that editing might provide a regulatory role in controlling maxicircle gene expression (Feagin and Stuart, 1988, Souza et al., 1992) (Table 1.1). Stage-specific editing may also contribute to maintaining the diversity of the kDNA, to prevent loss of genes unnecessary in one life cycle stage but necessary in another (Speijer, 2006, Lukes et al., 2009).

Editing is performed by the 20S editosome and a multitude of accessory proteins (Stuart and Panigrahi, 2002, Maslov and Simpson, 1992, Aphasizheva et al., 2011). The gRNAs encoded on minicircles facilitate this process, these are sequences that are partially complementary to sections of a maxicircle transcript. A region of 8–12 nucleotides on the 5′-end of the gRNA, the ‘anchor’, anneals to its cognate transcript. Editing occurs at the first
base pair mismatch within the RNA duplex, adding or deleting a uridylyl base to extend the complementarity between mRNA molecule and gRNA sequence. Editing is a stepwise process and occurs in the 3'-5' direction (Maslov and Simpson, 1992), preventing translation occurring before editing. Many gRNAs are redundant within the kDNA population, making the process more robust in case not all minicircles are inherited by both daughter cells during the cell cycle (Savill and Higgs, 2000, Corell et al., 1993).

1.4 Cell cycle of trypomastigote stages

During the cell cycle, kinetoplast DNA is replicated during G1, with the nucleus replicated during S phase. The duplication of the single copy organelles occurs before cellular segregation (Figure 1.4). There is coordination between the cell cycle and the parasitic life cycle (Matthews and Gull, 1994b, Matthews and Gull, 1994a) (section 1.5), allowing, when required, population synchrony in differentiation, asymmetric division and alternation between cell cycle arrested and proliferative stages.

In G₁, the parasite does not divide, and has one kinetoplast and one nucleus (1K1N). In S phase, DNA replication occurs, increasing the DNA content of both the kDNA and nucleus. The pro basal body elongates and matures to a daughter basal body, lying in close proximity to the existing basal body (Sherwin and Gull, 1989). From here, a daughter flagellum starts to protrude from the daughter basal body, generating a new flagellar pocket (Lacomble et al., 2010). As it extends, the daughter flagellum follows the course of the parent flagellum, with a flagellar connector linking the two in the first insect PCF stage (Moreira-Leite et al., 2001). There is no flagellar connector in the BSF, allowing more flexibility in the path of growth (Briggs et al., 2004). The daughter basal body is pushed more posterior due to the tethering of the daughter basal body by FAZ between the flagellar connector and base of flagellum, and by growth and movement of the daughter flagellum (Absalon et al., 2007). The daughter
kDNA separates from the parent kDNA as it is attached to the daughter basal body via the TAC (Ogbadoyi et al., 2003). This can occur before or after the end of S phase, but before mitosis (Siegel et al., 2008).

G₂ phase is entered in a 2K1N configuration. Chromosomes separate, allowing the nucleus to begin M phase (Sharma et al., 2008), and a 2K2N configuration to be generated. In the insect stage the arrangement is KNKN, whereas in the BSF there is a KKNN arrangement (Briggs et al., 2004). Cytokinesis then occurs, with cleavage occurring first at the anterior end (Kohl et al., 2003), and moving towards the posterior end between the two flagella (Vaughan and Gull, 2003). The independent beating of the two flagella aid this. As mentioned above, the flagellar connector is absent in the BSF, and the daughter flagellum can beat independently (Briggs et al., 2004).

In the PCF, checkpoints exist to prevent entry into mitosis unless the nucleus has undergone S phase, and to prevent cytokinesis in the absence of kDNA segregation (Ploubidou et al., 1999). However, cytokinesis can occur in the absence of kDNA in BSF (Dean et al., 2013). The directed movement of organelles integral to the cell cycle is not fully understood (Ooi and Bastin, 2013).

1.5 The *T. brucei* life cycle

As a parasitic organism, trypanosomes have adapted to survive in different environments during their life cycle (Figure 1.5). Differentiation is required in order to function successfully within each niche environment, with differing metabolic substrates present, and differing host immunological tactics threatening the parasite’s survival. Changes thus include gene expression, metabolism, morphology and cell cycle progression or arrest.
1.5.1 Bloodstream stages

BSF cells express the essential VSG protein on their surface. The VSGs mask the invariant membrane proteins on their surface, the receptors and transporters needed to take up nutrients (Schwede and Carrington, 2010). Hence, these VSGs are constantly targeted by the host immune system. With a delay, the immune system raises an antibody response to the specific VSG expressed, allowing the clearance of cells expressing that VSG. To evade the immune system, slender BSF cells undergo antigenic variation (section 1.3.1.1), but stumpy BSF cells do not (Amiguet-Vercher et al., 2004, Perez-Morga et al., 2001). VSG is also constantly internalised via the flagellar pocket, allowing stripping of any bound antibodies (Engstler et al., 2007).

In the bloodstream of the mammalian host, the cell population cycles between proliferative slender BSFs, the intermediate form and the cell cycle-arrested stumpy form (Figure 1.6). The stumpy form is the insect-transmissible form, preadapted to survive within the tsetse fly midgut (sections 1.5.2, 1.9.2). Differentiation from the slender BSF is triggered upon high slender parasite numbers. This density dependent mechanism has been shown to be induced by a stumpy induction factor (SIF), a form of quorum sensing (Vassella et al., 1997). Although the identity of SIF is not known, it can pass through a 500 Da cut-off filter, and is stable for 27 days at 37°C. It is proposed to be produced and received by slender BSFs; as the number of slender BSFs increases over time, SIF accumulates, inducing stumpy formation.

The emergence of cell cycle-arrested stumpy forms prevents parasitaemia increasing further, prolonging host survival. Stumpy cells have a limited lifespan of around 3 days (Turner et al., 1995), hence cell senescence and immune system action causes the infection to be cleared. Not all parasites do differentiate. Each time, a few remaining slender BSFs with a
switched VSG are sustained during clearance and able to proliferate, resulting in repeated waves of infection.

VSG switching is hypothesised to occur early in the infection, allowing a probing of the immune system for prior antigen exposure preventing herd immunity (Macgregor et al., 2011). Stumpy forms dominate in the later stages of an infection. As these do not switch their VSG, this prevents VSG repertoire overwhelming the host immune system, while increasing the chance of transmission. A low level of slender cells remain and switch VSGs, allowing maintenance of the infection into chronic stages.

BSF cells are able to clear the antibody bound to their surfaces by hydrodynamic flow, with swimming action aiding the sweeping of antibody backwards for internalization at the flagellar pocket (Engstler et al., 2007). Slender cells seem to be more sensitive to antibody-dependent complement-mediated lysis compared with stumpy cells: there is a 7.3x difference in the lysis rate between cells in slender and stumpy stages, with stumpy cells more able to withstand the antibody response of the immune system (Mclintock et al., 1993). Supporting this, stumpy cells have a faster rate of endocytosis at the flagellar pocket and a faster antibody clearance rate (Engstler et al., 2007). This would allow stumpy cells to dominate at the peak of parasitaemia, when the antibody responses of the immune system have been activated, allowing for efficient tsetse transmission.

Monomorphics slender BSFs also exist, for example those generated by serial *in vitro* passage. Unlike pleomorphic parasites, these forms are unable to differentiate to the stumpy form. Monomorphic cells invariably kill a mouse within a few days (Turner, 1990), whereas a pleomorphic parasitaemia rises over a longer period of time and is sustained through repeated waves of infection. Monomorphic cells are unable to sense SIF (Vassella et al., 1997). Treatment of monomorphic cells with cell permeable analogs of cyclic AMP (cAMP)
can generate a ‘stumpy-like’ phenotype, suggesting that these cells can undergo some of the morphological and genetic changes required for the stumpy form (Laxman et al., 2006).

Some of the stumpy induction pathway has been elucidated using a genome-wide RNAi screen (Mony et al., 2014). The identified components included kinases, phosphatases and RNA binding proteins. Several hypothetical proteins were also identified as playing a role, including some potential mitochondrial proteins that could contribute to differentiation and/or influence mitochondrial elaboration (Mony and Matthews, 2015). As well as the components that are already recognised as playing a role in differentiation (Vassella et al., 2001b, Barquilla et al., 2012), these potential players and their binding partners need to be ordered into a linear or branched signalling cascade to fully elucidate the molecular events influencing the slender to stumpy transition.

1.5.2 Insect stages

Stumpy forms differentiate to the PCF synchronously within the tsetse midgut. This differentiation can also be performed in vitro. The study of the differentiation effectors has been compounded by use of different strains and there has been confusion as to their physiological relevance.

As activation of the Krebs cycle could trigger the metabolic changes that are characteristic of BSF to insect form differentiation, Brun and Schönenberger (1981) screened Krebs cycle intermediates in order to investigate whether they were able to stimulate stumpy to procyclic transformation. Adding citrate or cis-aconitate (CA) at concentrations of 3-10 mM to SDM79 was able to drive in vitro differentiation of stumpy forms. They postulated that as insect hemolymph contains high concentrations of Krebs cycle intermediates (Wyatt, 1961), this could stimulate the transformation observed in the midgut in vivo. The in vivo relevance
of this data was questioned due to the high non-physiological concentration of CA required (Overath et al., 1986).

There has been controversy surrounding the requirement for the stumpy form to generate PCF as some monomorphic strains are able to differentiate to the PCF in vitro (Bass and Wang, 1991, Mutomba and Wang, 1995). This differentiation is asynchronous however (Ziegelbauer et al., 1990, Roditi et al., 1989). A dividing population of monomorphic cells have a mixture of cell cycle stages present, whereas peak pleomorphic T. brucei parasitaemia in vivo contains mostly cells arrested in G0, hence for synchrony the signal for differentiation needs to be initiated in stages G1/G0. Increased transformation efficiency in the stumpy form was explained by the discovery that a cell surface protein specifically expressed in the stumpy form, named ‘proteins associated with differentiation 1’ (PAD1), was a carboxylate transporter (Dean et al., 2009). Stumpy form cells are preadapted to life within the midgut as they express the CA/citrate receptor that can receive this differentiation signal.

Trypanosomes experience a temperature shift between their two hosts. The mammalian bloodstream is 37°C, whereas the optimum temperature for tsetse flies is ~27°C. Flies can experience temperature fluctuations however, for example they tend to feed around dusk and dawn where there is a cooler ambient temperature of around 20°C (Makumi et al., 1998, Torr and Hargrove, 1999). Parasites respond to environmental cues via the genetically-defined developmental changes during their life cycles. Temperature shifts are used as a signal for environmental changes in many organisms, including other parasites (Zilberstein and Shapira, 1994, Fang and Mccutchan, 2002). Stumpy form cells incubated at 20°C prior to CA treatment are sensitive to more physiological concentrations of CA (down to 0.6 µM) (Engstler and Boshart, 2004). The internal environment of the fly contains low concentrations (~16 µM) of CA/citrate (Hunt et al., 1994). Carboxylate transporter PAD2 also has upregulated expression at 20°C compared with 37°C, with its localisation also altering from close to the flagellar pocket to surface expression upon the temperature
decrease (Dean et al., 2009). Cold shock within the fly midgut therefore induces the expression of PAD on stumpy forms and lowers the concentration of CA required to cause differentiation, preventing premature differentiation whilst in the mammalian bloodstream.

The hypersensitivity to CA produced during cold shock can be overridden by induction with a high, non-physiological concentration of CA. Cells with repressed PAD expression are not responsive to low levels of CA (0.1 mM), but are responsive to high concentrations of CA (1 or 6 mM) (S. Dean, thesis). CA may be able to be transported by other lower affinity transporters when PAD transporters are absent and CA is present at high concentrations.

The intracellular signalling pathway that responds to CA has been deduced. TbPTP1, a protein phosphatase acts downstream of PAD1 to prevent differentiation of stumpy forms to the PCF whilst in the bloodstream (Szoor et al., 2006, Szoor et al., 2010). Once CA has been perceived, this block is removed: the presence of CA inhibits activation of TbPTP1, preventing the dephosphorylation of its substrate, TbPIP39. The active and phosphorylated TbPIP39 is then targeted to the glycosomes where it promotes differentiation. The molecular targets of TbPIP39 once within the glycosomes are not known, but some of the downstream effects caused by the change in metabolism are known (Vassella et al., 2004, Morris et al., 2002).

There are multiple other triggers that are also able induce stumpy to procyclic differentiation. The action of proteases has been shown to induce differentiation to the PCF (Yabu and Takayanagi, 1988, Hunt et al., 1994). Digestive proteases are present within the fly midgut (Aksoy et al., 2003), hence are a factor of the environment that trypanosomes have to be able to tolerate. The effect of mild acid stress on the differentiation of pleomorphic AnTat1.1 BSF cells has also been investigated (Rolin et al., 1998). This treatment killed slender cells by stimulating VSG loss, unlike in stumpy forms (Rolin et al., 1993, Rolin et al., 1996, Nolan et al., 2000). Slender BSFs are unable to regulate their cytoplasmic pH (Nolan and Voorheis,
whereas stumpy forms were shown to sustain an estimated constant cytoplasmic pH of 6.9 despite a high external proton concentration (Nolan et al., 2000). These adaptations are required for tolerance of the unbuffered and protease-rich environment of the midgut. Acidic stress has also been implicated in the differentiation of *Leishmania* and *T. cruzi* promastigotes to amastigotes *in vitro* and *in vivo* (Zilberstein et al., 1991, Bates et al., 1992, Tomlinson et al., 1995).

Multiple environmental signals for differentiation would allow a strong, irreversible differentiation response selectively within the stumpy form, preventing premature and lethal transformation within the bloodstream of the mammalian host. The molecular basis of these other differentiation signals are being probed (Szoor et al., 2013). Mild acid signals via the TbPTP1/TbPIP39 pathway, as does CA, whereas protease treatment of the cells is independent of this pathway.

### 1.5.3 Differentiation within a fly

BSF trypanosomes are taken up into the midgut of the fly during a blood meal. The slender BSFs are not well adapted to survive in the midgut, and therefore die (Dyer et al., 2013). Stumpy forms differentiate into proliferative PCFs, which are able to infect the tsetse fly midgut. Many factors can influence the success rate of the infection, including the age of the fly, number of blood meals a fly has had, the tsetse immune response and gut conditions (Dyer et al., 2013). By day 3 post infection, PCF are found in the ectoperitrophic space from where they migrate forward towards the proventriculus (PV) to the anterior of the gut by day 6 (Sharma et al., 2009)(Figure 1.5A, B). Long mesocyclic trypomastigotes appear that migrate efficiently due to their long flagella. Parasites start to differentiate to long epimastigotes in the lumen of the PV. This differentiation involves the reversal of the positions of the kinetoplast and nucleus (Sharma et al., 2008). Long epimastigotes divide
asymmetrically into long and short epimastigotes, aiding the delivery of short, less motile epimastigotes to the salivary gland (Van Den Abbeele et al., 1999). Few parasites are able to complete this migration (Oberle et al., 2010), and only a small proportion of experimentally-infected tsetse flies are found with infected salivary glands (Peacock et al., 2012); in the field, salivary gland infection rates are also very low (Harley, 1966, Njiru et al., 2004, Lehane et al., 2000). Epimastigotes attach to the epithelium of the salivary glands, proliferate and thus colonise the salivary gland (Tetley and Vickerman, 1985, Sharma et al., 2009). Epimastigotes undergo further asymmetric division to continuously yield trypomastigotes that are able to develop into metacyclics (Rotureau et al., 2012). Metacyclics appear around 3 weeks post infection. These cell cycle-arrested forms can be injected into the mammalian host upon a tsetse bite to begin the life cycle again. Metacyclogenesis in the salivary gland induces modification of the composition of saliva to increase the number of vector and host contact events to improve the chance of transmission (Van Den Abbeele et al., 2010).

The bottlenecks experienced by the parasites are thought to allow clonal expansion of rare variants in individual flies to maintain diversity within the population of mammalian host (Oberle et al., 2010). Additionally, meiosis and genetic exchange can occur in the tsetse salivary glands (Jenni et al., 1986, Peacock et al., 2011). This can be intraclonal and interclonal, allowing genetic diversity to be obtained for the selective advantage of the population (Peacock et al., 2009). The mechanism for this is not yet understood, but some proteins involved have begun to be characterised (Peacock et al., 2011). Meiosis does not appear to be necessary for metacyclogenesis.

The sensory mechanisms that are involved in the complex migration pattern through the fly are not fully understood. Trypanosomes are able to respond as a community to environmental cues in the mammalian bloodstream, for example, in response to SIF, and in the insect vector for example migration between tissues. Social motility is currently being explored as a theory to explain these migrations within the fly (Oberholzer et al., 2010,
Lopez et al., 2015). This coordinated movement has been shown to occur in early PCFs in vitro, when in vivo migration is required from the midgut lumen into the ectoperitrophic space (Imhof et al., 2014).

The surface proteins of the forms found in the fly stages can be used as differentiation and stage specific markers. A glutamate-proline repeat containing protein, EP procyclin, is found in the midgut and PV stages, until asymmetric division begins. A related glycine-proline-glutamate-glutamate-threonine repeat containing protein, GPEET procyclin, is also expressed on early PCFs (Imhof et al., 2014, Vassella et al., 2000). These are both resistant to midgut proteases (Acosta-Serrano et al., 2001) and are not essential: T. b. brucei unable to express EP or GPEET procyclins is fly transmissible, but does have a reduced midgut colonisation rate (Vassella et al., 2009). A protein misnamed bloodstream alanine-rich protein (BARP) is expressed on attached epimastigotes in the salivary gland (Urwyler et al., 2007). VSG is expressed on metacyclic forms, with a population of cells expressing a range of metacyclic VSG, allowing the infected population to be resistant to any existing humoral immunity to VSGs (Barry et al., 1998). It is not known what surface molecules are expressed on epimastigotes moving from the PV.

1.5.4 Difficulty studying certain stages of the trypanosome life cycle

Only the proliferative trypomastigote stages, slender BSF and PCF, are able to be grown in vitro, in HMI-9 and SDM-79/80 medium, respectively (Hirumi and Hirumi, 1989, Brun and Schonenberger, 1979, Lamour et al., 2005). They can be transfected with DNA plasmids, and are thus experimentally tractable. Stumpy-like forms can be generated via treatment of monomorphomic cells with cell permeable analogs of cAMP in vitro (Breidbach et al., 2002).
These cells are cell cycle-arrested and have a gene expression profile similar to stumpy forms, but are not fully representative of the stumpy form due to aberrant morphology (Maegregor et al., 2014). Stumpy forms can be generated in large numbers in mice and purified from blood, but without the ability to proliferate and with a short lifespan, stumpy forms are notoriously difficult to work with. Given these difficulties, there is much still to be understood about stumpy form biology.

Other than the PCF, other fly stages are extremely hard to obtain: not all tsetse flies that feed on infected blood establish a midgut procyclic infection, and only a small proportion of flies with infected midguts establish a salivary gland infection (Peacock et al., 2012). A large number of flies are required to obtain a useful number of parasites for further experiments, dissections are laborious and time consuming and parasites are difficult to purify from fly material.

Recently it was shown that overexpression of an RNA-binding protein, RBP6, caused the \textit{in vitro} differentiation of PCF cells of the laboratory adapted strain Lister 427 to metacyclic forms that were able to infect mice (Kolev et al., 2012). This suggests the developmental program within the fly is integral to the cell biology of the parasite, not requiring signals from the internal environment of the tsetse fly. This may open up the study of other fly stages to experimental manipulation, and will hopefully allow a greater understanding of the biology of these stages.

\subsection*{1.5.5 Mitochondrial function through the life cycle}

One of the characteristics of the \textit{T. brucei} life cycle is the regulation of mitochondrial activity. Control of mitochondrial activity through the life cycle, in particular with respect to energy metabolism, is central to fulfilling the metabolic requirements of the trypanosome.
Alternating between extreme niche environments requires vast morphological and metabolic adaptations. The slender BSF of the parasite uses ATP generated by glycolysis, with oxidative phosphorylation absent (section 1.9.1). The mitochondrion of the slender BSF therefore is downregulated in terms of its role in ATP production compared to the mitochondrion found in the insect stages. It still retains functions in iron-sulphur centre (Fe/S) biogenesis (Kovarova et al., 2014), fatty acid biosynthesis (Stephens et al., 2007, Clayton et al., 2011, Mazet et al., 2013) and production of thymidine monophosphate (dTMP), a DNA nucleotide (Roldan et al., 2011), meaning mitochondrial activity is still present and essential. Mitochondrial membrane potential, ΔΨm, is essential for mitochondrial import of tRNAs needed for mitochondrial translation, and of polypeptides for the metabolic processes occurring within the mitochondrion and for the replication, expression and editing of the mitochondrial genome. Mitochondrial remodelling and activation begins upon differentiation to the stumpy form in preparation for the procyclic stage (Priest and Hajduk, 1994, Vickerman, 1965). The mitochondrion increases in volume and becomes less tubular in morphology, with membrane invaginations or cristae beginning to form (Bohringer and Hecker, 1975, Vickerman, 1965) that are required for respiratory chain efficiency (Gilkerson et al., 2003, Mannella et al., 2013).

The activated mitochondrion plays vital roles in the metabolic network established in the PCF, allowing metabolic flexibility (section 1.9.2). There is a need to increase the mitochondrial ATP output at this stage. Mitochondrial oxidative phosphorylation functions in the PCF to generate ATP, but the degree to which oxidative phosphorylation is essential varies between strains (section 1.9.2.1). Mitochondrial activity in the other fly stages has not been experimentally probed, although it is inferred that during the metacyclic stage there must be a down regulation of mitochondrial activity and a de-elaboration of the mitochondrial architecture in preparation for the slender BSF (Bohringer and Hecker, 1975, Brun et al., 1984).
1.6 Mitochondrion

1.6.1 The mitochondrial respiratory chain

The major players of a classical respiratory chain are present in the *T. brucei* genome (Figure 1.3B). A number of the protein complexes involved are able to transfer electrons and pump protons, regenerating redox equivalents for metabolic pathways and manufacturing the essential $\Delta \psi_m$. In a typical respiratory chain, reduced nicotinamide adenine dinucleotide (NADH) is oxidised to NAD$^+$ by complex I, the NADH:ubiquinone oxidoreductase. The electrons are removed from NADH by a flavin mononucleotide (FMN) centre, and ferried up the complex by a chain of Fe/S centres with increasingly positive redox potentials (Sazanov, 2007). This complex is the largest of the respiratory chain, and the movement of electrons through the complex provides the energy for the translocation of protons into the IMS, contributing to the $\Delta \psi_m$. The complex translocates 4 protons per 2 electrons (Janssen et al., 2006). The electrons reduce ubiquinone to ubiquinol within the inner mitochondrial membrane.

Complex II, succinate dehydrogenase, is an enzyme of the Krebs cycle that catalyses the conversion of succinate to fumarate via a covalently bound flavin adenine dinucleotide (FAD). The electrons are transferred through Fe/S centres to reduce ubiquinone (Maklashina and Cecchini, 2010), but the complex does not translocate protons due to the similar redox potentials of the metal cofactors (Ackrell, 2000).

Complex III, the $bc_1$ complex, reoxidises ubiquinol. Electrons are passed through Fe/S centres and heme groups (Crofts, 2004), driving the translocation of protons through the inner mitochondrial membrane at a stoichiometry of four protons per 2 electrons but two positive charges ($q^+$) per two electrons. Mobile cytochrome *c* shuttles electrons from
complex III across to complex IV, cytochrome oxidase, through the IMS. From here the electrons pass through metal ion centres to the O$_2$ electron sink (Belevich and Verkhovsky, 2008). Complex IV translocates protons at a stoichiometry of two protons per two electrons, but uses an additional two protons in the matrix during the reduction of oxygen to water (Kaila et al., 2010).

Complex V of the mitochondrial respiratory chain, the F$_1$F$_{c}$-ATP synthase, is comprised of multiple subunits that are arranged as two moieties (Figure 1.7). The F$_o$ domain is hydrophobic, and is embedded in the mitochondrial inner membrane, acting as an ion channel, whereas the hydrophilic F$_1$ domain is positioned extending into the mitochondrial matrix, containing the active sites for ATP synthesis/hydrolysis. The $\gamma$ subunit connects these two moieties, along with the external stator or peripheral stalk.

In the simplest *Escherichia coli* version of the complex, there are five subunits in the F$_1$ moiety, with the stoichiometry $\alpha_3\beta_3\gamma\delta\epsilon$, and three in the F$_o$, $ab_{2}c_{9-12}$ (Foster and Fillingame, 1982) (Figure 1.7A). Nomenclature in mammalian complexes is slightly different, with the equivalent of bacterial subunit $\delta$ being named oligomycin sensitivity-conferring protein (OSCP), and the equivalent of bacterial subunit $\epsilon$ being named $\delta$ (Figure 1.7B). Additional subunits are also present in the mammalian complex: F$_1$ subunit $\epsilon$, F$_o$ subunits A6L, $d$, F6, $e$, $f$, $g$ (Walker, 2013). Of these, only subunit $d$ influences ATP synthase activity. Some of these subunits allow dimerisation of the enzyme, and the functions of the others are presently unclear.

The $\alpha$ and $\beta$ subunits alternate around the central $\gamma$ core (Abrahams et al., 1994). The active site for ATP synthesis is the $\beta$ subunit, with three active sites per complex. All three active sites need to be occupied for optimal catalysis (Amano et al., 1996, Weber et al., 1993). The proton channel is formed between subunit $a$ and a $c$ monomer. The movement of protons down their concentration gradient into the matrix, through the F$_o$ segment, via sequential
protonation and deprotonation of aspartate 61 in subunit c, drives rotation of the c ring (Girvin and Fillingame, 1995, Assadi-Porter and Fillingame, 1995, Dimroth, 1995, Angevine and Fillingame, 2003, Angevine et al., 2007, Kuruma et al., 2012). This is coupled to rotation of γ within the α/β hexamer, forcing conformational changes in the active sites of β via asymmetric interactions, fuelling ATP synthesis (Figure 1.8A) (Ariga et al., 2007, Rastogi and Girvin, 1999, Fillingame et al., 2003). One complete 360° rotation of the c ring causes a complete rotation of γ within the α3β3 core, and the synthesis of three ATP molecules (Stock et al., 1999).

The crystal structure of the bovine heart mitochondria F₁ moiety in the presence of non-hydrolysable ATP analogue suggested the alternating site mechanism of catalysis (Kagawa et al., 2004, Menz et al., 2001, Abrahams et al., 1994). The β hinge domain containing catalytic residues changes conformation drastically after binding of ATP, propagating through β to a short DELSEED amino acid sequence that interacts with a short 18 residue helix on γ (Abrahams et al., 1994, Yagi et al., 2009). The three different β conformations each differently interact with the central asymmetrically-structured γ, with sequential conformational changes in β hypothesised to provide the torque that drives the rotation of γ in ATP hydrolysis/proton pumping mode. The rotation of γ within the α3β3 core, along with δ/ε and c subunits, has been shown experimentally (Noji et al., 1997, Adachi et al., 2000, Yasuda et al., 1998, Kato-Yamada et al., 1998, Sambongi et al., 1999). The rotation during ATP synthesis occurs in the opposite direction to that required for ATP hydrolysis (Itoh et al., 2004), suggesting that the torque for γ rotation in the hydrolysis direction is provided by ∆Ψₘ. The stator formed by a, b and δ/OSCP subunits is thought to prevent the α3β3 core rotating with γ, thereby forcing the conformational changes within F₁ and preventing proton movement through Fₖ without coupled F₁ ATP synthesis/hydrolysis (Collinson et al., 1996, Rees et al., 2009).
The complex can function in both directions. ATPase activity predominates in absence of respiratory activity in yeast (Cox et al., 1971, Kanner and Gutnick, 1972, Schairer and Haddock, 1972, Yamamoto et al., 1973); the F\textsubscript{o} moiety acts as an ion pump to maintain $\Delta \Psi m$, which is no longer generated by the movement of electrons through the inner mitochondrial membrane respiratory chain. Here F\textsubscript{1}-ATPase activity produces the energy for the movement of protons through F\textsubscript{o}, against their concentration gradient (Figure 1.8B). The activities of F\textsubscript{1} and F\textsubscript{o} are therefore still coupled together.

1.6.2 Supercomplexes of the inner mitochondrial membrane

In some organisms it has been shown that complexes of the respiratory chain interact physically within the inner mitochondrial membrane. These controversial ‘supercomplexes’ have been suggested to improve efficiency of respiration by increasing the local concentration and decreasing diffusion times of substrates and products (Dudkina et al., 2010, Blaza et al., 2014). Additionally, ‘ATP synthasomes’ have been observed in mammalian and yeast mitochondria; a physical interaction of F\textsubscript{1}F\textsubscript{o} ATP synthase, AAC, the ADP/ATP carrier and the phosphate carrier (Chen et al., 2004, Wittig and Schagger, 2008b).

Similarly, this has been suggested to increase the efficiency of the phosphorylation event at the F\textsubscript{1}-ATPase moiety. Although supercomplexes have not been observed in T. brucei (Gnipova et al., 2015), the ATP synthasome was detected in L. mexicana (Detke and Elsabrouty, 2008).

Complex V has been shown to self-organise into rows of dimers that have been proposed to be important for generating the cristae of the mitochondria (Davies et al., 2012). The angle of the dimer causes the curvature of the inner mitochondrial membrane. Dimers of ATP synthase have been seen in many eukaryotic organisms, including many protists (Acestor et al., 2011, Chaban et al., 2014, Balabaskaran Nina et al., 2010, Balabaskaran Nina et al.,
Cristae are seen in some *T. brucei* life cycle stages, for example the PCF, and not seen in others, for example the slender BSF. Whether regulation of complex V expression or assembly could be involved in this mitochondrial remodelling in trypanosomes is still to be investigated.

1.6.3 The *T. b. brucei* respiratory chain

The BSF and insect stages of the *T. brucei* life cycle are aerobic (Figure 1.3B). The *T. brucei* respiratory chain contains three additional enzymes that communicate with the ubiquinone pool in the inner mitochondrial membrane. Glycerol-3-phosphate dehydrogenase (FAD-G3PDH) is able to oxidise glycerol-3-phosphate (Gly-3-P), producing dihydroxyacetone phosphate (DHAP). This and the alternative NADH dehydrogenase (NDH2) (section 1.6.4.1) reduce ubiquinone to ubiquinol. The single subunit trypanosome alternative oxidase (TAO) is an additional electron sink, catalysing the reduction of O$_2$ to H$_2$O. These enzymes do not pump protons, and therefore do not contribute to ΔΨm. In the BSF, these three complexes along with complexes I and V (section 1.6.4) have been shown to be present in the inner mitochondrial membrane (Figure 1.3B) (Surve et al., 2012, Schnaufer et al., 2005, Clarkson et al., 1989).

1.6.3.1 TAO

The cytochrome-containing enzymes are not expressed until the stumpy form differentiates into the PCF form (Tielens and Van Hellemond, 2009), hence TAO is the terminal oxidase in slender and stumpy forms (Bienen et al., 1991). In the BSF the amount of TAO protein is ~100x higher than in PCF (Chaudhuri et al., 2002). As the only terminal oxidase in BSF, an oxidase absent in the mammalian host, the TAO is being targeted as a potential therapy for sleeping sickness (Shiba et al., 2013). The enzyme is essential in BSF, knockdown causes a
growth effect and inhibition with salicylhydroxamic acid (SHAM) kills the parasites in 24 hr (Helfert et al., 2001).

The protein is present in the PCF, however. The presence of two terminal oxidases (TAO and complex IV) allows the system flexibility in PCF (Figure 1.3B), required for survival in fluctuating oxygen and nutrient levels in the fly midgut and salivary glands. In normal PCF cells, the complex IV inhibitor potassium cyanide (KCN) inhibits 70% of oxygen uptake, showing that most oxygen consumption occurs via the classical pathway (Gnipova et al., 2012). However, when complex IV is disrupted, there is a shift towards the KCN-insensitive alternative oxidase, with KCN inhibiting much less of the oxygen uptake. Inhibition of both pathways simultaneously has a lethal phenotype, suggesting that electron transport is necessary for the survival of the parasite (Coustou et al., 2003, Van Weelden et al., 2003), presumably due to the regeneration of reducing equivalents or production of the proton gradient.

1.6.3.2 G3PDH

The glycerol-3-phosphate:dihydroxyacetone phosphate shuttle is a connection between glycolytic steps in the glycosomes and the mitochondrial respiration (Figure 1.2). An FAD-dependent G3PDH is located on the outer side of the inner mitochondrial membrane (Allemann and Schneider, 2000), and an NAD-dependent G3PDH is located in glycosomes (Opperdoes et al., 1977). In the BSF, reducing equivalents leave the glycosomes in the form of Gly-3-P, with conversion of Gly-3-P to DHAP occurring at the mitochondrion (Clarkson et al., 1989). DHAP can then return to the glycosomes, allowing the regeneration of glycosomal NAD⁺.

In PCF, the FAD-G3PDH is present, but it is not essential (Guerra et al., 2006, Skodova et al., 2013). It is linked to mitochondrial respiration; Gly-3-P can stimulate ATP production (Allemann and Schneider, 2000). Glycosomal NAD⁺ is regenerated by the production of
succinate (section 1.9.2.3) (Michels et al., 2006, Ebikeme et al., 2010). The shuttle in PCF is hypothesised to allow an upregulation in glucose and glycerol metabolism in situations when succinate formation in the glycosome, i.e. CO$_2$ levels or pyruvate demand, is limiting flux through other pathways (Guerra et al., 2006, Besteiro et al., 2002).

1.6.3.3 Complex II

Complex II has been shown as non-essential to PCF in glucose-rich conditions, but essential in glucose-depleted conditions (Coustou et al., 2008). In the absence of glucose, proline catabolism, of which succinate is an intermediate, is required to generate mitochondrial ATP (section 1.9.2.1). Due to the presence of additional, non-canonical subunits T. brucei complex II is larger than typical (Acestor et al., 2011). Complex II also may be present in the stumpy BSF, although its activity or role has not been probed (Gunasekera et al., 2012).

1.6.3.4 AAC

MCP5 has been characterised as the mitochondrial ADP/ATP carrier (AAC) in PCF T. b. brucei (Pena-Diaz et al., 2012). The AAC functions in the PCF to import ADP$^3$ while exporting ATP$^4$ (Figure 1.8A). Thereby this enzyme fuels the substrate level phosphorylation and oxidative phosphorylation in the mitochondrion and provides the rest of the cell with mitochondrially produced ATP. AAC activity in PCF is driven by the $\Delta\psi_m$. Knockdown caused a decrease in cytosolic ATP, an increase in $\Delta\psi_m$ and a reduction in O$_2$ consumption (Gnipova et al., 2015).

In the BSF, both complex V and the AAC are reversed in function (Figure 1.8B). F$_1$F$_{\sigma}$-ATPase generates the $\Delta\psi_m$ and the AAC supplies the mitochondrion with ATP (Nolan and Voorheis, 1992, Schnaufer et al., 2005). The AAC contributes to the $\Delta\psi_m$, as the exchange of ATP$^5$ for ATP$^4$ is electrogenic in nature. There is a possibility that there could be another transporter in the inner mitochondrial membrane able to import ATP as a knockdown of
AAC caused only a 50% decrease in ATP-induced ATPase activity (Gnipova et al., 2015). The ATP detected could however have been cytosolic contamination as crude mitochondrial preparations were used.

1.6.4 The respiratory complexes containing maxicircle gene products

1.6.4.1 Complex I

There is much controversy about the significance of complex I in *T. brucei* energy metabolism (Tielens and Van Hellemond, 1998, Tielens and Van Hellemond, 1999, Turrens, 1999). Sequence homology identified a number of nuclear and maxicircle encoded genes of the complex (Figure 1.3B) (Opperdoes and Michels, 2008). Two core subunits thought to be essential for proton pumping, NADH dehydrogenase subunits 4L (ND4L) and 6 (ND6), were not found using bioinformatic or proteomic means, suggesting that complex I does not participate in the generation of the \( \Delta \psi m \) (Opperdoes and Michels, 2008) or that the subunits could be present in diverged form. This is in contrast to the trypanosomatid *Phytomonas serpens*, devoid of complexes III and IV (Nawathean and Maslov, 2000, Maslov et al., 2002), which uses complex I for generation of \( \Delta \psi m \) (Cermakova et al., 2007). Interestingly, the expression and/or editing of some of the genes is upregulated in the BSF (Table 1.1) (Jasmer et al., 1985, Bhat et al., 1992). Indeed ND7 is only completely edited in BSF (Koslowsky et al., 1990, Read et al., 1994), suggesting a distinct importance in this life cycle stage.

The complex has been detected in PCF and BSF (Fang et al., 2001, Panigrahi et al., 2008, Surve et al., 2012, Verner et al., 2011). Knockdown of key electron transferring subunits presented no *in vitro* growth defect in PCF, and no effect on the \( \Delta \psi m \) (Verner et al., 2011); it seems that these electron transfer capabilities are not essential. However, these cell lines had
a reduction of around 20% of their total mitochondrial NADH oxidation activity. Knockout of key subunits of complex I in monomorphic BSF similarly showed no growth phenotype in vitro or in vivo (Surve et al., 2012). Unlike in PCF, no reduction was seen in NADH oxidation activity; thus, surprisingly, complex I is not essential and does not contribute to proton pumping in BSF either. Additionally, complex I may not be an entry point of electrons into the respiratory chain (Turrens, 1989, Allemann and Schneider, 2000). Why energy is expended into the biogenesis of the largest of the respiratory complexes in both PCF and BSF is uncertain. Other life cycle stages have yet to be studied.

The activity of this complex has been observed via inhibitor studies. Rotenone-sensitive respiration was detected in BSF and PCF (Beattie et al., 1994, Beattie and Howton, 1996, Fang et al., 2001), and rotenone-sensitive Δψm was detected in the stumpy BSF (Bienen et al., 1991). However the concentrations of rotenone used were extremely high, meaning there may have been off-target effects on other mitochondrial dehydrogenases (Hernandez and Turrens, 1998, Turrens, 1999).

The situation is further complicated by the presence of NDH2 (Fang and Beattie, 2003), as in bacteria, fungi, plants and other protozoa. This enzyme is nuclearly-encoded and rotenone-insensitive, and therefore contributes to some or all rotenone-insensitive respiration (Fang et al., 2001, Fang and Beattie, 2003, Horvath et al., 2005, Verner et al., 2010). It has been hypothesized that NDH2 may facilitate rapid growth of PCF when complex I activity limits growth, and may allow NAD+ regeneration for other mitochondrial dehydrogenases (Fang and Beattie, 2003).

However, there is some controversy as to whether the NADH binding site of NDH2 has the correct orientation to contribute to mitochondrial NAD+ regeneration. When NADH was applied externally to digitonin-permeabilised PCF cells, oxygen consumption was detected (Verner et al., 2013). As the mitochondrial membrane is impermeable to NADH, this
suggests that NDH2 is positioned in the mitochondrial membrane facing the cytosol. It is therefore uncertain whether NDH2 is able to compensate for complex I activity.

1.6.4.2 Complex III

Subunit cytochrome $b$ (CyB) is mitochondrially-encoded in kinetoplastids (Figure 1.3B). In the BSF, the levels of this transcript are very low (Michelotti and Hajduk, 1987) and translation of this subunit cannot occur as unedited CyB mRNA lacks an AUG codon that is formed by the editing of the transcript in PCF (Feagin et al., 1987) (Table 1.1).

Two nuclearily encoded subunits of complex III have been knocked down in PCF: Rieske protein and cytochrome $c_1$ (Horvath et al., 2005). Both knockdowns slowed growth of the cells, and disrupted complex III. Cytochrome $c$ reductase activity and $\DeltaΨm$ decreased, in concurrence with complex III having a classical role in the PCF respiratory chain.

1.6.4.3 Complex IV

Cytochrome oxidase (COX) has three mitochondrially-encoded subunits (Figure 1.3B). Two of these, COX II and III, are edited in the PCF (Table 1.1) (Priest and Hajduk, 1994). The complex is large and diverged, with at least 15 nuclearily-encoded core subunits and multiple accessory proteins with currently unknown function (Zikova et al., 2008b, Speijer et al., 1996a, Speijer et al., 1996b, Horvath et al., 2000). Three non-canonical subunits of complex IV have been knocked down in PCF, all of which generated non-lethal growth defects (Gnipova et al., 2012). Complex IV has a traditional role in the PCF respiratory chain; knocking down subunits of the complex reduced the $\DeltaΨm$ and the cytochrome $c$ oxidase activity of the cells.

In the BSF, complex IV is absent (Figure 1.3B), with nuclearily encoded COX transcripts being destabilised (Mayho et al., 2006). COX mitochondrial transcripts are also downregulated in the BSF (Priest and Hajduk, 1994).
1.6.4.4 Complex V

Subunit \( a \) (A6) of the \( F_o \) moiety has been predicted to be encoded by the previously named mitochondrial maxicircle unidentified reading frame 4 (MURF4) gene in \( T. brucei \) (Figure 1.3A) (Bhat et al., 1990). This transcript is extensively edited, resulting in an amino acid sequence and hydropathy profile with homology to subunit \( a \) found in other organisms. A6 is thought to be essential for PCF and BSF cells (Schnaufer et al., 2005, Schnaufer et al., 2002, Schnaufer et al., 2001).

1.6.4.5 Ribosome

One mitoribosomal protein, ribosomal protein S12 (RPS12), is encoded in kDNA and is edited in both PCF and BSF. Like most other kDNA-encoded proteins, RPS12 has not yet been detected by mass spectrometry (Zikova et al., 2008a, Acestor et al., 2011). The homolog of this protein plays an essential role in the decoding centre of a bacterial ribosome (Demeshkina et al., 2012). The mitochondrial ribosome is required for translation of kDNA-encoded transcripts in both BSF and PCF. Interestingly, it was recently shown that both A6 and RPS12 mRNA are associated with ribosomes in PCF and BSF stages due to a long A/U tail that is added as a post-editing 3’ processing event, unlike the maxicircle-encoded transcripts known to not be required in BSF (Aphasizheva et al., 2013, Aphasizheva et al., 2011). It has been shown that a disruption in editing capacity causes a global mitochondrial protein synthesis inhibition, suggesting that RPS12 production could be a controllable link between editing and translation, allowing synchronisation in levels of edited and non-edited transcripts (Aphasizheva et al., 2013).
1.7 *T. b. brucei* complex V structure

The ATP synthase is present in all stages of the *T. brucei* life cycle; it is more abundant in the PCF (Williams, 1994). The transcripts for α, β and c subunits were shown to be less stable in the BSF of the cells than in the PCF, with α and c increasing in stability at the stumpy BSF stage. This suggests a method for regulation of gene expression of F₁F₉ subunits (Brown et al., 2001).

Only the homologs of *Saccharomyces cerevisiae* α, β, γ, δ, ε, c and OSCP are readily detectable in the nuclear genome of *T. brucei* (Zikova et al., 2009)(Figure 1.7). In the PCF stage of the life cycle, the complex acts as an ATP synthase (Figure 1.8A), and the composition of this complex has been analysed thoroughly (Zikova et al., 2009). A total of 22 proteins were found to make up the complex, a similar complexity to other eukaryotic ATP synthases. Of these, 14 were novel proteins that have no recognisable homologs in yeast or mammalian complexes. RNAi of two of these novel proteins (7760 and 2930, later renamed Tb1 and Tb2) in PCF gave a growth phenotype that was not lethal (Zikova et al., 2009), agreeing with the fact that F₁F₉ ATP synthase is not the only complex generating mitochondrial ATP (Bochud-Allemann and Schneider, 2002). Knockdown of Tb1 and Tb2 caused a disruption of the complex (Zikova et al., 2009). Although required for ATP synthase activity, these two novel subunits were shown to be non-essential for ATPase activity in vitro, a demonstration that *T. brucei* complex V can have different functional constituents depending on its direction of activity.

The F₁ moiety from *T. b. brucei* PCF has been characterised (Gahura et al., personal communication). Interestingly, *T. b. brucei* F₁ shows some Euglenozoan-specific features (Perez et al., 2014). A stoichiometry of αβγδε1p181,3 was seen (Gahura et al., personal communication). Subunit α is split into two fragments between the N-terminal crown domain and the nucleotide binding domain, with both being found associated with F₁.
The crown facilitates binding of the catalytic core to the peripheral stalk, mediated by OSCP (Rees et al., 2009). The significance of the cleavage of α is not understood. Subunit p18 was originally thought to be a homolog of F_{o}-subunit b (Speijer et al., 1997), but this has been disproved (Zikova et al., 2009, Hashimi et al., 2010) (Gahura et al., personal communication). Subunit p18 is now thought to be a dimer or a trimer, but its function within F_{1} is still unknown (Gahura et al., personal communication).

The respective functions of Tb1 and Tb2 were analysed in BSF (Subrtova et al., personal communication) (Subrtova et al., 2015). Tb2 was found to contain a putative transmembrane domain and homology to bovine subunit d, a hydrophilic essential component of the peripheral stalk (Fig. 1.7B), and was suggested to be part of a divergent form of the peripheral stalk. RNAi against this protein in BSF caused a growth effect, decrease in ΔΨm, and disrupted F_{1}F_{o} complexes when analysed on native gels, although the knockdown did not affect the presence of F_{1} moieties or F_{1}-ATPase activity. The decrease in ΔΨm is likely a direct consequence of disruption of the complex. Immunogold labelling suggested that ATPase-F_{1} is not associated with the inner mitochondrial membrane when Tb2 was depleted, consistent with the role of its putative subunit d homolog as a peripheral stalk protein (Norais et al., 1991).

Tb1 was shown to be present in the inner mitochondrial membrane, and was shown to be a component of F_{1}F_{o}, but not F_{1} (Subrtova et al., personal communication). A reduction in ΔΨm was caused almost immediately after induction of Tb1 knockdown, suggesting an impairment of the proton pore function. RNAi knockdown gave an immediate growth response in BSF that was not caused by complex instability, which appeared almost a day later. It was hypothesised that this subunit is an accessory factor of the F_{o} moiety, or for A6 subunit stability.
There are three different subunit c genes present in the *T. b. brucei* genome, unlike in other organisms (Gulde et al., 2013). The sequences show divergence towards the 5’ end of the genes. The signatures of c subunit in other organisms, including residues essential for proton movement, are present in all three isoforms. Pull down assays indicated that the c ring in PCF contains all 3 isoforms. The significance of having 3 isoforms in a heteromeric complex is not known. A change in composition of the c ring during the life cycle could change the function of the complex, for example by inducing the ATPase/ATP synthase reversal between BSF and PCF. The number of c monomers in the ring is also unknown. This number is known to determine the ratio of protons translocated per ATP molecule in either direction (Dimroth et al., 2006).

### 1.7.1 Function of complex V in BSF

Complex V is essential for maintaining viability of the slender BSF of the parasite, despite energy metabolism not involving classical oxidative phosphorylation through the mitochondrial electron transport chain. The complex acts as an ATPase to maintain $\Delta \Psi_m$ (Figure 1.7C, 1.8B) (Nolan and Voorheis, 1990, Nolan and Voorheis, 1992, Divo et al., 1993).

RNAi knockdown of the complex V α subunit showed a decrease in $\Delta \Psi_m$ that began a few hours before a growth effect was seen (Schnaufer et al., 2005). The loss of viability could be due to the dependence of mitochondrial protein import on this $\Delta \Psi_m$ (Priest and Hajduk, 1996, Bertrand and Hajduk, 2000, Nolan and Voorheis, 1992, Nolan and Voorheis, 1990, Vercesi et al., 1992). The loss of viability in the absence of complex V, and the essentiality of kDNA in the BSF (Schnaufer et al., 2001) suggests that kDNA-encoded A6 is essential in BSF.
1.8 How is the $\Delta \Psi m$ generated in cells without kDNA?

It has been long known that facultative anaerobic petite positive yeast (e.g. *S. cerevisiae*) can survive loss of some or all mtDNA (mtDNA$^-$ and mtDNA$^0$, respectively), as they are viable in the absence of oxidative phosphorylation. Petite negative yeast (e.g. *Kluyveromyces lactis*) cannot tolerate the loss of mtDNA (Clark-Walker and Chen, 1996). As mtDNA in yeast encodes subunits of respiratory subunits essential for generating $\Delta \Psi m$, including subunit $a$, mtDNA$^0$ cells cannot generate their essential $\Delta \Psi m$ using either direction of the F$_1$F$_o$ ATPase/synthase.

Similarly, *T. b. evansi* and *T. b. equiperdum* are *T. brucei* DK subspecies that are characterised by a complete or partial loss of functional kDNA (complete loss of kDNA is also called AK). The gene for subunit $a$ in these subspecies is missing (loss of the maxicircle), or cannot be edited (loss of minicircles with essential gRNAs). In *T. b. evansi*, a knockout of RNA ligase 1 (REL1), a protein essential for RNA editing of mitochondrial transcripts in *T. brucei*, had no effect on growth rate, indicating that RNA editing of maxicircle transcripts is not necessary in DK cells (Schnaufer et al., 2005). However, a knockdown of the ATPase $\alpha$ subunit was still lethal in *T. evansi*, with an effect on $\Delta \Psi m$ and cell growth; hence, even in the absence of a functional F$_o$ moiety the F$_1$ moiety still has a direct role in generating the $\Delta \Psi m$.

The electrogenic AAC is the direct producer of the $\Delta \Psi m$ in petite positive yeast and DK trypanosome cells (Clark-Walker and Chen, 2001, Schnaufer et al., 2005, Dean et al., 2013): ATP$^+$ is imported while ADP$^-$ is exported, making the matrix more negatively charged (Figure 1.8C). The direction of the exchange of ADP$^-$ for ATP$^+$ is driven by differences in substrate concentration (Schnaufer et al., 2005, Clark-Walker and Chen, 2001). The F$_1$-
ATPase acts to maintain the ATP/ADP ratio by depleting mitochondrial ATP and generating more ADP substrate. This is in contrast to WT BSF *T. brucei*, where the AAC acts more indirectly, by providing ATP for proton pumping, which makes up the majority of the contribution towards $\Delta \Psi m$ (Figure 1.8B) (Brown et al., 2006). *S. cerevisiae* with mutated $\gamma$ has a decreased $\Delta \Psi m$ consistent with this model (Wang et al., 2007, Schnaufer et al., 2005, Dunn and Jensen, 2003).

Interestingly, in *T. b. evansi*, knockdown of the peripheral stalk protein Tb2 caused a growth effect, with a decrease in $\Delta \Psi m$ (Subrtova et al., 2015), in accordance with the knockdown effects seen in BSF *T. b. brucei*. Maintaining Tb2 bound to the membrane seems to allow maintenance of the $\Delta \psi m$ in *T. b. evansi*, keeping the enzyme within a close proximity to AAC. This could ensure that the product of the ATPase reaction, ADP, is generated close to the AAC complex that requires it as a substrate, ensuring efficient coordination between the complexes for $\Delta \psi m$ production. The close localisation of these complexes is reminiscent of the ATP synthasomes seen in other organisms that are hypothesised to perform the same function (Ko et al., 2003, Chen et al., 2004). This arrangement could be particularly useful in tubular BSF mitochondria lacking the cristae that traditionally enhance respiratory chain function (Demongeot et al., 2007).

### 1.8.1 Uncoupling F$_{1}$F$_{0}$-ATPase mutations

Mutations were found in ATPase-F$_{1}$ subunits in mutant petite negative yeast that survived loss of mtDNA (Chen and Clark-Walker, 1995, Chen and Clark-Walker, 1996). These mutations compensated for the loss of mtDNA. This prompted a comparison of the sequences of $\gamma$ subunits between *T. brucei*, naturally occurring DK subspecies of *T. brucei*,
and an acriflavine-induced DK *T. brucei* strain, which revealed several polymorphisms in the ATPase γ subunit (Table 1.2) (Schnaufer et al., 2005, Lai et al., 2008).

The effect of these mutations on the ability of *T. b. brucei* BSF cells to survive kDNA loss was tested by knocking out one or both alleles of endogenous ATPase γ and expressing a mutated γ (Dean et al., 2013). Cells expressing γ subunit with a lysine to proline substitution at amino acid 262 (L262P) or an alanine to proline substitution at amino acid number 273 (A273P) were able to remain viable after kDNA loss upon acriflavine treatment, with no effect on Δψm. These mutations therefore fully compensate for kDNA loss, meaning these cells are kDNA-independent. This is unlike WT cells, which stop growing after 24 hrs of acriflavine treatment, have an obliterated Δψm by 48 hrs, and are dead by 72 hrs. Similarly, cells expressing the L262P or A273P-mutated γ subunit show no requirement for kDNA gene products; for instance, REL1 knock out causes no growth effect.

The mechanism for this compensation was probed, and these cells were shown to be more dependent on AAC activity. They were shown to be manufacturing their Δψm independently of Fₐ, meaning they had no requirement for A6 (and therefore RPS12), and thus were surviving without kDNA gene products:

- Cells expressing mutated γ were as oligomycin resistant as the AK versions of these cell lines. Similarly, *T. b. evansi* is more resistant to oligomycin than *T. b. brucei*, as are petite mutants of yeast compared with WT yeast (Kovac, 1974, Opperdoes et al., 1976, Schatz, 1968). In BSF, F₁-ATPase activity actively pumps protons through Fₒ, thereby controlling the direction of proton flow through Fₒ; the actions of F₁ and Fₒ are coupled. Cells with mutated γ subunits do not use Fₒ to generate Δψm and therefore have uncoupled activities of F₁ and Fₒ, even in the presence of kDNA. The role of F₁ in these cells has not yet been defined, but is thought to be essential, as is the case in *T. b. evansi* (Schnaufer et al., 2005).
• Increased sensitivity to bongkrekic acid, an AAC inhibitor, occurred upon expression of a mutated γ subunit in the presence or absence of kDNA. This suggests that it is the presence of the uncoupling mutation that allows survival dependent on AAC-Δψm generation.

Interestingly, a methionine to lysine substitution at amino acid position 282 (M282L) γ mutation was shown to not compensate for kDNA loss in T. brucei (Dean et al., 2013). Thus it is still unexplained how the strain of T. b. evansi with this γ compensates for kDNA loss.

The mechanism of kDNA independence for the A281- γ mutation is not clear-cut. Cells expressing A281- γ were unable to proliferate in the presence of acriflavine, but, in contrast to WT cells, not all cells in the population died. Growth recovery occurred after removal of acriflavine, and after a short recovery period the cells grew well in the presence of acriflavine. The mutation thus seemed to require a secondary adaptation to produce full kDNA independence. Subsequent studies have shown that replacement of one endogenous γ gene with A281- γ did cause full kDNA independence in multiple clones (C. Dewar and L. Woodcock, unpublished). Although the expression levels of mutant protein in these studies have not been compared directly, these observations could possibly be explained by nonsufficient expression of the mutant gene in the Dean et al. study.

The alanine 281 deletion (A281-) γ mutation is found in many DK isolates (Dean et al., 2013). Interestingly, the mutation is usually only found in one allele in nature (Table 1.2)(Dean et al., 2013, Lai et al., 2008). Analysis of γ subunit cDNA from T. b. evansi STIB810 suggested that while both ATPase γ alleles are transcribed, 90% of transcripts originate from the mutated A281- γ allele. Thus it seemed that the mutated γ allele is somehow transcribed in preference to the WT allele, possibly to increase the mutated allele dosage. Appendix A contains an investigation into whether this could be part of the mechanism that allows survival in the absence of kDNA in heterozygous A281-γ expressing
T. b. evansi strains. Pyrosequencing showed that the mRNA expression levels for the A281- and wt subunit γ alleles are approximately equivalent for all three T. b. evansi strains investigated, disagreeing with the finding in Lai et al., 2008. This strongly suggests that upregulation of the mutated ATPase γ transcript is not part of the compensatory mechanism for heterozygous γ mutations. However whether an upregulation of the mutated γ subunit occurs at the protein level has not yet been investigated.

Structural studies in S. cerevisiae have indicated how compensatory mutations in the C-terminus of γ subunit could functionally uncouple the action of the yeast F₁F₀-ATPase (Figure 1.7D). These mutations are seen to cluster in the hydrophobic collar region that is formed by loops in the α and β subunits (Wang et al., 2007). The collar provides structural support, securing the axis of rotation of γ in the α/β core. The collar tilts 45° due to the asymmetric nature of the F₁ moiety. The L262Pγ mutation of lab-induced DK T. brucei is equivalent to L254P in S. cerevisiae, and is at the beginning of the coiled-coil region of γ. The helix-breaker proline is thought to kink the γ helix, altering the axis of rotation, and as such altering the functional interactions with α and β subunits. A hydrogen bond network in this collar region has been shown to be distorted in crystal structures of collar region ATPase mutants, preventing β binding an inorganic phosphate group (Arsenieva et al., 2010); this is thought to allow γ to rotate despite substrate not being attached to the F₁-β active site. The free movement of γ would allow F₀-c ring rotation uncontrolled by F₁. This could cause a proton leak (Mueller, 2000), as proton could move down their concentration gradient from the IMS, through F₀, into the more negative matrix, dissipating the ΔΨm. The loss of the ΔΨm would be lethal.

The survival of S. cerevisiae cells is therefore facilitated by preventing this leak. The F₀ complex is encoded by mtDNA, therefore partial or complete deletion of mtDNA prevents
the leak occurring (Lai-Zhang et al., 1999). Thus, the same mutations that permit loss of mtDNA in petite-negative yeast force its loss in petite-positive yeast.

However, studies from our lab in *T. brucei* suggest that introduction of the compensatory mutations that permit loss of kDNA usually do not cause gross effects on kDNA stability (personal observations and unpublished results by Matt Gould). However, in *T. brucei*, minicircles encoding gRNAs specific for A6 are needed for production of the mature transcript, hence loss of a few minicircles by asymmetric division of the kDNA during cell division could prevent daughter cells from generating fully edited A6. Loss of A6 protein would be expected to prevent the proton leak. This is yet to be proved.

The structure of the *T. brucei* ATPase in cells expressing mutated γ subunit is yet to be probed. On native protein gels, an active *T. b. evansi* F1-ATPase moiety is detectable, but an F1F0 complex is not (Hashimi et al., 2010). Similarly, an indirect knockdown of A6 in PCF cells through ablation of editing showed an accumulation in free soluble F1 moiety, with a decrease in F1F0 complex (Hashimi et al., 2010). These knock-down cells showed increased resistance to F0-inhibitor oligomycin, suggesting that in the absence of A6, there is decreased stability of the incompletely-assembled F0 moiety, but the ATPase activity of the F1 is maintained.

Kinetic studies have been carried out using *K. lactis* mutants expressing mutated F1 subunits. In comparison with WT *K. lactis* and other petite negative yeasts, the Km value for the F1 ATPase reaction is lower, showing that the mutant complex has a higher affinity for its substrate (Clark-Walker, 2003). ATPase γ with the equivalent of the L262P mutation has also been expressed in yeast; likewise this mutation reduced the Km (Schnaufer et al., 2005). Rate of reaction analysis showed that, at low ATP concentrations, cells expressing mutant γ have a greater rate of ATP hydrolysis. In yeast this is thought to be an adaptation allowing growth during hypoxia, as in the absence of oxygen the electron transport chain that
generates the $\Delta \Psi_m$ cannot function. The ATP synthase reverses in function to maintain this $\Delta \Psi_m$.

**Detailed examination on the molecular mechanism of the compensatory $\gamma$ mutations in *T. brucei* is required to understand how these mutations uncouple BSF $F_1F_0$-ATPase and how trypanosomes are able to combat the proton leak to maintain their $\Delta \psi_m$. These objectives are investigated in Chapter 2 of this thesis.**

### 1.8.2 Impact of independence from kDNA

Many positively charged compounds are actively accumulated in the mitochondrion due to the active $\Delta \psi_m$ (Mathis et al., 2006, Lanteri et al., 2008). A number of established and candidate drugs for diseases caused by trypanosomatids target the unique but essential kinetoplast by binding to it, or inhibiting its replication (Deterding et al., 2005, Shapiro and Englund, 1990, Roy Chowdhury et al., 2010). Decreased sensitivity to multiple drugs is seen in kDNA-independent *T. brucei*, notably in the diamidines and phenanthridines classes: diminazene, DB829, isometamidium and EtBr (Gould and Schnaufer, 2014). This correlates with resistance to isometamidium seen in *T. b. evansi* strains (Brun and Lun, 1994).

As naturally occurring DK subspecies of *T. brucei* have evolved multiple times previously (section 1.10.1), these findings should encourage intelligent and sparing use of these drugs in the field, particularly on animals. Drug dosage at non-lethal concentrations could cause adaptation to survival in the presence of the drug (Matovu et al., 2001); for example, continued use of prophylactic drugs could select for cells that have become kDNA-independent via ATPase $\gamma$ mutation, which would provide cells with a mechanism to tolerate multiple drugs. **Additionally, if such kDNA-independent cell lines were able to complete the life cycle within the tsetse fly, the trait of increased resistance to multiple drugs**
could be spread. Whether kDNA-independent cells can be transmitted by tsetse flies is investigated in Chapter 4 of this thesis.

1.9 Metabolism

1.9.1 ATP production in the slender stage

Glucose is readily obtained from the blood of the mammalian host, and glycolysis is used to generate ATP (Figure 1.2). Even a 50% inhibition of glycolytic flux causes death of the parasites (Eisenthal and Panes, 1985, Albert et al., 2005, Haanstra et al., 2011), one reason why glycolysis in BSF is an attractive drug target (Haanstra et al., 2011). Glucose is converted to mostly pyruvate (Haanstra et al., 2012), with some production of succinate, acetate and alanine (Mazet et al., 2013). The steps converting glucose to 3-phosphoglycerate occur within the glycosomes, with the final conversion to pyruvate occurring in the cytosol.

Under aerobic conditions, there is a fine balance between ATP production and use, and the NADH/NAD⁺ ratio needs to be maintained within the glycosome. All carbon present in glucose is used to produce pyruvate. The action of aldolase generates two 3-carbon (3C) intermediates, glyceraldehyde 3-phosphate (G-3-P) and DHAP. DHAP is converted to Gly-3-P within the glycosome, regenerating glycosomal NAD⁺ to maintain the metabolism of G-3-P to pyruvate. Gly-3-P leaves the glycosome and is reoxidised to DHAP by the action of mitochondrial G3PDH (section 1.6.3.2). Once back in the glycosome, DHAP is converted to G-3-P, allowing the continuation of glycolysis through to pyruvate. The two ATP molecules used by hexokinase and phosphofructokinase are regenerated by two 3C entities passing through phosphoglycerate kinase. The two ATP generated by pyruvate kinase from a single glucose molecule are the output of glycolysis used by the cells for growth and motility.
In anaerobic conditions, the DHAP is metabolised to glycerol, producing one ATP molecule within the glycosome. The G-3-P pathway produces two ATP molecules, one inside the glycosome, regenerating the ATP required for the first steps of glycolysis, and one outside by pyruvate kinase. Hence one ATP is produced per glucose, 50% of the ATP produced in aerobic conditions.

1.9.2 Metabolism in PCF

The study of metabolism in PCFs is compounded by the use of different strains with different passage histories. Some studies are performed with culture-adapted PCF strains, some with midgut-isolated PCF, and some with strains in vitro differentiated from BSFs that again have varied passage histories in vitro and in vivo.

The metabolic network present in PCF cells enables utilisation of a range of substrates. PCF trypanosomes are able to utilise proline, an amino acid abundant in the fly midgut, as a carbon source. However, in in vitro culture, PCF tend to be grown in SDM79 medium (Brun and Schonenberger, 1979), rich with glucose (at a concentration of 6 mM or 10 mM). This is non-physiological compared to in vivo conditions, as although glucose is present within blood meals, it is not thought to remain in the midgut longer than 15-30 minutes after a blood meal (Vickerman, 1985).

1.9.2.1 ATP production

Mitochondrial ATP can be generated by oxidative phosphorylation and substrate phosphorylation (Figure 1.9). There is some controversy about the requirement for oxidative phosphorylation in the PCF. Succinate seems to be the main substrate feeding into the respiratory chain at complex II (Turrens, 1989, Bochud-Allemann and Schneider, 2002). RNAi of succinate dehydrogenase (complex II) abolishes oxidative phosphorylation, but this
has no growth effect in the presence of glucose (Bochud-Allemann and Schneider, 2002). Strains EATRO1125 and Lister 427 are >1000x more sensitive to $F_0$ inhibitor oligomycin when grown in the absence of glucose compared to in the presence of glucose (Lamour et al., 2005). Similarly, addition of oligomycin has no effect on intracellular ATP levels in EATRO1125 in glucose-rich SDM79 (Coustou et al., 2003). Hence oxidative phosphorylation appears to be required in the absence of glucose, but not in the presence of glucose. However the electron transport chain is still essential in the presence of glucose, presumably for regeneration of redox intermediates (Coustou et al., 2003, Van Weelden et al., 2003). The effect of glucose could be strain-specific; Lister 427 29.13 cells have been shown to be sensitive to oligomycin in the presence of glucose (Zikova et al., 2009). Oxidative phosphorylation uses the electron-transferring and proton-pumping complexes of the inner mitochondrial membrane and complex V to synthesise ATP, including those with subunits encoded by kDNA (Figure 1.3B). Very few studies have investigated the metabolism occurring in in vivo cultivated PCF cells. As the midgut is thought to be lacking in glucose, it is thought that oxidative phosphorylation and therefore kDNA is essential in PCF (Vickerman, 1965, Opperdoes et al., 1976). However the requirement for kDNA in the fly midgut has never been formally tested in vivo. The requirement for kDNA in the PCF is assessed in Chapter 4 of this thesis.

The acetate:succinate CoA-transferase/SCoAS cycle (ASCT cycle) produces ATP, coupling ATP generation with acetate formation (Bochud-Allemann and Schneider, 2002). It can function in anaerobic conditions, a possible adaptation to hypoxic conditions in the fly. This cycle is found in trypanosomatids, some parasitic helminths and some hydrogenosomes (Steinbuchel and Muller, 1986, Marvin-Sikkema et al., 1993, Saz et al., 1996, Van Hellemond et al., 1998). ASCT is expressed in PCF, and is not essential in the presence of glucose due to acetyl-CoA thioesterase being able to generate acetate, but with reduced energy yield (Riviere et al., 2004, Millerioux et al., 2012). It is estimated that the net
production of ATP using substrate level phosphorylation is around 3 times higher in glucose-rich media than in glucose-depleted media due to ASCT (Bringaud et al., 2006).

SCoAS, a Krebs cycle enzyme, generates ATP rather than GTP as in most higher eukaryotes. Proline metabolism with α-ketoglutarate as an intermediate causes flux through this pathway (Bochud-Allemann and Schneider, 2002). This enzyme is essential in the presence of glucose; the interruption of SCoAS eliminates both mitochondrial substrate level phosphorylation ATP production pathways (Figure 1.9)(Bochud-Allemann and Schneider, 2002, Zhang et al., 2010b). Oxidative phosphorylation is not eliminated as mitochondrial succinate can be produced from malate (Coustou et al., 2005), but apparently cannot sustain PCF cells.

In glucose-depleted conditions the AAC has an essential role in distributing ATP generated via substrate-level and oxidative phosphorylation from proline catabolism to the rest of the cell (Pena-Diaz et al., 2012). In the presence of glucose, there is slower growth effect of AAC depletion. Glycolysis is able to provide the cytosol with some ATP, but the loss of the AAC means that mitochondrion is unable to sustain ATP production (Pena-Diaz et al., 2012, Coustou et al., 2003).

### 1.9.2.2 Growth in the absence of glucose

Proline is a major source of energy in PCF (Evans and Brown, 1972, Ter Kuile, 1997). Proline metabolism is used to generate α-ketoglutarate, functioning in a partial Krebs cycle, via CO₂-producing α-ketoglutarate dehydrogenase and the ATP-producing SCoAS, to produce succinate (Figure 1.10) (Van Weelden et al., 2003). Succinate does not proceed further in the Krebs cycle, but is oxidised by complex II of the respiratory chain, contributing to oxidative phosphorylation. In low glucose in vitro conditions, the end products of ¹⁴C-proline metabolism are CO₂ and succinate in equal proportions, with a fraction of acetate (Van Weelden et al., 2005). CO₂ is the product of α-ketoglutarate
dehydrogenase activity, with proline only degraded to succinate (Figure 1.10). Proline metabolism has not been investigated within the fly midgut.

Threonine can also be utilised as an energy source by the PCF; it is the most readily consumed metabolic substrate in PCF when available (Lamour et al., 2005). Acetate is the major end product (Figure 1.11) (Cross et al., 1975, Linstead et al., 1977). Threonine does not sustain growth of the parasite alone as not enough ATP is produced (Lamour et al., 2005). Threonine is converted to acetyl-CoA, conversion of which to acetate can generate ATP via the ASCT cycle (Millerioux et al., 2013). Both glucose and threonine can contribute to fatty acid biosynthesis as the pathways merge at acetyl-CoA production (Millerioux et al., 2013, Riviere et al., 2009). Threonine is the preferred carbon source for lipid biosynthesis, but the pathway is not essential in the presence of glucose (Millerioux et al., 2013). It seems that the presence of glucose downregulates the threonine pathway by reducing threonine dehydrogenase levels.

1.9.2.3 Growth in the presence of glucose

The glycolytic pathway acts to metabolise glucose in PCF (Figure 1.10). There are some differences in this process in PCF compared to BSF. The enzyme phosphoglycerate kinase is not present in the glycosome, as in BSF, but in the cytosol (Alexander and Parsons, 1991, Opperdoes and Cottem, 1982). Hence 1,3-bisphosphoglycerate (1,3-BPG) leaves the glycosome for further metabolism. In the glycosome, the succinate-producing pathway allows ATP levels to be regenerated by phosphoenolpyruvate carboxykinase and pyruvate phosphate dikinase (Michels et al., 2006) (Deramchia et al., 2014), and NAD+ levels to be regenerated by malate dehydrogenase and fumarate reductase (Besteiro et al., 2002, Opperdoes et al., 1981).

In PCF the metabolic end product of glycolysis, pyruvate, is metabolised further to acetyl-CoA, unlike in the slender BSF (Figure 1.10). From here it was widely thought that acetyl-
CoA contributed to the Krebs cycle, generating CO$_2$ (Durieux et al., 1991, Clayton and Michels, 1996, Opperdoes, 1987). However, van Grinsven et al., (2009) reported there was no labelled CO$_2$ detected as a glucose metabolic end product in strain TREU 927. This agrees with other literature that states that this classical complete Krebs cycle, despite all components being present within the genome, surprisingly does not function as a cycle in PCFs (Van Weelden et al., 2003, Van Weelden et al., 2005, Bochud-Allemann and Schneider, 2002). Unlike in yeast (Carlson, 1999, Trumbly, 1992), in T. brucei the Krebs cycle does not even seem to be activated to function as a cycle when the growth medium is depleted of glucose and glycerol, both glycolytic substrates (Van Weelden et al., 2005). Instead of oxidation of carbon-containing compounds to CO$_2$, parts of the cycle are reported to be functioning in amino acid degradation, gluconeogenesis and fatty acid production (Van Weelden et al., 2005). The Krebs cycle could perhaps have a role functioning as a cycle in another insect stage of the parasite or in depleted nutrient conditions. Hence glucose is not an essential substrate for PCF. There does not seem to be an increase in amino acid catabolism to compensate for the loss of glucose metabolism (Van Weelden et al., 2005).

In SDM79, the end products of $^{14}$C-glucose metabolism are 56% acetate and 44% succinate (Van Weelden et al., 2003). Succinate is produced within the glycosomes and mitochondrion (Figure 1.10) (Cross et al., 1975, Besteiro et al., 2002, Bringaud et al., 2006). In SDM79, the end products of $^{14}$C-proline metabolism are 50% CO$_2$ and 50% succinate (Van Weelden et al., 2003), similar to in glucose-depleted conditions (Van Weelden et al., 2005).

Strains preferentially use D-glucose as the main carbon source in vitro (Cross et al., 1975) (Cazzulo et al., 1985, Van Weelden et al., 2003). The rate of glucose metabolism is three times faster than that of proline (Van Weelden et al., 2003). This is despite proline metabolism being more efficient in ATP yield than D-glucose metabolism. PCF grown in +/-6 mM glucose prior to fly infections show the same rate of infectivity (Ebikeme et al., 2008), hence this glucose preference in vitro does not seem to affect adaption to life in vivo. Within
the fly midgut, the end products of $^{14}$C-glucose metabolism in TREU 927 are 50% acetate, with 30% pyruvate and 20% succinate (Van Grinsven et al., 2009). The increase in pyruvate production in conditions of depleted glucose could suggest metabolism to pyruvate is more significant under physiological conditions, or could have been contamination from the midgut tissue. Labelled CO$_2$ was not produced; an active Krebs cycle is not present in *in vivo* conditions.

Glucose depletion has no effect on the growth rate of established PCF cell lines: the rates of growth in SDM80 (5.2 mM proline, 0.15 mM glucose) and SDM79 (5.2 mM proline, 6 mM glucose) for culture-adapted strains EATRO1125 and Lister 427 are the same (Lamour et al., 2005). Proline consumption becomes essential once glucose is removed as a possible substrate. The rate of proline consumption increases 6-fold in the absence of glucose compared to in the presence of glucose. This is due to an increase in the activity of proline dehydrogenase in the absence of glucose. The presence of glucose or glucose analogues represses proline metabolism: there is a decrease in the $V_{\text{max}}$ of proline uptake and a decrease in pyruvate dehydrogenase (PDH) activity, suggesting proline metabolism and uptake are negatively affected by the presence of glucose. It could be that in the fly midgut immediately after a blood meal, glucose is utilised as the main carbon source, and as glucose is depleted the repression of proline metabolism is relinquished. There does not seem to be an on-off switch for proline metabolic repression caused by a threshold of glucose concentration being reached, rather a dynamic adjustment between proline and glucose metabolism (Ebikeme et al., 2008). Cells adapted to growth without glucose retain high levels of proline consumption in the presence of glucose, hence the effect does not seem to be due to a direct action of glucose itself, more an adjustment of global cellular metabolism. Why the presence of glucose is used as a negative signal for metabolic regulation rather than the presence of proline being used as a positive signal is uncertain.
1.9.3 Metabolism in the stumpy life cycle stage

The stumpy life cycle stage is preadapted to differentiation to the PCF in the midgut of the tsetse fly. There is a corresponding shift in metabolism during differentiation from slender to stumpy forms. Stable isotope labelling by amino acids in cell culture (SILAC)-quantitative proteomic analysis through the life cycle from BSF to the PCF has shown that there is an increase in the abundance of many mitochondrial proteins in the stumpy life cycle form compared to the slender BSF (Gunasekera et al., 2012). Most of the changes in abundance occurred during slender to stumpy differentiation rather than during the transition from stumpy form to the PCF, although this does not ultimately mean that the activity of these proteins is present or required in the stumpy form. All complexes of the respiratory chain are upregulated in the stumpy form compared to the slender BSF, with complex I, II and V peaking in levels at the stumpy stage. Complex III and IV peak in PCF, hence their expression could control the activation of the respiratory chain in the PCF.

In the slender BSF, key mitochondrial metabolic enzyme activities are missing (α-ketoglutarate dehydrogenase, citrate synthetase and succinate dehydrogenase) (Vickerman, 1965, Flynn and Bowman, 1973), whereas these enzymes are expressed in the subsequent stumpy BSF (Flynn and Bowman, 1973, Gunasekera et al., 2012). Slender BSFs mainly excrete pyruvate as they rely on glycolysis for ATP production (Flynn and Bowman, 1973). When incubated with glucose, stumpy forms excrete pyruvate and a higher proportion of acetate than slender BSF (Van Grinsven et al., 2009, Mazet et al., 2013). This is thought to be due to increased ASCT expression and activity (Figure 1.9) (Van Grinsven et al., 2009, Gunasekera et al., 2012). Acetate is generated from acetyl-CoA by either ASCT or acetyl-coA thioesterase (Figure 1.11) (Mazet et al., 2013). Mitochondrial acetyl CoA production causes NADH production in the mitochondrion, meaning either complex I or NDH2 activity...
would be required in the stumpy form to regenerate mitochondrial NAD⁺ (Opperdoes and Michels, 2008).

Stumpy forms can utilise glucose and α-ketoglutarate as energy sources (Vickerman, 1965, Bienen et al., 1993, Flynn and Bowman, 1973). The lack of dependency on glucose highlights the shift in metabolism towards the glucose-deficient environment of the tsetse midgut. Indeed there is a positive correlation between intracellular ATP concentration and the proportion of stumpy form cells within a mixed population when using α-ketoglutarate as a sole substrate (Bienen et al., 1993). This ATP production is mitochondrial, being bongkrekic acid- and SHAM-sensitive. However, the effect of oligomycin was not tested, so we do not know which mitochondrial ATP production pathway is being used. Complex V is thought to act in the ATP synthase direction in the stumpy stage (Bienen et al., 1991), meaning that oxidative phosphorylation can also contribute to ATP production. SCoAS and ASCT are present in the stumpy form at levels comparable to the PCF (Gunasekera et al., 2012) (section 1.9.2.1, Figure 1.9). As long as these enzymes are active, it seems that ATP can be generated in stumpy forms through glycolysis in the presence of glucose, and by the ASCT cycle, SCoAS or oxidative phosphorylation in the presence of α-ketoglutarate. Interestingly, SCoAS appears to also have a function in slender BSF (Zhang et al., 2010b), although how it contributes to metabolism remains to be determined.

The classical diaphorase assay has been used to measure mitochondrial activity. Blue-black formazan deposits occur localised to the mitochondrion upon incubation of cells with NADH and nitro-blue tetrazolium salt, indicating unspecific NADH dehydrogenase activity. Whereas slender BSF and T. b. evansi show no clear activity, stumpy forms do stain blue-black (Vickerman, 1965, Tyler et al., 1997); there is an upregulation of some mitochondrial NADH dehydrogenase activity during slender to stumpy differentiation. It is uncertain which protein generates this upregulated activity. LipDH is a candidate. α-LipDH immunofluorescence was seen to have the same staining pattern as the diaphorase deposits.
(Tyler et al., 1997), with clear signal being seen only in stumpy forms. However, some α-LipDH staining has been detected in slender BSF mitochondria in other studies (Roldan et al., 2011, Stephens et al., 2007). Upregulation in expression and/or dehydrogenase activity of four LipDH-containing complexes during slender to stumpy differentiation could explain the diaphorase signal: LipDH is a subunit of PDH, α-ketoglutarate, branched chain ketoacid dehydrogenase and glycine cleavage (GCC) complexes (Roldan et al., 2011), of which GCC and PDH are present in the slender BSF (Roldan et al., 2011, Mazet et al., 2013). The activity could also be complex I activity. However, in-gel NADH dehydrogenase activity used traditionally to investigate complex I activity also seems to stem from a LipDH-containing complex in *T. b. brucei* (Surve et al., 2012). Hence it is more likely that LipDH activity is the cause of diaphorase staining. The requirement to regenerate mitochondrial NAD+ consumed by increased LipDH activity in the stumpy form again indicates a need for complex I or NDH2.

### 1.9.4 The possible role of kDNA gene products in the stumpy stage

As discussed, kDNA is known to be essential in the slender BSF and the PCF. It is expected that only two maxicircle genes are required for the BSF stage, A6 and RPS12 (Dean et al., 2013, Aphasizheva et al., 2013, Schnaufer et al., 2005). *Interestingly DK subspecies of T. brucei are generally thought to be monomorphic* (section 1.10.2), which could suggest that the lack of kDNA prevents differentiation to the stumpy life cycle stage. Chapter 3 of this thesis investigates whether kDNA is essential for the stumpy form.
It is not known which, if any, mitochondrial genes are essential in the stumpy form. Stumpy forms have an increased sensitivity to rotenone, a complex I inhibitor, compared to slender BSFs. Rotenone was seen to reduce mitochondrial accumulation of the cationic dye rhodamine 123 by 90%, suggesting that the ΔΨm is dependent on complex I activity in stumpy forms (Bienen et al., 1991). Hence it would be expected that stumpy forms require RPS12, and ND1, 2, 3, 4, 5, 7, 8 and 9 to be expressed and edited in order to remain viable. Cytochrome-associated complexes of the respiratory chain are not expressed at the protein level in the stumpy form (Gunasekera et al., 2012), hence COX1, 2 and 3 and CyB would not be required. As ΔΨm production in stumpy forms is reported to be oligomycin-insensitive, this suggests that complex V acts as an ATP synthase (Bienen et al., 1991), with A6 therefore expected to be required. As the functions of the proteins encoded by MURF 2 and 5 and cytosine-rich template (CR) 3 and 4 are unknown, it is not possible to postulate the possible requirements for these genes through the life cycle. There is also potential for numerous products of alternative editing that could be generated from maxicircle transcripts.

1.10 DK subspecies of *T. brucei*

There are two DK subspecies of *T. b. brucei*: *T. b. evansi* and *T. b. equiperdum*. Interestingly these do not cause sleeping sickness or Nagana, instead causing separate and distinct diseases in a wide range of animals. *T. b. evansi* causes surra, a disease of horses, camels, cattle and other mammals (Desquesnes et al., 2013, Brun et al., 1998). This parasite is mechanically transmitted from the blood of a host via the mouthparts of hematophagous flies, including *Tabanus spp*, and via the saliva of vampire bats. *T. b. equiperdum* causes dourine, a sexually transmitted disease of horses, donkeys and mules, and as such the parasite is only found in immunoprivileged zones. These trypanosomes were traditionally classified as different species due to the differences in the mode of transmission and disease
caused (Hoare, 1972). More recently, this classification has been called into question due to heterogeneity between strains of *T. b. equiperdum* and an overlap of serological and molecular markers between *T. b. evansi* and *T. b. equiperdum* strains (Claes et al., 2005). A number of studies have now recommended that these organisms should be considered subspecies of *T. brucei* (Carnes et al., 2015, Lai et al., 2008).

The only observed molecular difference between these two parasites is in their kDNA (Brun et al., 1998): *T. b. equiperdum* can contain maxicircles, or maxicircles with some deletions present (Lai et al., 2008, Frasch et al., 1980, Shu and Stuart, 1994), whereas *T. b. evansi* has no maxicircle present (Brun et al., 1998), and also can be devoid of minicircles. Both subspecies have reduced minicircle diversity, with editing capacity therefore much decreased (Schnaufer et al., 2002). If these differences are related to the occupation of such diverse ecological niches is not known.

1.10.1 Evolution of DK trypanosomes

It is hypothesised that after relocation of an infected animal into a non-tsetse area, the loss of particular minicircles required for the PCF occurs. This begins the transition to dyskinetoplasmy, caused by possibly a mutation in a component of the kDNA replication or maintenance machinery increasing the likelihood of asymmetrical division of kDNA (Simpson et al., 2000, Jensen et al., 2008). As more edited mRNAs are required for survival in the PCF than the BSF, this would not necessarily affect life in the bloodstream. However, these mutations would prevent survival in the fly, and therefore the possibility of cyclic transmission is eliminated. Locked in the BSF, genetic drift in the absence of genetic exchange and further asymmetrical division could cause homogeneity in minicircle classes and thus further loss of gRNAs (Lun et al., 2010). Hence, the lack of selective pressure in the bloodstream would mean that maxicircle deletions and minicircle loss could be tolerated as
long as these do not affect the two genes thought to be the only ones required for survival in the BSF, A6 and RPS12. A compensatory mutation in ATPase γ would eventually allow complete loss of the maxicircle, allowing survival independently of ATPase-Fo, and thus kDNA. Remaining minicircles could then be eliminated due to a lack of selection to maintain them. It is also conceivable that loss of gRNAs required to edit A6 could select for cells with ATPase γ mutations.

It is not known if there is a selective advantage in losing the ability to express A6 and generating Δψm independently of ATPase-Fo. Presumably there could be a replication advantage in not sustaining a population of kDNA. However DK and AK strains still expend large amounts of energy needlessly in the expression of proteins required for the editing, replication and maintenance of kDNA (Lai et al., 2008, Domingo et al., 2003).

The evolutionary origin of these naturally occurring DK subspecies of *T. b. brucei* is therefore uncertain. A number of hypotheses have emerged:

1) *T. b. evansi* diverged from *T. b. brucei* when this was introduced to camels that had entered tsetse-infested sub-Saharan Africa. The parasite adapted to mechanical transmission and spread to areas outside of sub-Saharan Africa (Hoare, 1956). *T. b. equiperdum* evolved from a strain of *T. b. evansi* able to infect horses by colonizing and adapting to life in genital tissues (Hoare, 1972). However, it is now known that most strains of *T. b. equiperdum* possess at least a partial maxicircle (Lai et al., 2008), therefore could not have evolved from *T. b. evansi*.

2) *T. b. evansi* evolved from a rare clone of *T. b. equiperdum* that had lost its maxicircle and had a preference for life in the bloodstream, allowing adaptation to mechanical transmission (Brun et al., 1998).

3) Evolution of DK subspecies is continuous from *T. b. brucei* (Lai et al., 2008, Lun et al., 2010). *T. b. brucet* has a full kDNA repertoire and is therefore able to complete
the life cycle within the fly and be transmitted cyclically. AK *T. b. evansi* is the terminal state of this evolution, with complete kDNA loss. *T. b. equiperdum* and DK *T. b. evansi* are intermediate states of this evolution, with homogenous populations of minicircles and a gradual build-up of maxicircle deletions over time. Analysis of SL RNA intragenic region showed that these 3 subspecies intermingle with respect to SL RNA sequence, whereas distinct species tend to cluster away from each other. Additionally, it was suggested that without continuing evolution, only the terminal AK state would be seen in nature. Any *T. b. brucei* strain can be the source of a new AK lineage, with strains able to arise independently. However, even with continuing emergence of AK strains from DK strains over time, AK strains would still dominate unless these AK strains had then been selected against (Schnaufer, 2010). There is no biological basis for this negative selection.

4) DK and AK strains have arisen from a limited number of separate ancestors (Schnaufer, 2010, Carnes et al., 2015). It has been identified that there are at least four distinct groupings of DK and AK subspecies. This was initially based on the observation that, despite their evansi or equiperdum ‘status’, the DK/AK strains containing the same ATPase γ mutations also had the same dominant minicircle class (Schnaufer, 2010). The analysis was later expanded and the presence of particular γ mutations was found to be consistent with the presence of SNPs in other genes (Carnes et al., 2015). It seems likely that these groups arose from genetically distinct *T. b. brucei* ancestors at least four independent times. Crucially these groups include both *T. b. equiperdum* and *T. b. evansi* strains. Hence their original evolutionary divergence produced cells unable to be transmitted by tsetse flies, and these groups subsequently evolved to the strains that occupy diverse ecological niches.
These evolutionary schemes do not however account for the need for adaptation towards mechanical transmission (Schnaufer, 2010). The success of surra and dourine in the field demonstrates that effective and efficient mechanical transmission between hosts does occur. Mechanical transmission appears to be correlated with kDNA loss, but this raises further questions. Can this be explained by genetic drift? Or is there a selective advantage of kDNA loss to mechanical transmission? What adaptations are required for cells to be efficiently mechanically transmitted? It is also uncertain whether adaptations towards mechanical transmission in *T. brucei* would need to occur before or after kDNA loss. For example, if mechanical transmission evolved first, the parasite could escape the African tsetse belt. Without cyclical transmission, kDNA would become redundant and could be lost with the help of a γ mutation. Alternatively, kDNA loss could be hypothesised to somehow increase the chances of mechanical transmission. If the loss of kDNA affects the parasites’ ability to differentiate to the stumpy BSF, the innate density controlling mechanism of the parasite would be breached, allowing, theoretically, an increased level of parasitaemia that could increase the likelihood of mechanical transmission (Desquesnes et al., 2009, Schnaufer, 2010).

*Trypanosoma vivax* (*T. vivax*) is a fascinating example of the correlation between kDNA status and method of transmission. Certain strains of *T. vivax* are transmitted cyclically by an insect vector in Africa, but American strains are found locked in the BSF and are thus transmitted mechanically. Recently the kDNA from genetically related strains of both groups of *T. vivax* was sequenced (Greif et al., 2015). The African strain, able to fulfil the entire life cycle of the parasite, unsurprisingly had a full repertoire of kDNA. In the two American strains however, there was massive kDNA degradation, with multiple maxicircle deletions and frameshifts affecting all genes but A6, RPS12 and MURF2. Their minicircle repertoire was found to be highly enriched for gRNAs for A6 and RPS12. Correlating with presumed functionality of A6, the ATPase γ subunit genes were WT at the C terminus. The authors of
the study concluded that after arrival in tsetse-free America, the kDNA of the cells was rapidly dissembled, with the strains losing all but the BSF-essential maxicircle genes and gRNAs. What prevented *T. vivax* going a step further and adopting complete kDNA independence via ATPase γ mutations like *T. brucei* is not certain. There seems to be a selective advantage to retaining expression of A6 in *T. vivax* BSFs that is not present in the equivalent stage in *T. brucei*. Similarly, stable AK cells could not be generated by acriflavine treating other trypanosomatid species (Trager and Rudzinska, 1964, Strauss, 1972, Steinert and Van Assel, 1967, Muhlpfordt, 1963, Muhlpfordt, 1959). It is not known why these species seem to lack the ability to compensate for kDNA loss. It could be due to differences in metabolism between species, or in the structure of their F1F0 complexes for example.

### 1.10.2 Monomorphism in DK trypanosomes

The naturally occurring AK and DK strains of *T. b. evansi* and *T. b. equiperdum* are found in the slender BSF and are widely reported to be monomorphic, a characteristic potentially caused by their lack of kDNA. Even laboratory-generated DK forms of pleomorphic *T. brucei* strains have more limited ability to differentiate into the stumpy form (Stuart, 1971), supporting this theory.

There have been a few historical examples of stumpy forms being seen in field samples: Lavier (1933) quantified a population of *T. b. evansi* cells as being comprised of 0.03-0.05% stumpy forms, a proportion designated as ‘negligible’ (Hoare, 1972). Bruce (1911), Fry (1911) and Balfour (1914) all noted that stumpy forms were so rare that the term ‘monomorphic’ was justified to be used for *T. b. evansi* (Hoare, 1972). Investigations on animals reinfected with field-harvested strains showed between 0.005-0.07% (Blacklock and Yorke, 1913) and 0-0.40% (Hoare, 1956) incidence of stumpy forms with *T. b. equiperdum* and *T. b. evansi*, respectively.
There was also investigation into whether stumpy forms occurred sporadically during these infections (Hoare, 1956). An equine Bulgarian strain generated stumpy forms on four occasions in 4 years, with the proportion of the population being stumpy once reaching 25%. A strain isolated from a camel in Sudan generated stumpy forms eight times in 5 years, with a 2% stumpy form maximum. In a second Sudanese cameline strain stumpy forms were detected 250 times over 17 years, once with a stumpy proportion of over 60% being seen. It was concluded that *T. b. evansi* was ‘practically monomorphic’, with pleomorphism being an ‘inconsistent feature’ only in certain strains.

No surviving laboratory-grown strains of DK trypanosomes show any ability for pleomorphism. Whether this is due to culturing methods promoting monomorphism, as with *T. brucei*, is uncertain. Some strains were evidently once capable of sporadically differentiating to a form described as morphologically stumpy despite inconsistency between studies as to which characteristics a true stumpy form shows.
1.11 Aims

The aims of this thesis are to investigate some key outstanding questions about the requirement for kDNA through the life cycle. We have generated cell lines expressing mutant L262P ATPase γ subunit, in monomorphic and pleomorphic strain backgrounds, with and without kDNA. These kDNA-independent cell lines are used to probe the following questions.

1. **What is the molecular mechanism for kDNA independence in the slender BSF?**
   Namely, what is the effect of ATPase γ mutations on the structure of the F\(_1\)F\(_{\text{o-}}\) ATPase and how do ATPase γ mutant cells tolerate the presumed production of a lethal proton leak?

2. **Is kDNA required in the stumpy life cycle stage?**
   Does a pleomorphic strain retain this characteristic after removal of kDNA? Does the lack of kDNA have any effect on the resulting parasitaemia?

3. **Are kDNA and a functional F\(_1\)F\(_{\text{o-}}\)ATP synthase required in the tsetse fly?**
   Are AK cells able to differentiate into the PCF? Can ATPase γ-mutant cells exhibiting decreased sensitivity to multiple drugs complete their life cycle within a tsetse fly?
1.12 Figures

Figure 1.1  Schematic of cellular architecture of *T. brucei*
From (Matthews, 2005)

1. Nucleus
2. Kinetoplast
3. Flagellar pocket
4. Basal body and probasal body
5. Axoneme and paraflagellar rod
6. Golgi

7. Lysosome
8. Endosomes
9. Mitochondrion
10. Microtubule cytoskeleton
11. Glycosomes
Pyruvate is produced from glucose under aerobic conditions. In anaerobic conditions the reaction shaded in blue occurs, producing equimolar amounts of pyruvate and glycerol from glucose. Key: 1,3 BPGA = 1,3 bisphosphoglycerate, DHAP, dihydroxyacetone phosphate, F6P = fructose-6-phosphate, G3P = glyceraldehyde-3-phosphate, G6P = glucose-6-phosphate, Gly-3-P = glycerol-3-phosphate, PEP = phosphoenolpyruvate, 3PGA = 3-phosphoglycerate, Pi = inorganic phosphate, UQ = ubiquinone pool. Enzymes: 1. hexokinase, 2. G6P isomerase, 3. phosphofructokinase, 4. aldolase, 5. triose-phosphate isomerase, 6. G3P dehydrogenase, 7. glycerol kinase, 8. G3P dehydrogenase, 9. phosphoglycerate kinase, 10. phosphoglycerate mutase, 11. enolase, 12. pyruvate kinase, 13. glycerol-3-phosphate dehydrogenase, 14. alternative oxidase. From (Michels et al., 2006)
Figure 1.3A  Schematic of the maxicircle element of *T. brucei* kDNA
VR = variable region containing the origin of replication. The function of MURF1, MURF2 and 5 and CR3 and 4 are not known. Adapted from (Opperdoes and Michels, 2008) using (Kannan and Burger, 2008), (Bhat et al., 1990), (Read et al., 1994).
Figure 1.3B  The composition of the respiratory chain alters through the life cycle of *T. b. brucei*
Complexes I, III, IV and V contain kDNA-encoded subunits. These are labelled, black = non edited, white = edited. Key: GPO = glycerol-3-phosphate oxidase, NDH2 = alternative complex I, TAO = trypanosome alternative oxidase, AAC = ADP/ATP antiporter, IMS = intermembrane space. Figure produced by L. Jeacock and A. Schnaufer
Figure 1.4  Cell cycle of *T. brucei* procyclic form
I. 1K1N cells in G1 phase. Basal body is in red. II. kDNA and nuclear DNA replicates. Probasal body matures into second basal body, where new flagellum begins to grow from. III. 2K1N cells entering G2 phase. The daughter flagellum has grown in contact with the parental flagellum at its distal tip. The kDNA has separated. IV. Nucleus divides, forming 2K2N cells. V. Both flagella can beat freely, allowing cytokinesis from the anterior pole of the cell to form two daughter cells. From (Ooi and Bastin, 2013)
The life cycle of *T. brucei*

A) The morphological changes occurring during the *T. brucei* life cycle. B) The direction of migration of *T. brucei* within the tsetse fly. A bloodstream form mammalian infection is initiated when a metacyclic-infected tsetse fly bites a host. Metacyclic forms differentiate into the slender bloodstream form, which is proliferative. As the infection progresses, these differentiate into the cell cycle-arrested short stumpy form. These are transmissible to the fly. Once in the midgut, stumpy forms differentiate into proliferative procyclic forms. These migrate through the midgut lining, the ectoperitrophic matrix, along the foregut up into the proventriculus. Here procyclic forms elongate and go through asymmetric division to generate a long and short epimastigote. From the proventriculus, epimastigotes migrate to the salivary glands, where the short forms attach. These replicate and differentiate into detached, cell cycle-arrested metacyclics, which are the mammalian transmissible form. From (Langousis and Hill, 2014)
Figure 1.6 The dynamics of *T. brucei* mouse infection

Slender forms proliferate and secrete SIF, inducing stumpy formation in a concentration-dependent manner. As slender form number falls, due to stumpy formation and immune clearance, SIF concentration falls, preventing further differentiation. Stumpy forms are present in high concentrations at the peak of the infection. Stumpy numbers then fall due to cell senescence and immune system clearance. The next wave of slender forms will have a new VSG coat, allowing the parasites to evade the immune system. From (Mony and Matthews, 2015)
Figure 1.7  Structure of respiratory chain complex V
The subunit composition of A) Prokaryotic F₁F₀ ATP synthase. B) Eukaryotic mitochondrial F₁F₀ ATP synthase. From (Walker, 2013). C) Schematic of T. brucei complex V acting in ATPase direction. ATPase activity at the β subunits induces conformational changes, forcing the γ subunit to rotate. As this subunit is physically connected to the F₀ moiety, this forces the rotation of the c ring. This in turn forces protons to move against their concentration gradient towards the IMS, generating a Δψm. Subunits b and OSCP prevent rotation of F₁ in the absence of ATP substrate. D) Crystal structure of S. cerevisae F₁ enzyme. Mutations in the C-terminal region of the γ subunit mutations (spheres) are shown that interact deep in the α/β core and permit survival of yeast and BSF T. brucei in the absence of mtDNA. In yellow are the mutations found in petite positive yeast. DK T. brucei mutations are shown as spheres, colour coded by atom type. From top to bottom: M282L, A281L, A273P, L262P. Subunits colour coded as in C. From (Dean et al., 2013)
Figure 1.8  Complex V activity and Δψm generation through the life cycle
A) In PCF, complex V acts as ATP synthase and AAC pumps ATP out of the mitochondrion, both using Δψm. Δψm is generated by the respiratory chain of the inner mitochondrial membrane. B) In slender BSF, complex V generates Δψm, with the AAC providing substrate. C) In DK bloodstream forms AAC acts to generate Δψm, fuelled by the ATPase activity of F₁. From (Clark-Walker, 2003) which describes the analogous situation for yeast.
Figure 1.9  Mitochondrial ATP production in PCF
I. Oxidative phosphorylation via succinate dehydrogenase. II. SCoAS from α-ketoglutarate. III. ASCT cycle using acetyl-CoA and mitochondrial succinate. Adapted from (Schneider et al., 2007)
Figure 1.10  Proline and glucose metabolism in PCF
Substrates are in blue boxes, with end products in black boxes. Key: AA= amino acids; α-KG, α-ketoglutarate, AOX= alternative oxidase, c= cytochrome c, CI-IV= complexes I-IV of the electron-transport chain, OA= 2-oxoacids, Q= ubiquinone. From (Tielens and Van Hellemond, 2009)
Figure 1.11  Acetate production in the presence of threonine and glucose
Black arrows are steps of glucose and threonine metabolism, and double lines are steps of de novo fatty acid biosynthesis from acetyl coA. Excreted end points are in boxes. PYR = pyruvate, from glycolysis AOB = amino oxybutyrate, AcCoA = acetyl coA, Suc = succinate. Enzymes (encircled) = 1. threonine dehydrogenase, 2. 2-amino-3-ketobutyrate coenzyme A ligase, 3. pyruvate dehydrogenase, 4. ASCT, 5. acetyl coA thioesterase, 6. SCoAS, 7. AMP-dependent acetyl coA synthetase. Adapted from (Millerioux et al., 2013)
1.13 Tables

Table 1.1 RNA editing is life cycle stage specific
Adapted from (Schnaufer et al., 2002)

<table>
<thead>
<tr>
<th>Maxicircle transcript</th>
<th>Mitochondrial complex</th>
<th>Life cycle stage edited in</th>
<th>Editing site</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>ND3</td>
<td>I</td>
<td>Bsf and pcf</td>
<td>Pan</td>
<td>Read et al., 1994</td>
</tr>
<tr>
<td>ND7 5'</td>
<td>I</td>
<td>Bsf and pcf</td>
<td>-</td>
<td>Koslowsky et al., 1990</td>
</tr>
<tr>
<td>ND7 3'</td>
<td>I</td>
<td>bsf</td>
<td>-</td>
<td>Koslowsky et al., 1990</td>
</tr>
<tr>
<td>ND8</td>
<td>I</td>
<td>bsf</td>
<td>Pan</td>
<td>Souza et al., 1992</td>
</tr>
<tr>
<td>ND9</td>
<td>I</td>
<td>bsf</td>
<td>Pan</td>
<td>Souza et al., 1993</td>
</tr>
<tr>
<td>Cyb</td>
<td>III</td>
<td>pcf</td>
<td>5'</td>
<td>Feagin et al., 1987</td>
</tr>
<tr>
<td>COXII</td>
<td>IV</td>
<td>pcf</td>
<td>3'</td>
<td>Benne et al., 1986</td>
</tr>
<tr>
<td>COXIII</td>
<td>IV</td>
<td>Bsf and pcf</td>
<td>Pan</td>
<td>Feagin et al., 1988</td>
</tr>
<tr>
<td>A6</td>
<td>V</td>
<td>Bsf and pcf</td>
<td>Pan</td>
<td>Bhat et al., 1990</td>
</tr>
<tr>
<td>RPS12</td>
<td>Ribosome</td>
<td>bsf</td>
<td>Pan</td>
<td>Read et al., 1992</td>
</tr>
<tr>
<td>MURF2</td>
<td>Unknown</td>
<td>Bsf and pcf</td>
<td>5'</td>
<td>Feagin and Stuart, 1988</td>
</tr>
<tr>
<td>CR3</td>
<td>Unknown</td>
<td>bsf</td>
<td>Pan</td>
<td>Stuart et al., 1997</td>
</tr>
<tr>
<td>CR4</td>
<td>Unknown</td>
<td>bsf</td>
<td>Pan</td>
<td>Corell et al., 1994</td>
</tr>
</tbody>
</table>
Table 1.2  Point mutations in ATPase γ subunit in lab-generated and naturally occurring DK subspecies
From (Dean et al., 2013)

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Source (origin, host, year of isolation)</th>
<th>Genotype of strains</th>
</tr>
</thead>
<tbody>
<tr>
<td>L262P</td>
<td><em>T. b. brucei</em> 164DK</td>
<td>Homozygous</td>
</tr>
<tr>
<td>CTT→CCT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A273P</td>
<td><em>T. equiperdum</em> BoTat1.1 (Morocco, horse, 1924)</td>
<td>Homozygous</td>
</tr>
<tr>
<td>GCG→CGG</td>
<td><em>T. equiperdum</em> STIB784 (unknown)</td>
<td></td>
</tr>
<tr>
<td>A281del</td>
<td><em>T. equiperdum</em> ATCC30019 (France, horse, 1903)</td>
<td>Heterozygous</td>
</tr>
<tr>
<td>TCTGCTATG→TCT−ATG</td>
<td><em>T. evansi</em> Antat 3/3 (South America, capybara, 1969)</td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>T. evansi</em> C13 (Kenya, camel, 1982)</td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>T. evansi</em> E9/CO (Columbia, horse, 1973)</td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>T. evansi</em> SS143M (Philippines, water buffaloes, 2006)</td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>T. evansi</em> SS73M (Philippines, water buffaloes, 2006)</td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>T. evansi</em> STIB805 (China, water buffaloes, 1985)</td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>T. evansi</em> STIB807 (China, water buffaloes, 1979)</td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>T. evansi</em> STIB810 (China, water buffaloes, 1985)</td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>T. evansi</em> Stock Kazakh (Kazakhstan, camel, 1995)</td>
<td></td>
</tr>
<tr>
<td>M282L</td>
<td><em>T. evansi</em> KETRI2479</td>
<td>Heterozygous</td>
</tr>
<tr>
<td>ATG→TG</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
2. Investigation into the mechanism for survival of BSF *T. brucei* in the absence of kDNA
2.1 Introduction

The kinetoplast (kDNA) is essential in slender BSF *T. brucei* (Schnaufer et al., 2001). In fact, several drugs target the kDNA of *T. brucei* and contribute to killing of the parasite (Gould and Schnaufer, 2014, Motta, 2008). In the long slender BSF, A6 of the F₅ part of F₁F₅-ATPase is required for the generation of the essential Δψm and RPS12 of the mitochondrial ribosome is thought to be required for the translation of A6. However, DK subspecies of *T. brucei* are able to survive without kDNA, therefore must survive without these kDNA gene products. How do they produce Δψ without subunit A6?

Presently, certain characteristics of kDNA-independent cells are known. Single point mutations in the C-terminus of the F₁F₅-ATPase γ subunit are found in DK subspecies of *T. brucei* (Schnaufer et al., 2005, Lai et al., 2008). When expressed in *T. brucei*, some of these mutations confer resistance to acriflavine (Dean et al., 2013), a DNA intercalator that interferes with kDNA replication (Manchester et al., 2013). Despite the loss of kDNA in these cells, they are viable, and grow as before. Cells also become oligomycin resistant when mutated γ is expressed (Dean et al., 2013). Oligomycin is an inhibitor of F₅, (Symersky et al., 2012), thus these cells manufacture Δψm independently of F₅, circumventing the requirement for A6 and therefore possibly RPS12. The F₁F₅-ATPase is therefore functionally uncoupled. F₁ is still essential in DK trypanosomes (Schnaufer et al., 2005). AK cells are hypersensitive to bongkrekic acid (Dean et al., 2013), a selective inhibitor of the AAC (Henderson and Lardy, 1970). These cells therefore use the electrogenic capabilities of the AAC to generate Δψm. Interestingly this method of generating Δψm is the same as in petite yeast mutants (Clark-Walker and Chen, 2001).
In the presence of an ATPase-F₁γ subunit mutation in yeast, the axis of rotation of γ within the α/β-F₁ core is altered (Wang et al., 2007). A rearranged hydrogen bonding network reduces the coupling of F₁ to F₀ (Arsenieva et al., 2010). This alters the ability of F₁ to control activity of F₀, allowing F₀ to become a permeant pore in the inner mitochondrial membrane. This would dissipate the essential Δψm in a proton leak into the mitochondrial matrix (Mueller, 2000). Thus, introduction of such mutations into petite positive yeast selects for mtDNA⁻ or mtDNA⁰ cells (Chen and Clark-Walker, 1993, Lai-Zhang et al., 1999). Mitochondrial DNA in yeast contains subunits a, 8 (A6L) and c of F₀ (Foury et al., 1998), so deletion of parts or all of mtDNA prevents the formation of F₀ and therefore prevents the proton leak (Chen and Clark-Walker, 1993, Lai-Zhang et al., 1999).

However in T. brucei, by microscopic examination, there typically seems to be no gross effect on the kDNA network in the presence of an ATPase γ mutation (personal observation; Matt Gould, unpublished observation). How T. brucei deals with this proton leak is therefore uncertain. However a loss of kDNA would not necessarily be required to prevent F₀ forming. RNA editing is an extra layer of processing required to generate fully translatable transcripts from encrypted maxicircle-encoded genes. Disruption of RNA editing would prevent A6 translation, and therefore prevent the proton leak through F₀.

### 2.1.1 Hypothesis

Firstly, the L262Pγ compensatory mutation results in changes to the structure and function of the F₁F₀ ATPase, permitting survival in the absence of the kDNA-encoded A6 subunit. Secondly, like yeast, uncoupling the T. brucei F₁F₀-ATPase via L262Pγ mutation will cause a proton leak that has to be stopped for cells to retain viability. The A6 editing capacity of cells is expected to be decreased in the presence of a γ subunit mutation, preventing F₀
causing a proton leak. $F_\theta$ is therefore expected to be absent in cell lines expressing a $\gamma$ mutation.

### 2.1.2 Study design

The L262P$_\gamma$ mutation was originally induced by long-term acriflavine treatment of a BSF *T. brucei* cell line that generated a clonal DK cell line (Stuart, 1971). The mutation was subsequently characterised as being an uncoupling mutation that allowed viability in the absence of kDNA (Schnaufer et al., 2005, Dean et al., 2013). To gain a better understanding of the mechanism of independence from kDNA in BSF *T. brucei*, one or both endogenous $\gamma$ alleles were replaced by L262P$_\gamma$ versions in monomorphic Lister 427 ‘single marker’ (Wirtz et al., 1999) and pleomorphic AnTat1.1 90:13 (Engstler and Boshart, 2004) cell lines, and acriflavine treated to generate AK cell lines. After verifying that multiple clones were kDNA-independent, the structural consequences of i) the L262P$_\gamma$ mutation and ii) the loss of kDNA was probed. Subunit composition of $F_1F_\theta$ complexes was analysed after affinity purification and in mitochondrial extracts. The degree of structural uncoupling of $F_1F_\theta$-ATPase complex attained in the presence of the L262P$_\gamma$ mutation was assessed by high-resolution clear native electrophoresis (hrCNE). The effect of the mutation on RNA editing was assessed by quantitative RT-PCR (qRT-PCR) and DNA sequencing. Functional uncoupling of $F_1F_\theta$-ATPase was judged by sensitivity to inhibitors of the complex. The effect of the mutation and loss of kDNA on production of $\Delta\psi\text{m}$ was quantified. These analyses allow a more detailed understanding of how $\Delta\psi\text{m}$ is maintained in the absence of kDNA, and in the absence of a proton gradient.
2.2 Materials and Methods

2.2.1 Generation of cell lines with TAP-tagged ATPase subunits

*T. brucei* strain Lister 427, ‘single marker’ (427SM) cell line, transgenic for T7 RNA polymerase and the tetracycline repressor (Wirtz et al., 1999), was transfected with either plasmid pLew79-MH-β-TAP or pLew79-MH-p18-TAP (kind gift of Zikova lab, (Zikova et al., 2009)). These plasmids allow the expression of tandem affinity purification (TAP)-tagged ATPase subunit β or p18. Expression is controlled by a tetracycline-inducible EP1 promoter. The construct inserts into a ribosomal RNA (rRNA) spacer sequence, allowing transfected cell lines to have an additional copy of either subunit β or p18.

5 µg plasmid DNA was used per transfection. DNA was linearised by restriction enzyme NotI. After confirming linearization via gel electrophoresis, DNA was ethanol precipitated and the final pellet was resuspended in 5 µl MilliQ water. Cells in the logarithmic stage of growth were centrifuged at 1300 g for 10 min. 30 x 10⁶ cells were used per transfection; pellets were resuspended in 100 µl nucleofection solution (90 mM NaH₂PO₄, 5 mM KCl, 0.15 M CaCl₂, 50 mM HEPES, pH 7.3) (Schumann Burkard et al., 2011). A mock transfection of parental cells without DNA was also carried out as a control to confirm the selective drug killed untransfected cells. For the transfection, the AMAXA Nucleofector® II was used with Program Z-001 (Schumann Burkard et al., 2011). After nucleofection, cells were plated in three 24-well plates in 10-fold serial dilutions. Transfectants were selected using 5 µg/ml phleomycin (Invivogen) that was added 6 hrs after transfection. After 3 days,
an additional 5 µg/ml of drug was added to the media, and clones were selected after another 3 days. Lister 427SM β-TAP or p18-TAP clones were maintained in 2.5 µg/ml G418 (Sigma) and 5 µg/ml phleomycin.

2.2.2 Generation of heterozygous γL262P cell lines

Cell lines Lister 427SM, 427SM β-TAP, 427SM p18-TAP and a culture-adapted pleomorphic T. brucei strain AnTat1.1 90:13 (Engstler and Boshart, 2004) were transfected with plasmid pEnT6-γL262P-PURO or pEnT6-γWT-PURO (manufactured by Dr Matt Gould, then of University of Edinburgh)(Kelly et al., 2007). This plasmid contains either L262P ATPase γ or WT ATPase γ, allowing the replacement of one endogenous ATPase γ allele in order to generate cell lines containing a single allele γL262P (ATPγ/Δatpγ::atpγL262P PURO) or, as a control, γWT (ATPγ/Δatpγ::atpγWT PURO) replacement (Appendix B). The replaced gene is under the control of its endogenous promoter and 5’ UTR, but contains the aldolase 3’UTR. The transfection protocol from section 2.2.1 was used. Transfectants were selected using 0.1 µg/ml puromycin (Sigma) added 6 hrs after transfection. AnTat1.1 90:13 clones were selected after 4 days and were maintained in 2.5 µg/ml G418, 5 µg/ml hygromycin (Calbiochem) and 0.1 µg/ml puromycin. Lister 427 SM clones were selected after 3 days and were maintained in 2.5 µg/ml G418, 5 µg/ml phleomycin and 0.1 µg/ml puromycin.

2.2.2.1 Confirmation of γ genotype in transfected clones

The genomic DNA (gDNA) of these clones was extracted using GenElute Mammalian gDNA miniprep kit (Sigma), and the γ subunit gene was amplified via PCR, allowing direct Sanger sequencing of the gel-extracted PCR product to confirm the presence or absence of the L262Pγ mutation.
Standard PCR conditions: 95°C 5 min, [95°C 1 min, 54°C 1 min, 72°C 2 min] for 30 cycles, 72°C for 10 min.

Reaction conditions per 25 µl reaction: 14.25 µl dH2O, 5 µl 5x GoTaq reaction buffer (Promega), 0.2 µl 25 mM dNTPs, 1.5 µl MgCl2, 0.5 µl 10 µM primer #1 and #2, 1 µl ~10 µg/ml gDNA template, 0.25 µl GoTaq polymerase (Promega).

Sanger sequencing big dye reactions were carried out by Edinburgh Genomics, as per their protocol.

2.2.3 Generation of homozygous γL262P/L262P cell lines

Both 427SM and AnTat1.1 90:13 WT/L262Pγ cell lines were then transfected with plasmid pEnT6-γL262P-BSD or pEnT6-γWT-BSD (manufactured by Dr Matt Gould, then of University of Edinburgh) in order to generate homozygous L262P/L262Pγ cell lines (Δatpγ::atpγL262P BSD/Δatpγ::atpγL262P PURO) (or the corresponding control with WTγ alleles) (Appendix B). The same protocol as in Section 2.2.1 was used. Transfectants were selected using 5 µg/ml blasticidin (Invivogen) that was added 6 hrs after transfection. AnTat1.1 90:13 clones were selected after 4 days and were maintained in 2.5 µg/ml G418, 5 µg/ml hygromycin, 0.1 µg/ml puromycin and 5 µg/ml blasticidin. Lister 427SM clones were selected after 3 days and were maintained in 2.5 µg/ml G418, 5 µg/ml phleomycin, 0.1 µg/ml puromycin and 5 µg/ml blasticidin. The genotype of these clones was then assessed as Section 2.2.2.1.
2.2.4 Generation of AK cell lines

Cells were treated with 10 nM acriflavine (Sigma) over 3 days in order to induce the loss of kDNA in WT/L262Pγ or L262P/L262Pγ clones (WT/WTγ were treated in parallel and died, as expected). The loss of the kinetoplast was assessed by preparing microscope slides and mounting with a cover slip using 50 µl Prolong Gold Antifade with 4’, 6-diamidino-2-phenylindole (DAPI) (Life Tech.).

The loss of maxicircle genes and a representative minicircle (type A-like) (Dean et al., 2013, Njiru et al., 2006) was assessed via PCR amplification from cellular genomic DNA (gDNA) using primers combinations detailed in Table 2.1. All primers were manufactured by Sigma and are listed in Appendix B. Gel electrophoresis was used to assess the amplicons from these PCR reactions.

Table 2.1 Primer combinations used to amplify kDNA and nuclear components from gDNA

See Appendix B for primer sequences.

<table>
<thead>
<tr>
<th>Amplicon</th>
<th>Forward primer # in this chapter</th>
<th>Reverse primer # in this chapter</th>
</tr>
</thead>
<tbody>
<tr>
<td>A6</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>ND4</td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td>ND7</td>
<td>7</td>
<td>8</td>
</tr>
<tr>
<td>ND5</td>
<td>9</td>
<td>10</td>
</tr>
<tr>
<td>Minicircle type A-like</td>
<td>11</td>
<td>12</td>
</tr>
<tr>
<td>LipDH</td>
<td>13</td>
<td>14</td>
</tr>
</tbody>
</table>

Reaction mix and cycle conditions for maxicircle and LipDH sequences: as Section 2.2.2.1.

Cycle conditions for minicircle sequence: 94 °C for 5 min and [94 °C 40 s, 59.4 °C 1 min, 72 °C 90 s] for 35 cycles and 72 °C for 10 min.
2.2.5 Growth analysis in vitro

Cell lines were grown to measure the growth effect of the L262Pγ allele and the effect of absence of kDNA in vitro. Cells were grown in HMI-9 media (Invitrogen) (Hirumi and Hirumi, 1989) containing 10% fetal calf serum (FCS) (Invitrogen), with supplemented drugs as appropriate. To analyse whether the cells were able to survive without kDNA, growth analysis was performed with cells grown in HMI-9, 10% FCS plus 10 nM EtBr (Sigma). Cell counts were performed daily using a Beckmann Z2 Coulter counter, and cells were split to a concentration of 1x10^5/ml after counting.

2.2.6 Pyrosequencing

2.2.6.1 Design of pyrosequencing primers and PCR of pyrosequencing templates

PCR and sequencing primers were designed using the Biotage PSQ Assay Design software (Fig. 2.9A), and were ordered from Sigma. PCR reactions were performed on 60 ng T. b. brucei gDNA and cDNA to amplify the ~60 bp sequence surrounding the polymorphism, using primers #31 and #32. PCR conditions and reaction conditions were as described in section 2.2.2.1.

A volume of 5 µl of each reaction was analysed via gel electrophoresis using a 2% agarose gel.

2.2.6.2 Pyrosequencing reaction

Pyrosequencing was performed using Qiagen reagents and a Biotage Pyromark instrument (Figure 2.8). 0.5 µl pyrosequencing primer (#33) was added to 11.5 µl annealing buffer in
each well of a Biotage pyrosequencing plate. To each well of a round bottomed microtitre plate, 30 µl water, 38 µl binding buffer, 2 µl streptavidin beads and 5 µl PCR product were added and the plate was placed on a shaker to mix. Nucleotides, enzyme and substrate solutions were added into dispensing tips in volumes advised by the PSQ Assay Design programme. Using the hedgehog vacuum (Biotage), the contents of the wells containing the PCR products were washed in 70% ethanol for 5 s, denatured in 0.2 M NaOH for 5 s and washed in 10 mM Tris acetate pH 7.6 for 5 s. The vacuum was switched off, and the hedgehog vacuum holding the beads with PCR product bound was lowered into the pyrosequencing plate and shaken. This plate was incubated at 80°C for 2 min, cooled and then placed in the instrument for the pyrosequencing run to commence.

Three individual pyrosequencing runs were carried out with each sample in triplicate. Each pyrosequencing run was carried out with a separate PCR reaction providing the template. Peak height assessment was performed by Biotage PSQ software.

2.2.7 Pulldown analysis of mitochondrial F$_1$F$_0$-ATPase

2x10$^8$ cells were harvested by centrifugation at 1300 g for 10 min. Cells were washed with PBS-G (137 mM NaCl, 2.7 mM KCl, 10 mM Na$_2$HPO$_4$, 1.8 mM KH$_2$PO$_4$, 10 mM glucose) and resuspended in 100 µl cold IPP150 (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1% NP40, plus protease inhibitors). Triton X-100 was added to 1% and cells were incubated on ice for 20 min, and the lysate was centrifuged in table top at 4°C, 10000 g for 15 min.

For the tandem affinity purification (TAP) protocol, 10 µl IgG-conjugated magnetic beads (Invitrogen Dynabeads M-270 Epoxy, with IgG conjugation by Laura Jeacock, (Oeffinger et al., 2007)) per cell line were blocked with 100 µl IPP150 1% bovine serum albumin (BSA)
for 30 min while constantly mixing, and then washed twice in IPP150 using a magnetic rack to remove the beads from the solution.

Cleared lysate (supernatant) was added to the beads and rotated for 2 hrs at 4°C. A magnetic rack was used to remove the flow through (the solution not bound to the beads), and the beads were washed three times with cold IPP150. The beads were then washed once with 100 µl TEV cleavage buffer (TEVCB: 10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1% NP40, 0.5 mM EDTA, 1 mM DTT). The beads were resuspended in 30 µl TEVCB plus 1 µl AcTEV protease (to cleave the protein from the beads) for 2 hrs at 16°C. The protein was eluted by removing supernatant using a magnetic rack.

Fractions were run on a denaturing protein gel and western blotted to analyse the content of the eluate.

### 2.2.8 Western blot

When expression of a TAP tagged construct was required, this was induced by addition of 1 µg/ml tetracycline to the cell culture for 48 h. Cells were harvested by centrifuging 2x10⁶ cells, washing the cells once with PBS, and lysing the cell pellet in 10 µl 2x sodium dodecyl sulphate (SDS) solution (250 mM Tris pH 6.8, 20% (v/v) glycerol, 4% (v/v) SDS, 200 mM 1 M DTT, 0.01% (v/v) bromophenol blue).

Samples were boiled for 10 min at 95°C. Denatured samples were loaded onto a Novex NuPAGE Bis-Tris 10% precast gel (Invitrogen), which was run at 150 V with 500 ml ice-cold 1x MOPS SDS running buffer (25 ml 20x MOPS SDS running buffer, 475 ml dH₂O). The gel was blotted onto a methanol-equilibrated polyvinylidene fluoride (PVDF) membrane using 1 L ice-cold transfer buffer and a Biorad Criterion blotter at 90V for 45 min, and blocked overnight at 4°C in 1x TBST (10 mM Tris-HCl, 150 mM NaCl and 0.05% Tween-
20) with 10% milk powder. Antibody incubations were performed in 1x TBST with 5% milk powder (Table 2.2), and washes were performed with 1x TBST. For detection, Enhanced Chemiluminescence reagents 1 and 2 (Amersham) were mixed in equal volumes and incubated with the membrane. The membrane was placed into an X-ray film cassette and exposed using Kodak BioMax light film, with exposure time depending on intensity of signal produced. Developing was performed by a SRX-101A X-ray developer (Konica Minolta).

Table 2.2 Antibodies used for western blotting in this chapter

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Concentration</th>
<th>Secondary</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-β</td>
<td>1:2000</td>
<td>Anti-rabbit 1:2000</td>
<td>(Subrtova et al., 2015)</td>
</tr>
<tr>
<td>Anti-α</td>
<td>1:500</td>
<td>Anti-rabbit 1:2000</td>
<td>(Dean et al., 2013)</td>
</tr>
<tr>
<td>Anti-p18</td>
<td>1:2000</td>
<td>Anti-rabbit 1:2000</td>
<td>(Subrtova et al., 2015)</td>
</tr>
<tr>
<td>Anti-Tb1</td>
<td>1:2000</td>
<td>Anti-rabbit 1:2000</td>
<td>(Subrtova et al., 2015)</td>
</tr>
<tr>
<td>Anti-Tb2</td>
<td>1:2000</td>
<td>Anti-rabbit 1:2000</td>
<td>(Subrtova et al., 2015)</td>
</tr>
<tr>
<td>Anti-EF1α</td>
<td>1:7000</td>
<td>Anti-mouse 1:2000</td>
<td>Millipore</td>
</tr>
<tr>
<td>Anti-CBP</td>
<td>1:1000</td>
<td>Anti-rabbit 1:2000</td>
<td>Millipore</td>
</tr>
<tr>
<td>Anti-PAP</td>
<td>1:5000</td>
<td>-</td>
<td>Sigma</td>
</tr>
</tbody>
</table>

2.2.9 Mitochondrial extraction

2.2.9.1 Preparation of crude mitochondrial extracts

2x10^8 cells were harvested from logarithmic growth by centrifugation at 1300 g for 10 min. The cell pellet was washed in PBS-G, and the pellets were frozen at -80°C immediately. Cell
pellets were thawed on ice and resuspended in 100 µl ice-cold SHE buffer (250 mM sucrose, 25 mM HEPES pH 7.4, 1 mM EDTA, 10 mM glucose, plus complete, mini, EDTA-free protease inhibitor (1 in 5 ml) (Roche)). This was then supplemented with 387.5 µl ice-cold HBSS (136.89 mM NaCl, 5.37 mM KCl, 0.81 mM MgSO₄, 1.26 mM CaCl₂, 0.44 mM KH₂PO₄, 4.17 mM NaHCO₃, 5.55 mM glucose plus complete, mini, EDTA-free protease inhibitors (1 in 5 ml) (Roche), pH 7.3), and 12.5 µl 10 mg/ml digitonin was mixed in for a final concentration of 0.025%. This was incubated for 5 min at 25°C.

The crude mitochondrial fraction (pellet 1, P1, see Figure 2.12) was collected by centrifugation at 16000 g for 5 min, and resuspended in 90 µl ice-cold HBSS and 10 µl 10% n-Dodecyl β-D-maltoside (DDM). This was incubated on ice for 10 min, and centrifuged at 16000 g for 10 min at 4°C. For hrCNE, 80 µl organellar lysate (supernatant, S2) was supplemented with 1 µl DNase I (1 U/µl, Promega) and 1 µl 1 M MgCl₂ and incubated for 10 min on ice. For analysis on a denaturing protein gel, 20 µl of mitochondrial extract was resuspended in 60 µl 2x SDS.

### 2.2.9.2 hrCNE

DNase-treated crude mitochondrial lysates from Section 2.2.9.1 (82 µl volume) were mixed with 10 µl 1% Ponceau S and 10 µl 50% glycerol, and 20 µl of each sample was loaded per well onto pre-cast 3-12% Bis-Tris NuPAGE gels (Invitrogen). Gels were run using ice-cold buffers at 4°C for 1 hr at 150V, and then at 300V until the dye front ran out of the bottom of the gel.

Anode buffer: 25 mM imidazole, pH 7.0.

Cathode buffer: 50 mM tricine, 7.5 mM imidazole, 0.05% sodium deoxycholate, 0.02% DDM, pH 7.0.
Gels were incubated in 1x MOPS running buffer for 30 min. The gel was blotted onto a methanol-equilibrated PVDF membrane at 4°C using 1 L ice-cold transfer buffer and a Biorad Criterion blotter at 90 V for 1 hr, and blocked overnight in 1x TBST with 10% milk powder at 4°C. Antibody incubations and detection occurred as per the western protocol above.

2.2.10 cDNA preparation

2.2.10.1 RNA extraction

RNA extraction was performed via the Life Technologies TRI reagent protocol. The protocol was carried out in a fume hood, double layered gloves were worn, benches and pipettes were cleaned thoroughly prior to the protocol with 70% ethanol and RNaseZAP (Ambion), and filter tips were used to reduce RNase contamination. Pellets of 1x10^9 cells were lysed in 10 ml ice-cold TRI reagent solution (Ambion), and frozen at -80°C. The lysate was thawed for use at room temperature, 1 ml bromo-chloro-3-propanol (BCP) was added and the solution was vortexed. After a 15 min incubation at room temperature, the lysate was centrifuged at 12000 g for 15 min at 4°C. The aqueous phase was collected and 5 ml isopropanol was added. After vortexing and a 10 min incubation at room temperature, the mixture was centrifuged at 12000 g at 4°C for 15 min. The pellet was washed in 10 ml 75% ethanol and centrifuged at 12000 g for 10 min at 4°C, and the final pellet was air dried before resuspension in 100 µl DEPC water. The solution was stored at -80°C.
2.2.10.2 DNase treatment of extracted RNA

Removal of contaminating DNA was performed as via the Ambion DNA-free DNase kit. 40 µl RNA was mixed with 4 µl DNase I 10x buffer and 0.5 µl DNase I, and incubated at 37°C for 30 min. An additional 0.5 µl DNase I was then added, and the mixture was incubated for a further 30 min at 37°C. After vortexing, 4 µl inactivation reagent was added and the solution was mixed for 2 min, and then centrifuged at 10000 g for 90 s. 40 µl of supernatant was transferred to a fresh 1.5 ml tube.

2.2.10.3 Nano Agilent analysis of RNA

The integrity of the DNase-treated RNA was assessed using the Agilent Bioanalyzer Nano chip kit. The chip priming station was set to ‘C’, and an RNA Nano chip was positioned in the station. 1 µl Nano dye concentrate was mixed with 65 µl of spin filtered (1500 g for 10 min) Nano gel matrix, and vortexed thoroughly. 9 µl was pipetted into the ‘G’ well for the RNA Nano chip, and was dispensed by pressing down of the plunger for 30 s. 9 µl of the gel-dye mix was then pipetted into the two wells above well ‘G’, 5 µl Nano marker was pipetted into all remaining wells and 1 µl heat-denatured RNA ladder (70°C for 2 min) was pipetted into the ladder well. 1 µl of each sample was loaded into each of the sample wells before the chip was vortexed for 60 s. The chip was inserted into the Agilent 2100 Bioanalyzer to start the run.

2.2.10.4 Reverse transcription of T. b. brucei RNA

Reverse transcription was performed using the protocol for Life Technologies SuperScript III. 25 ng RNA in 11 µl dH₂O was mixed with 1 µl 10 mM dNTPs and 1 µl 200 ng/µl random hexamer primers (NEB), and the solution was incubated at 65°C for 5 min and then put on ice for at least 1 min. 4 µl 5x first strand buffer, 1 µl 0.1 M DTT, 1 µl RNaseOUT (40 units/µl) (Invitrogen) and either 1 µl Superscript III Reverse Transcriptase (+RT) or 1 µl
dH₂O (-RT) were then added. The following reaction conditions were used: 25°C 5 min, 50°C 60 min, 70°C 15 min.

1 µl RNase H (NEB) was then added, and the mixture was incubated at 37°C for 20 min to remove RNA. To stop the reaction, the temperature was increased to 90°C for 10 min.

2.2.11 Quantitative analysis of expression levels of maxicircle gene transcripts

The SYBR Green quantitative PCR (qPCR) protocol was used (Carnes and Stuart, 2007). Briefly, to each well of a 96 well plate the following components were added and mixed: 12.5 µl 2 x SYBR Green PCR Master Mix, 10 µl 1.75 µM primer mix, 2.5 µl diluted cDNA.

Template cDNA from Section 2.2.10.4 was diluted 1/7 for all maxicircle primers and 1/50 for the 18S rRNA internal control, allowing the Ct values to be closer in value to the Ct values for the less abundant kDNA-encoded transcripts. ‘-RT cDNA’ was used as a negative control with each primer set.

The endogenous transcript control used was 18S rRNA, a stable housekeeping transcript. The internal reference was the WT/WTγ cell line of the same strain for each analysis, therefore all results were normalised against the results for this cell line. Each reaction was done in triplicate.

Thermocycler conditions: 50°C 2 min, 95°C 10 min, [95°C 15 s, 60°C 1 min] x 40.

Melt curve stage: 95°C 15 s, 60°C 1 min, 95°C 15 s.

Results were analysed via the ΔΔCt method (Carnes and Stuart, 2007), assuming comparable primer efficiencies.
2.2.12 Amplification of A6 transcripts

Pre-edited, editing intermediates and fully edited A6 were amplified by PCR using non-discriminatory A6 primers #3 and #4 (Appendix B). These anneal to the very 5’ and 3’ ends of all A6 transcripts as these ends are unaffected by editing. Reaction mix as Section 2.2.2.1.

Cycle conditions: 95 °C for 5 min and [95 °C 1 min, 44 °C 1 min, 72 °C 30 s] for 40 cycles, and 72 °C for 8 min.

2.2.13 In vitro analysis of sensitivity to F1F0-ATPase inhibitors

This Alamar Blue protocol was adapted from (Raz et al., 1997). 100 µl HMI-9, 10% FCS was added to each well of a white-bottomed 96-well plate (Greiner), except for the first column. 200 µl of test compound at 2 x desired starting concentration in HMI-9, 10% FCS was added to the empty first well. A serial dilution of the drug was performed by removing 100 µl from first well and mixing it with the 100 µl of HMI-9, 10% FCS in 2nd well. This was repeated for subsequent wells, leaving the last well in the row un-mixed as the no drug control well. 2x starting concentrations of inhibitors: sodium azide (F1 inhibitor) 20 mM, oligomycin (F0 inhibitor) 20 µg/ml.

Cells were taken at logarithmic phase of growth. Cell density was measured using a Coulter Counter and adjusted to 1x10^4/ml in HMI-9/FCS. 100 µl parasites in media were added to each well of diluted test compound. For monomorphic cell lines, the plates were incubated for 72 hrs at 37°C and 5% CO2. 20 µl 0.5 mM resazurin sodium salt in PBS was then added to each well, followed by a further 4 hr incubation under the same conditions before measurement of fluorescence. For pleomorphic cell lines, plates were incubated for 72 hrs
before addition of 20 µl 0.5 mM resazurin sodium salt. A 24 hr incubation was then carried out, and the fluorescence was measured.

Fluorescence was measured using a FLUOstar OPTIMA fluorimeter (BMG Labtech) with excitation and emission filters of 544 nm and 590 nm, respectively. Data was analysed using GraphPad Prism software and ED$_{50}$/EC$_{50}$ values were derived from sigmoidal dose-response curves with variable slopes (four parameter nonlinear regression).

### 2.2.14 $\Delta \psi_m$ measurement

The Abcam TMRE Mitochondrial Membrane Potential kit was used. Two sets of 1x10$^6$ cells from each sample were resuspended in 1 ml HMI-9, 10% FCS, and one set was supplemented with 20 µM carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP) to uncouple $\Delta \psi_m$. All samples were pre-incubated for 10 min at 37°C, supplemented with 100 nM tetramethylrhodamine (TMRE), and left at 37°C for 20 min. Cells were pelleted at 2000 g for 5 min in 5 ml polystyrene round bottom tubes (BD Falcon 352052), and washed three times in 5 ml 0.2% BSA in PBS-G. Cell pellets were resuspended in 500 µl 0.2% BSA in PBS containing 5 µg/ml Hoechst 33342 DNA staining dye (Life Tech.) and left for 30 min in foil. Analysis was performed on BD LSRII instrument, with peak excitation at 549 nm, and peak emission at 575 nm.
2.3 Results

In order to investigate the effects of the L262Pγ mutation and kDNA loss on the structure and function of the *T. brucei* F₁F₀-ATPase, novel cell lines were generated as detailed in Section 2.2. We introduced tagged versions of F₁ subunits, allowing affinity-purification of the complex for studying its structure and function in more detail. In each tagged cell line, one or both endogenous ATPase γ alleles were replaced with L262Pγ to allow investigation into the effect of the mutation on the complex. kDNA was then deleted from these cells by acriflavine treatment to investigate the effects of loss of kDNA on the complex. This process generated multiple clones to act as replicates, with which to study clonal differences (Table 2.3). Pleomorphic cell lines that express L262Pγ and were depleted of kDNA by acriflavine were required for subsequent studies detailed in Chapters 3 and 4. As these cell lines had already been generated, they were also analysed in this Chapter as additional clones (Table 2.3).

2.3.1 Generation of TAP-tagged cell lines

The Lister 427 Single Marker BSF cell line (427SM; based on a monomorphic strain of *T. brucei*) allows tetracycline-inducible constructs to be expressed (Wirtz et al., 1999). This strain was transfected with linearised pLew79-MH-β-TAP or pLew79-MH-p18-TAP, tetracycline-inducible constructs that encode TAP-tagged copies of F₁F₀-ATPase subunits β or p18, respectively (Fig. 2.1A). This allowed the insertion of an additional tagged copy of these genes into the ribosomal spacer sequence (Fig. 2.1B). Cells that had taken up the plasmid and had undergone homologous recombination to incorporate the plasmid into the gDNA were selected with phleomycin, and 427SM β-TAP and p18-TAP clones were picked after 6 days (Table 2.3). Insertion of these constructs into the genome was assessed in clones
resistant to phleomycin by inducing expression of the construct with tetracycline for two
days and performing a western blot on cell lysate. Both β-TAP and p18-TAP could be
detected only in cells treated with tetracycline by anti-protein A (anti-PAP) and their
respective subunit antibodies, hence were shown to be inducible by tetracycline (Fig. 2.1C,
D).

2.3.2 Generation of WT/L262Pγ and L262P/L262Pγ cell lines

Kinetoplast DNA-independent cell lines were generated in four strains of T. b. brucei: Lister
strain AnTat1.1 90:13 (Engstler and Boshart, 2004) had been culture adapted previously in
the Matthews group in Edinburgh, UK.

Cell lines were transfected with plasmid pEnT6-γ-PURO containing either WT ATPase γ or
L262P ATPase γ (Fig. 2.2A), allowing the replacement of one endogenous ATPase γ allele
in order to generate cell lines containing a single WT γ allele replacement (WT/WTγ) or a
single L262Pγ allele replacement (WT/L262Pγ) (Table 2.3, Fig. 2.2B). The correct genotype
was confirmed by PCR (data not shown).

Clones of genotype WT/L262Pγ were subsequently transfected with plasmid pEnT6-γ-BSD
containing L262Pγ to generate their respective L262P/L262Pγ clones. After selection of
transfectants with blasticidin, correct integration was again confirmed by PCR (data not
shown; Table 2.3).
2.3.3 Generation of AK cell lines

Clonal cell lines with genotypes WT/WT\(\gamma\), WT/L262P\(\gamma\) and L262P/L262P\(\gamma\) were treated with 10 nM acriflavine to induce the loss of kDNA to generate AK versions of these cell lines (Table 2.3). The loss of maxicircle genes A6, ND4, ND5 and ND7, and a minicircle type upon acriflavine treatment was confirmed by PCR (Figs. 2.3, 2.4). DAPI staining confirmed the lack of a kinetoplast in AK clones (Fig. 2.5A). As in the literature (Gould and Schnaufer, 2014), cells expressing an L262P\(\gamma\) allele are resistant to EtBr treatment, unlike cells expressing solely WT\(\gamma\) (Fig. 2.6, 2.7B, dotted lines).

These analyses confirmed that despite cells expressing heterozygous and homozygous L262P\(\gamma\) being kDNA-independent, most non acriflavine-treated clones investigated had retained their kDNA (Fig. 2.3, 2.4), which was visible at an amount indistinguishable from the parental cell line by DAPI staining (data not shown). However, kDNA alterations in some clones were apparent. An example is AnTat1.1 90:13 L262P/L262P\(\gamma\) clone 3, which, despite normal kinetoplast staining by DAPI (Fig. 2.5B), had retained minicircle but not maxicircle kDNA (Fig. 2.4).
Table 2.3  Cell lines produced in Chapter 2

For genotypes as per (Clayton et al., 1998), see Appendix B.

<table>
<thead>
<tr>
<th>Cell line name</th>
<th>Cell line background</th>
<th>y allele 1</th>
<th>y allele 2</th>
<th>Tet-inducible construct?</th>
<th>Acriflavine treated?</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT/WTγ clone A</td>
<td>Lister 427SM</td>
<td>WT</td>
<td>WT</td>
<td>β-TAP</td>
<td>N</td>
</tr>
<tr>
<td>WT/L262Pγ clone A</td>
<td>Lister 427SM</td>
<td>WT</td>
<td>L262P</td>
<td>β-TAP</td>
<td>N</td>
</tr>
<tr>
<td>WT/L262Pγ (AK) clone A</td>
<td>Lister 427SM</td>
<td>WT</td>
<td>L262P</td>
<td>β-TAP</td>
<td>Y</td>
</tr>
<tr>
<td>L262P/L262Pγ clone A</td>
<td>Lister 427SM</td>
<td>L262P</td>
<td>L262P</td>
<td>β-TAP</td>
<td>N</td>
</tr>
<tr>
<td>L262P/L262Pγ (AK) clone A</td>
<td>Lister 427SM</td>
<td>L262P</td>
<td>L262P</td>
<td>β-TAP</td>
<td>Y</td>
</tr>
<tr>
<td>WT/WTγ clone B</td>
<td>Lister 427SM</td>
<td>WT</td>
<td>WT</td>
<td>p18-TAP</td>
<td>N</td>
</tr>
<tr>
<td>WT/L262Pγ clone B</td>
<td>Lister 427SM</td>
<td>WT</td>
<td>L262P</td>
<td>p18-TAP</td>
<td>N</td>
</tr>
<tr>
<td>WT/L262Pγ (AK) clone B</td>
<td>Lister 427SM</td>
<td>WT</td>
<td>L262P</td>
<td>p18-TAP</td>
<td>Y</td>
</tr>
<tr>
<td>L262P/L262Pγ clone B</td>
<td>Lister 427SM</td>
<td>L262P</td>
<td>L262P</td>
<td>p18-TAP</td>
<td>N</td>
</tr>
<tr>
<td>L262P/L262Pγ (AK) clone B</td>
<td>Lister 427SM</td>
<td>L262P</td>
<td>L262P</td>
<td>p18-TAP</td>
<td>Y</td>
</tr>
<tr>
<td>WT/WTγ clone C</td>
<td>Lister 427SM</td>
<td>WT</td>
<td>WT</td>
<td>-</td>
<td>N</td>
</tr>
<tr>
<td>WT/L262Pγ clone C</td>
<td>Lister 427SM</td>
<td>WT</td>
<td>L262P</td>
<td>-</td>
<td>N</td>
</tr>
<tr>
<td>WT/L262Pγ (AK) clone C</td>
<td>Lister 427SM</td>
<td>WT</td>
<td>L262P</td>
<td>-</td>
<td>Y</td>
</tr>
<tr>
<td>L262P/L262Pγ clone C</td>
<td>Lister 427SM</td>
<td>L262P</td>
<td>L262P</td>
<td>-</td>
<td>N</td>
</tr>
<tr>
<td>L262P/L262Pγ (AK) clone C</td>
<td>Lister 427SM</td>
<td>L262P</td>
<td>L262P</td>
<td>-</td>
<td>Y</td>
</tr>
<tr>
<td>WT/WTγ</td>
<td>AnTat1.1 90:13</td>
<td>WT</td>
<td>WT</td>
<td>-</td>
<td>N</td>
</tr>
<tr>
<td>WT/L262Pγ clone 1</td>
<td>AnTat1.1 90:13</td>
<td>WT</td>
<td>L262P</td>
<td>-</td>
<td>N</td>
</tr>
<tr>
<td>WT/L262Pγ clone 2</td>
<td>AnTat1.1 90:13</td>
<td>WT</td>
<td>L262P</td>
<td>-</td>
<td>N</td>
</tr>
<tr>
<td>WT/L262Pγ (AK) clone 1</td>
<td>AnTat1.1 90:13</td>
<td>WT</td>
<td>L262P</td>
<td>-</td>
<td>Y</td>
</tr>
<tr>
<td>WT/L262Pγ (AK) clone 2</td>
<td>AnTat1.1 90:13</td>
<td>WT</td>
<td>L262P</td>
<td>-</td>
<td>Y</td>
</tr>
<tr>
<td>L262P/L262Pγ clone 1</td>
<td>AnTat1.1 90:13</td>
<td>L262P</td>
<td>L262P</td>
<td>-</td>
<td>N</td>
</tr>
<tr>
<td>L262P/L262Pγ clone 2</td>
<td>AnTat1.1 90:13</td>
<td>L262P</td>
<td>L262P</td>
<td>-</td>
<td>N</td>
</tr>
<tr>
<td>L262P/L262Pγ clone 3</td>
<td>AnTat1.1 90:13</td>
<td>L262P</td>
<td>L262P</td>
<td>-</td>
<td>N</td>
</tr>
<tr>
<td>L262P/L262Pγ (AK)</td>
<td>AnTat1.1 90:13</td>
<td>L262P</td>
<td>L262P</td>
<td>-</td>
<td>Y</td>
</tr>
</tbody>
</table>
2.3.4 Assessment of the effect of L262P\(_\gamma\) alleles and kDNA loss on \textit{in vitro} growth

In order to test if the presence of L262P\(_\gamma\) or the loss of kDNA affected cell growth, cell lines expressing WT/WT\(_\gamma\), WT/L262P\(_\gamma\), WT/L262P\(_\gamma\) (AK) and L262P/L262P\(_\gamma\) were maintained in the logarithmic phase and cell counts were performed (Fig. 2.6, 2.7A, non-dotted lines). Cell lines homo- or heterozygous for L262P\(_\gamma\) grew at the same rate as the WT/WT\(_\gamma\) cell line, showing that the modifications had no effect on the \textit{in vitro} growth of the cells under these conditions. Cell line WT/L262P\(_\gamma\) (AK) clones A, B and C that had been treated with acriflavine to delete kDNA seemed to have a slight growth retardation in comparison to their respective WT/WT\(_\gamma\) cell lines, but exponential growth was nonetheless maintained (Fig. 2.6).

2.3.5 Expression levels of the L262P\(_\gamma\) allele in heterozygous cell lines

In order to see if WT\(_\gamma\) and L262P\(_\gamma\) alleles are both expressed in heterozygous WT/L262P\(_\gamma\) cell lines, pyrosequencing was used to assess the proportion of \(\gamma\) alleles expressed containing the L262P\(_\gamma\) mutation. This technique allows quantification of polymorphisms within a population of DNA molecules (Fig. 2.8). Via an enzyme-coupled DNA polymerase reaction, light is produced in proportion to the frequency of each nucleotide along a sequence, and as such allows quantitative mutation analysis between alleles of the same gene.

Using the Pyrosequencing Assay Design software, forward and reverse oligonucleotide primers were designed to amplify the section of the ATPase \(\gamma\) gene containing the L262P\(_\gamma\)
locus from cDNA (Fig. 2.9A). The dNTP dispersion order was selected by the Pyrosequencing software to generate pyrograms that could distinguish between wild type and mutant alleles (Fig. 2.9B). The L262Pγ mutation changes codon 262 (nucleotide 785) from CTT (leucine) to CCT (proline) (Fig. 2.9A, highlighted).

The analysis was performed on gDNA from WT/L262Pγ cell lines, with analysis on WT/WTγ and L262P/L262Pγ expressing cell lines as positive controls (Fig. 2.9B). This confirmed the genotypes of these cell lines. All WT/L262Pγ cell lines showed approximately a 50% WT and 50% L262P genotype, with the values for each cell line slightly varying (Fig. 2.9D). The L262Pγ allele level was consistently detected at slightly higher than 50%, there appears to be slight bias in the system.

RNA was extracted from the cell lines to be analysed, and a reverse transcriptase reaction was carried out to generate cDNA. The cDNA was assessed for gDNA contamination by performing reactions amplifying ATPase γ from plus and minus reverse transcriptase reactions. ATPase γ could not be amplified from any –RT samples, hence were negative for contaminating gDNA (data not shown).

The same series of PCR and pyrosequencing reactions was performed on this cDNA. For the homozygous control cell lines WT/WTγ and L262P/L262Pγ the expected signals for approximately 100% WT and mutated allele expression, respectively, were recorded (Fig 2.9D). As a control for heterozygosity with equal expression levels of both alleles, a 50:50 mixture of cDNA from cell lines expressing WT/WTγ and L262P/L262Pγ was used. As expected, this control showed approximately a 1:1 ratio between the WTγ and L262Pγ alleles, with the L262Pγ allele again detected at slightly higher levels than the WTγ allele (Fig. 2.9D).

For WT/L262Pγ cell lines in an AnTat1.1 90:13 background, as for the gDNA, approximately 50% of ATPase γ mRNA measured from cDNA was from the WTγ allele and
50% was from the L262Pγ allele, indicating similar mRNA abundance for the two alleles (Fig. 2.9C upper panel, D). For all three WT/L262Pγ cell lines in the 427SM background, there was a greater abundance of L262Pγ mRNA: for clone A 80% of expressed γ was from the L262Pγ allele, for clone B 75% of expressed γ sequences were L262Pγ, and for clone C 65% of expressed γ sequences were L262Pγ (Fig. 2.9C lower panel, D).

This quantification of ATPase γ allele expression occurs at the RNA level, and it remains to be determined how these changes in mRNA abundance affect protein levels. However it gives us some idea of expression of the L262Pγ allele in the two different T. brucei strains. AnTat1.1 90:13 cell lines seem to hold the replacement construct under tighter control than 427SM cell lines. In trypanosomes, the 3’ untranslated region (UTR) of mRNA transcripts have an important role in the regulation of their stability, for example (Furger et al., 1997, Walrad et al., 2012). The replacement construct containing the L262Pγ allele uses the aldolase 3’ UTR (a stable ‘housekeeping’ gene), whereas the endogenous allele has the endogenous 3’ UTR. Hence the higher detection of the mutant allele in 427SM cells could be due to AnTat1.1 90:13 cells regulating their aldolase mRNA more stringently. Additionally, as the γ gene in the replacement construct is from a different strain of T. brucei (EATRO 164), there may be SNPs present in this γ sequence when its sequence is compared to the CDS of the endogenous γ gene in 427SM cells. These synonymous mutations in L262Pγ could affect codon usage, which has been shown to potentially affect mRNA abundance and protein expression in multiple organisms (Gingold and Pilpel, 2011), including T. brucei (Horn, 2008)(Jeacock et al., de Freitas Nascimento et al., KMCBM 2015, abstracts 6C and 6D). Differing codon usage between the two γ alleles could therefore be contributing to the difference in the abundances of the two allelic γ transcripts in 427SM cells.
2.3.6 Probing the effect of L262Pγ mutation on F₁Fₒ–ATPase complex subunit composition

To investigate the subunit composition of the F₁Fₒ-ATPase in the presence of L262Pγ, we utilised antibodies specific for T. brucei F₁Fₒ-ATPase subunits α, β and p18. In addition, we used two antibodies that bind specifically to a putative Fₒ subunit (ATPTb1, Tb10.70.7760) and a stator protein (Tb2, Tb927.5.2930) (Subrtova et al., 2015). A TAP tag pull down analysis was performed on cell lines expressing an inducible TAP-tagged version of β subunit (Fig. 2.10). Cell lysates from cells expressing WT/WTγ, WT/L262Pγ, WT/L262Pγ (AK) or L262P/L262Pγ were incubated with IgG-conjugated magnetic beads. These beads bind the protein A part of the TAP tag, and can therefore be used to pull down the tagged bait protein and associated subunits. The beads were washed and treated with TEV protease that cleaves off the protein A part of TAP tag, eluting the calmodulin binding protein (CBP)-tagged bait protein and associated subunits from the beads. Fractions of the pull down were analysed with anti-CBP and various F₁Fₒ-ATPase subunit antibodies by western blot.

The band at 80 kDa in all four CL fractions is ATPase β-TAP subunit, showing the protein was expressed in all four cell lines (Fig. 2.11C). This band is detectable by all antibodies due to the protein A part of the TAP tag. In all four cell lines the pull down of β-TAP with the beads was effective (Fig. 2.11C). Some tagged protein was visible in all the flow through (FT) fractions; not all tagged protein bound to the beads (B). The protease treatment removed almost all the tagged protein from the beads: only a slight 80 kDa band is visible in the B fractions. A 65 kDa band corresponding to β-CBP is present in the eluates (E) for all four cell lines when the fractions were probed with anti-β. This band is detectable by anti-β and anti-CBP (Fig. 2.11C, D). A 55 kDa band also seen in the E fraction shows that the β-TAP has been able to integrate into F₁-α₃/β₃ cores that also contain untagged β (Fig. 2.11C).
As expected, Tb1 (putative subunit of F\textsubscript{o}) and Tb2 (putative subunit of the stator) are both associated with β-TAP in cell lines expressing WT/WT\textsubscript{γ} and WT/L262P\textsubscript{γ}, with bands at 40 kDa visible in the E fraction when probed with anti-Tb1 and anti-Tb2 (Fig. 2.11A, B). Neither Tb1 nor Tb2 are pulled down in cell lines expressing WT/L262P\textsubscript{γ} (AK) or, interestingly, L262P/L262P\textsubscript{γ}.

Subunits α and p18 are detectable in the eluate for all four cell lines (Fig. 2.11E). These subunits are therefore still associated with β-TAP in AK and L262P/L262P\textsubscript{γ} cell lines, despite Tb1 and Tb2 being much reduced or absent.

Affinity purified complexes from these TAP-tagged cell lines will prove useful for future kinetic studies, as discussed in Section 2.4.9. These cell lines were also used in subsequent studies in this Chapter with uninduced TAP-tag. These independently-generated cell lines were a convenient way of testing the effects of L262P\textsubscript{γ} in multiple replicates. The presence of an uninduced tagged subunit was not expected to affect the results (Fig. 2.6), and expression proved to be tightly controlled by tetracycline (Fig. 2.1).

### 2.3.7 Optimisation of crude mitochondrial extractions

In preparation for native protein electrophoresis in order to analyse the effect of the L262P\textsubscript{γ} mutation and acriflavine treatment on the structure of the F\textsubscript{1}F\textsubscript{o}-ATPase, crude mitochondrial extractions were performed from 2x10\textsuperscript{8} cells from each cell line (in cell lines with TAP tagged construct present, this was uninduced). Cells were subjected to digitonin detergent permeabilisation to solubilise the plasma membrane (Turrens, 1989, Vercesi et al., 1991). After centrifugation, the pellet (P1) containing membrane-bound organelles, including the mitochondrion-derived vesicles, was collected and then permeabilised with DDM (Fig. 2.12A). DDM is a mild non-denaturing detergent that does not distort protein-protein
interactions, hence can extract proteins from the mitochondrial membranes in their native state, allowing analysis of the structure of these membrane complexes (Seddon et al., 2004). This mitochondrial extraction protocol is crude as the final lysate (S2) will contain proteins from other lysed organelles, for example the ER and nuclear envelope (Mardones and Gonzalez, 2003, Furuta et al., 2014), and some cytosolic contamination.

The effective concentration of digitonin has to be determined per batch as digitonin is a natural product, with quality and purity varying. Hence published digitonin concentrations are difficult to reproduce. Therefore as an initial step, we performed a digitonin titration to see at which concentration our digitonin stock is most effective at disrupting the plasma membrane but not the mitochondrial membrane. 2x10^6 cells were harvested from culture, resuspended in the appropriate buffers and subjected to digitonin treatment at a range of concentrations. After centrifugation, pellet and supernatant were fractionated by SDS-polyacrylamide gel electrophoresis (PAGE), blotted, and probed with antibodies for a mitochondrial (ATPase subunit β) and a cytoplasmic marker (translation elongation factor 1, EF1) (Fig. 2.12B). Without digitonin, as expected, the majority of each protein was in the pellet. About 1/3 of both proteins were in the supernatant, indicating lysis of some cells and mitochondria even in the absence of detergent. It appears that some cells from the pellet were resuspended into the supernatant while the pellet and supernatant were being separated. Even at the lowest concentration of digitonin tested (0.025% (w/v)), virtually all EF1 was released into the supernatant, indicating efficient lysis of the cell membrane. At this concentration, less than 10% of subunit β was released into the supernatant (Fig. 2.12B). At higher than 0.025% (w/v) digitonin, there appeared to be more leakage of ATPase subunit β into the supernatant, hence 0.025% (w/v) was chosen as the final concentration of digitonin to be used.

We need to be able to probe the structure of the F₁F₅-ATPase complex of multiple cell lines at the same time. Since it is difficult to generate cultures of different cell lines that are all at
the correct density for harvesting at the same time, the effect on mitochondrial integrity of freezing the cell pellet at -80°C for storage, and subsequent thawing, was tested. In the freshly prepared cell pellet, S1, the cytoplasmic fraction, contains cytosolic EF1 and very little ATPase subunit β, showing, as before, that treatment with 0.025% digitonin did not rupture the mitochondrial membranes (Fig. 2.12C, left). As before, P1, the membrane-bound organelle fraction contained virtually all ATPase subunit β, and very little EF1 contamination was present. P2 contained non-permeabilised organelle membranes, with traces of ATPase subunit β and some cytosolic EF1 contamination. S2, the mitochondrial extract, contains a trace of contaminating cytosolic proteins EF1, but contains the vast majority of ATPase subunit β. Hence, DDM efficiently solubilised the mitochondrial membranes. In cell pellets frozen at -80°C before fractionation, this profile is very similar for P2 and S2, showing that there is no detrimental effect on the enrichment of mitochondrial proteins if the cell pellet is frozen (Fig. 2.12C, right). Cytosolic contamination levels are the same using fresh or frozen cell pellets. However, there is more ATPase subunit β present in S1 when the cell pellet was frozen before fractionation. This shows that freeze thaw does cause some leakiness of the mitochondrial membranes, with some loss of mitochondrial material into the cytosol. However as freezing allowed the analysis of multiple mitochondrial extracts simultaneously, this small loss of mitochondrial material was deemed acceptable, and this protocol was used from this point.

2.3.8 Putative F_0 and stator subunits are not detectable in L262P/L262Pγ cell line clones A, B and 3

F_1F_0 ATPase subunits Tb1 and Tb2 have been confirmed as F_1F_0-ATPase subunits for a WT complex (Subrtova et al., 2015)(Subrtova, personal communication), but are not associated
with the complex in L262P/L262Pγ and AK cell lines (Fig. 2.11). In order to investigate the consequences of the L262Pγ mutation, we assessed the expression of F₁Fₐ ATPase subunits Tb1 and Tb2 in all cell lines. Mitochondrial extracts for monomorphic and pleomorphic cell lines WT/WTγ, WT/L262Pγ, WT/L262Pγ (AK) and L262P/L262Pγ were run on denaturing protein gels and probed with antibodies for T. brucei subunits (Fig. 2.13).

All cell lines showed comparable ATPase β expression (data not shown). For Tb1, the subunit was not detected in any of the AK cell lines. The protein is expectedly absent in L262P/L262Pγ clone A (see Fig. 2.11), but is also absent in L262P/L262Pγ clone B (Fig. 2.13A). In cell line L262P/L262Pγ clone 3, the cell line that has lost the maxicircle (see Fig. 2.3), this subunit is also absent (Fig. 2.13B). The subunit is present in all WT/WTγ and WT/L262Pγ cell lines, and interestingly, also in clones C, 1 and 2 of the L262P/L262Pγ cell lines. The same general pattern is seen for Tb2; however, in the L262P/L262Pγ and AK cell lines where Tb1 was absent, Tb2 can still be detected, but at much lower levels than in WT/WTγ cell lines.

2.3.9 The L262Pγ mutation causes structural disruption of the F₁Fₐ-ATPase complex in most, but not all L262P/L262Pγ clones

In order to investigate the effect of the L262Pγ mutation on the structure of the F₁Fₐ-ATPase complex, high resolution clear native electrophoresis (hrCNE) was used (Wittig et al., 2007). Mitochondrial extracts from 427SM and AnTat1.1 90:13 cell lines were analysed on native gels by probing with antibodies specific for F₁, Fₐ and the stator part of the ATPase complex. Use of frozen cell pellets had no effect on banding patterns seen when using hrCNE (data not shown).
All 427SM cell lines containing at least one WTγ allele (cell lines expressing WT/WTγ, WT/L262Pγ) that had not been treated with acriflavine and that, by DAPI staining and PCR analysis, were kDNA+, show multiple bands (Fig. 2.14A). A band at 720 kDa is detectable with anti-β (F₁) but not with anti-Tb1 (Fo) or anti-Tb2 (stator), therefore this band most likely corresponds to an F₁ monomer (red arrows). This F₁ monomer was also present in T. b. evansi STIB 805 and in WT/L262Pγ (AK) cell lines.

The three upper bands detectable in cell lines WT/WTγ and WT/L262Pγ by anti-β (F₁) are also detected by anti-Tb1 (putative Fo) and anti-Tb2 (putative stator) (Fig. 2.14A). Hence these bands must be the physically coupled monomer and di- or oligomers of F₁Fo complexes, (blue brackets). The middle band at approximately 1048 kDa seems fainter than the others, so could be a stable intermediate between F₁Fo monomer and F₁Fo di- or oligomer. Alternatively it could be due to partial disruption of an oligomer due to experimental conditions. Results between replicate experiments were somewhat variable as the relative abundance of the three bands was not always the same. Also, it is not known to what extent the experimental conditions might disrupt the native state or result in aggregates. The levels of F₁Fo complexes in cell lines with WT/WTγ and WT/L262Pγ does seem to be relatively consistent.

The estimated band sizes here correlate with the work of Subrtova et al., 2015. Although the band pattern is similar in WT BSF cell lines, there is some discrepancy between the sizes of the complexes seen here by hrCNE and in the BNE experiments by Zikova et al., 2009. It is difficult to estimate the mass of complexes from bands on native gels as the protein markers used are invariably soluble proteins, which have a different migration rate from the membrane protein complexes being investigated. Differences in migration patterns of the marker also arise using different gradient PAGE gels, the time given for migration, and the detergents used in membrane permeabilisation and in the cathode buffer (Wittig et al.,
Size differences between bands are not that useful in predicting the constituents of the complex in each band.

From this hrCNE analysis we can conclude that cell lines with at least one WT γ allele and that are kDNA+ have physically coupled F₁F₀ complexes. F₁F₀ complexes are absent in T. b. evansi STIB 805 and acriflavine treated WT/L262Pγ as expected, due to their lack of kDNA and therefore lack of complete F₀. This suggests that A6 completes the stable assembly of the F₀ moiety, as in other organisms (Hadikusumo et al., 1988, Tzagoloff et al., 2004, Hermolin and Fillingame, 1995, Wittig et al., 2010b). Unstable assembly intermediate lacking subunit a have been seen in yeast (Rak et al., 2007, Wittig et al., 2010b). Faint F₁F₀ bands have also been seen in T. b. evansi AnTat3/3 and acriflavine-induced Dk T. b. brucei EATRO164 (Subrtova et al., 2015). Our protocol for mitochondrial extraction uses DDM to solubilise mitochondrial membranes. Unlike digitonin, used in Subrtova et al., 2015, DDM can cause dissociation of more weakly associating complexes (Wittig et al., 2007), hence the absence of unstable higher MW bands in our AK cell lines could be caused by the more stringent conditions used here.

In the absence of a WTγ allele (L262P/L262Pγ cell lines) the banding pattern changes. No coupled F₁F₀ complexes were observed for clones A and B (Fig. 2.14A, left hand side and central panel). Instead of the 720 kDa F₁ complex, probing with anti-β detected a somewhat larger complex, migrating just below the F₁F₀ monomer (green arrow). This F₁+x band was not detectable by anti-Tb1, but is faintly detectable with anti-Tb2. The F₁+x band is also detectable with anti-β in all WT/L262Pγ (AK) and L262P/L262Pγ (AK) cell lines. The band may represent F₁ in association with the c-ring (Subrtova et al., 2015, Zikova et al., 2009).
2.3.10 L262P/L262Pγ clones C, 1 and 2 have structurally coupled F₁F₀-ATPases

In L262P/L262Pγ clone C before acriflavine treatment, the three F₁F₀ bands, a faint F₁ band at 720 kDa and F₁+x were all detectable (Fig. 2.14A, right hand side panel). This clone therefore contained F₁F₀ complexes despite having homozygous L262Pγ.

For the parental AnTat1.1 90:13 cell line, three bands corresponding to physically coupled F₁F₀ complex are detectable by both anti-Tb1 and anti-Tb2 (Fig. 2.14B). These are at approximately the same size as F₁F₀ bands seen in 427SM cell lines. These bands are detectable in all clones with genotype WT/WTγ or WT/L262Pγ. In L262P/L262Pγ clones 1 and 2, F₁F₀ bands are also present, but at somewhat reduced levels. This analysis is not particularly quantitative, as levels are determined by the efficiency of mitochondrial extraction, not determined here. However, from Figure 2.13B, we can see that the levels of Tb1 and Tb2 in mitochondrial extractions from L262P/L262Pγ clone 1 and 2 are consistent with those found in WT/WTγ and WT/L262Pγ cell lines. This shows that the decrease in F₁F₀ complex levels in clone 1 and 2 is not due to decreased levels of subunits. F₁F₀ is absent from all acriflavine-treated cell lines, as expected, and additionally absent from non-acriflavine treated L262P/L262Pγ clone 3. The faint bands visible in the lanes for the acriflavine-treated cell lines are most likely due to spill-over from neighbouring lanes. The analysis on these cell lines does need to be repeated, as it has only been carried out once.

At the writing of this thesis, western blot data for anti-β for the AnTat1.1 90:13 cell lines were not available yet; hence no conclusions can be drawn on the presence of F₁ and F₁+x.
2.3.11 The impact of L262Pγ on RNA editing of subunit A6

In AK cell lines, there seems to be an absence of F₁ complexes larger than F₁+x and absence of complexes that can be detected with antibodies to putative F₉ and stator subunits (Fig. 2.13, 2.14). This could suggest that absence of the mitochondrially-encoded A6 subunit results in destabilization of F₁F₉ or even lack of assembly of F₉, leaving F₁+x. The same is seen in certain homozygous L262Pγ cell lines that do have visible kDNA (Fig. 2.3, 2.4, 2.5A). This would be consistent with the idea of L262Pγ causing a proton leak and the selection of cells that prevent this from happening, for example by partial or complete kDNA loss.

Unfortunately there are no antibodies available for F₉ subunits c or A6 with which to investigate the integrity of the proton pore subunits themselves. Since loss of fully edited A6 or RPS12 could potentially explain the loss of F₉ in the presence of seemingly intact kDNA, we investigated the levels of pre-edited and edited A6 and RPS12 mRNAs in L262Pγ-expressing cell lines. We also assessed the ability of the cells to edit other maxicircle genes and the levels of a never-edited mRNA.

RNA editing was assessed by qPCR on cDNA from cell lines expressing WT/L262Pγ or L262P/L262Pγ. Primers specific to pre-edited, edited and never-edited kDNA transcripts were used. Levels of transcripts were normalised to levels of 18S rRNA from each cell line as an endogenous control. Results in Figures 2.15A and B are shown relative to respective transcript levels found in WT/WTγ cell lines of the same genetic background.

As expected, in all acriflavine-treated cell lines levels of kDNA transcripts were either <1000-fold lower than the respective WT/WTγ cell line, or not detected.

427SM heterozygous WT/L262Pγ cell lines have fairly similar (< 3-fold higher or lower) levels of pre-edited, edited and never-edited transcripts as their respective WT/WTγ cell line
Hence, there is no indication of editing loss seen in the presence of one L262Pγ allele. The two AnTat1.1 90:13 WT/L262Pγ cell lines investigated had moderately (2- to 5-fold) decreased levels of pre-edited and edited A6 and RPS12. Never-edited ND4 and pre-edited ND7 were unchanged compared to the WT/WTγ cell line, while levels of edited ND7 were slightly (~3-fold) increased. From this, it seems that there is some loss of edited A6/RPS12 mRNA levels in the presence of one L262Pγ allele, but this loss is mostly due to a decrease in preedited mRNA levels.

Substantial changes were observed for several homozygous L262Pγ cell lines that had not been treated with acriflavine. 427SM L262P/L262Pγ clones A and B had a specific >20-fold decrease in levels of edited A6. There was no decrease in levels of pre-edited A6. This would be consistent with a decrease in A6 editing capacity or a loss in stability for the fully edited A6 transcript. Levels of the other pre-edited, edited and never-edited transcripts were consistent with the small levels of fluctuation seen between transcripts in WT/L262Pγ clones. L262P/L262Pγ clone C showed a slight (2- to 3-fold) but consistent increase in levels of all pre-edited and edited transcripts, including A6 (Fig. 2.15A). In AnTat1.1 90:13 L262P/L262Pγ clone 1 and 2, there was a decreased level of all pre-edited transcripts apart from RPS12 (Fig. 2.15B). Both clones had at least 8-fold lower levels of edited and pre-edited A6 and of edited RPS12, with clone 1 showing a >10 fold loss of edited A6. These cell lines showed a slight (2- to 4-fold) increase in edited ND7 levels, suggesting loss of editing activity was specific rather than general. Noticeably, kDNA transcript levels in L262P/L262Pγ clone 3 were as low as in acriflavine-treated cells.

In order to verify the loss of edited A6 in some L262P/L262Pγ clones, an RT-PCR analysis of transcript A6 from these cell lines was also performed. This amplified all species of A6 transcript present in each cell line using non-discriminatory A6 primers. A6 transcripts were able to be amplified in all cell lines, except L262P/L262Pγ clone 3 (Fig. 2.16) and all
acriflavine-treated cell lines (data not shown). In all cell lines except L262P/L262P γ clones A and B, a band corresponding to the size of fully edited A6 could be detected. These cell lines did contain A6 editing intermediates, but the editing process seemed to terminate prematurely, as the sizes of intermediates did not exceed ~ 600 bp. This suggests that particular gRNAs could have been lost from these cell lines, causing a halt in the stepwise editing process. Editing intermediates of variable sizes were also observed for all other cell lines, with the exception of L262P/L262P γ clone 3 and the acriflavine treated clones. L262P/L262P γ clone 1 showed an extremely faint band of a size consistent with fully edited A6, and fainter editing intermediate bands than other AnTat1.1 90:13 cell lines.

2.3.12 The effect of L262P γ on oligomycin sensitivity

Oligomycin is a specific inhibitor of F o moiety of the F 1 F o -ATPase (Symersky et al., 2012). It therefore inhibits ATP synthesis by the coupled F 1 F o -ATPase or, in BF T. brucei, Δψm generation (Nolan and Voorheis, 1992, Vercesi et al., 1992, Divo et al., 1993, Schnaufer et al., 2005). DK T. brucei no longer rely on proton pumping for Δψm generation and are therefore oligomycin resistant (see Section 1.8) (Opperdoes et al., 1976, Dean et al., 2013, Schnaufer et al., 2005). We exploited this inhibitor to investigate to what extent the various 427SM cell lines, homozygous or heterozygous for L262P γ alleles, and acriflavine-treated or not, depended on a functionally coupled F 1 F o -ATPase for survival. Alamar Blue assays were performed on recently selected transfectants as well as on cell lines that had been grown in vitro for 2 weeks, with sensitivity expressed as ED 50 values (as oligomycin is an imprecise mixture of Oligomycin A and Oligomycin B), the effective dose (in μg/ml) inhibiting 50% of cellular growth. The higher the ED 50 value, the more resistant the cell line is to oligomycin.
ED$_{50}$ values were extremely consistent between clones of the same $\gamma$ genotype (Fig. 2.17A). WT/WT$\gamma$ cells show sensitivity to oligomycin, with an ED$_{50}$ of \(~0.1\) $\mu$g/ml, as they depend on F$_{o}$ to generate $\Delta\psi$m (Schnaufer et al., 2005). The presence of L262P$_{\gamma}$ in general increases resistance to oligomycin. All L262P$_{\gamma}$-expressing cell lines, with or without acriflavine treatment, have an ED$_{50}$ of \(~0.8\) $\mu$g/ml, around 8 times higher than WT/WT$\gamma$. AK cells, where kDNA-encoded A6 is absent, generate their $\Delta\psi$m via the AAC, independently of F$_{o}$. Similarly, cell lines L262P/L262P$_{\gamma}$ clones A and B also lack A6 (Fig. 2.16). This suggested that L262P$_{\gamma}$ uncouples the F$_{1}$ moiety from F$_{o}$, resulting in a proton leak. F$_{o}$ has been lost in these cell lines (Fig. 2.14), and hence, as expected, oligomycin sensitivity of these clones is comparable to AK cell lines. In L262P/L262P$_{\gamma}$ clone C, fully edited A6 and structurally coupled F$_{1}$F$_{o}$ are present (Fig. 2.14, 2.16). However, this cell line is as oligomycin resistant as those that have lost A6, suggesting that L262P$_{\gamma}$ has functionally uncoupled the F$_{1}$F$_{o}$ complex present, and hence cells are utilising the AAC to generate $\Delta\psi$m. In WT/L262P$_{\gamma}$ cell lines, these are equally as oligomycin resistant as AK cell lines. In these cell lines, we assume there is a mixture of complexes competing for ATP, one containing WT$\gamma$ and one containing L262P$_{\gamma}$. There is no evidence for lack of (functional) A6 or F$_{o}$ in these cells, hence we must assume that there is a WT F$_{1}$F$_{o}$ complex that can use ATP to pump protons, and a mutated complex. The mutated complex will be able to generate a proton leak as L262P$_{\gamma}$ is present in the same complex as A6. It is unclear currently which complex dominates in the production of $\Delta\psi$m, it may be that $\Delta\psi$m is generated mostly by the more efficient classical F$_{1}$F$_{o}$ method, with the amount of WT complex present sufficient to manufacture enough $\Delta\psi$m despite a proton leak. There seems to be no loss of kDNA from these cell lines (which could close the proton leak), hence the proton leak in these cells must be compensated for by the manufacture of $\Delta\psi$m. The WT complexes will be inhibited by oligomycin, but the mutated complexes will be facilitated by the presence of oligomycin, as
this will close the proton leak by inhibiting $F_o$, hence in the presence of oligomycin, cell lines of this genotype are manufacturing $\Delta \psi m$ via the AAC.

The assay was repeated on cells that had been grown for a longer period of time allowing adaptation to their growth conditions in a clone specific manner. The same pattern is seen, with WT/WT cells sensitive to oligomycin, with an ED$_{50}$ of $\sim 0.1$ µg/ml. The presence of L262P$\gamma$ still increases resistance to oligomycin, however the degree of resistance to oligomycin is different between clones of the same $\gamma$ genotype. In cell lines with a genetic background of $\beta$-TAP (uninduced, clones A), all cell lines expressing L262P$\gamma$ had an approximately equivalent resistance to oligomycin in the presence or absence of kDNA, with a 40-fold increase in ED$_{50}$ values compared to WT/WT$\gamma$ cell lines (Fig. 2.17B, left panel). Hence for cells with an uninduced $\beta$-TAP construct, all L262P$\gamma$-expressing cell lines are able to generate $\Delta \psi m$ independently of $F_o$, in the manners discussed above.

In cell lines with a genetic background of p18-TAP (uninduced, clones B), acriflavine-treated WT/L262P$\gamma$ cells, and acriflavine-treated or untreated L262P/L262P$\gamma$ cells showed comparable resistance to oligomycin. They had a $\sim$10-fold increase in ED$_{50}$ value in comparison to WT/WT$\gamma$ cell lines (Fig. 2.17B, centre panel). Cell line WT/L262P$\gamma$B has a greater resistance to oligomycin, with an average ED$_{50}$ roughly 30-fold greater than WT/WT$\gamma$ cell lines.

In cell lines with a parental 427SM genetic background (clones C), cell lines WT/L262P$\gamma$ cells and acriflavine-treated WT/L262P$\gamma$ and L262P/L262P$\gamma$ cells had overlapping ED$_{50}$ value ranges, at $>20$ times the ED$_{50}$ values of WT/WT$\gamma$ cells (Fig. 2.17B, right panel). The resistance to oligomycin was more increased in the AK cell lines. Cell line L262P/L262P$\gamma$ maintained approximately 8 times higher resistance to oligomycin than WT/WT$\gamma$ cell lines, but a much lower resistance than other cell lines in this group C. Interestingly, the ED$_{50}$ value of L262P/L262P$\gamma$ clone C remains the same in cells grown for two weeks and for $>2$
months, suggesting that the presence of coupled F₁Fₒ-ATPase limits the ability of this cell line to adapt further to growth in culture.

Because of the slower growth rates of the AnTat1.1 90:13 cells *in vitro*, combined with a relatively slow effect of oligomycin on cell growth, sensitivity of those cell lines to the compound could not be measured via the Alamar Blue assay.

### 2.3.13 The effect of L262Pγ and kDNA loss on azide sensitivity

Sensitivity to sodium azide was also assessed by Alamar Blue assay in these cell lines. Sodium azide is an inhibitor of the F₁ moiety of F₁Fₒ-ATPase which is essential in generating the Δψm in BSF cells with and without kDNA (Schnaufer et al., 2005), hence we can assess the effect of L262Pγ and kDNA loss on the requirement for F₁-ATPase activity. Here EC₅₀ is used to express sensitivity to azide. This is the effective concentration (in µM) inhibiting 50% of cellular growth. The higher the EC₅₀ value, the more resistant the cell line is to azide.

In cells grown for 2 weeks, a general pattern of increased sensitivity to azide is seen upon the increase in number of L262Pγ alleles and then the removal of all kDNA by acriflavine treatment (Fig. 2.18A). This agrees with the increased azide sensitivity of *T. b. evansi* compared with *T. b. brucei* (Schnaufer et al., 2005), and with the requirement for active F₁-ATPase in human ρ⁰ cells that lack mtDNA (Buchet and Godinot, 1998). 427SM cells with WT/WTγ were the least sensitive to azide, with EC₅₀ values of around 600 µM. Cells with WT/L262Pγ have a comparable sensitivity, with the exception of WT/L262P clone C, which has an approximately 1.6-fold increase in EC₅₀ value compared to WT/WTγ clone C (Fig. 2.18A). L262P/L262Pγ cells generally have a lower EC₅₀ than WT/L262P cell lines,
showing increased sensitivity to the compound. However, L262P/L262Pγ clone C shows a comparable EC₅₀ value to WT/L262Pγ, again this homozygous cell line is more resistant to azide than WT/WTγ clone C (Fig. 2.18A). All AK L262P/L262Pγ cells are even more sensitive to azide than L262P/L262Pγ cells, with acriflavine-treated WT/L262Pγ cells being the most sensitive to azide.

Hence, these sodium azide assays indicate that both 427SM and AnTat1.1 90:13 cell lines become more dependent on F₁-ATPase activity upon addition of L262Pγ alleles, and upon kDNA deletion. This can be explained as removal of kDNA from cells changes the ratio of charge exchange per molecule of ATP hydrolysed. The ‘classical’ method of generating Δψₘ using F₀ involves the translocation of 3 protons through F₀ for every ATP molecule hydrolysed. The ‘alternative’ method only translocates 1 positive net charge per ATP through the AAC, hence is less efficient in producing Δψₘ (Fig. 1.8). Hence cells using the AAC have a greater dependence on F₁ for ATPase activity, and thus have a higher sensitivity to azide.

WT/L262Pγ expressing cells can use either method to generate Δψₘ productively (Fig. 2.20), depending on whether the coupled complex is inhibited, for example by oligomycin (Fig. 2.17). We do not know the ratio of WTγ to L262Pγ complexes, but taking into account the allelic expression quantification (Fig. 2.9) and assuming similar translation efficiency for the two alleles, approximately between a quarter and half of complexes are efficiently generating Δψₘ in the classical fashion, contributing to the majority of Δψₘ production.

In WT/L262Pγ (AK) cell lines we see no F₁F₀ complexes (Fig. 2.14), hence the classical method of generating Δψₘ cannot function. Roughly half the F₁ complexes contain L262Pγ, hence generate Δψₘ via the alternative method, and these L262Pγ-containing complexes would be predicted to have a lower Kₘ for ATP than the WT complex (Fig. 2.20)(Clark-Walker, 2003, Schnaufer et al., 2005). However, roughly half the F₁ complexes are less
productive at making $\Delta \psi m$ as they contain WT$\gamma$. Therefore the cell contains half as much relatively efficient $F_1$-ATPase activity as L262P/L262P$\gamma$ (AK) cells, and so less azide is required to inhibit $F_1$ activity (Fig. 2.18). WT/L262P$\gamma$ (AK) cells are therefore more sensitive to azide than L262P/L262P$\gamma$ (AK) cells.

We know that L262P/L262P$\gamma$ A and B cell lines do not have fully edited A6 (Fig. 2.15, 2.16), therefore they cannot perform the classical method of $\Delta \psi m$ generation. Hence complexes contribute to the less efficient alternative method to generate $\Delta \psi m$, meaning these cells are more sensitive to azide than WT/L262P$\gamma$ cells (Fig. 2.20). However they are more resistant to azide than L262P/L262P$\gamma$ (AK) cells, the cause of which is unclear.

Equally, it is presently unclear why L262P/L262P$\gamma$ clones 1, 2 and C have higher azide resistance than their AK counterparts. These cell lines contain $F_1F_o$ complexes (Fig. 2.14), but are functionally uncoupled (Fig. 2.17), hence must manufacture $\Delta \psi m$ via the alternative method like their AK counterparts.

After $>2$ months of continuous culturing, in 427SM cells with a $\beta$-TAP genetic background (uninduced, clones A), (Fig. 2.18A, left panel), the same general pattern of azide sensitivity is present. However all EC$_{50}$ values are approximately halved compared to EC$_{50}$ values in cells grown in vitro for 2 weeks (Fig. 2.18A, B). In cells with a p18-TAP genetic background (uninduced, clones B) and with a standard 427SM background the general pattern is also the same (Fig. 2.18B, centre and right panels). However, WT/L262P$\gamma$ clone B has an increased sensitivity to azide than WT/WT$\gamma$ cells after 2 months of culturing compared with after only 2 weeks in culture. WT/L262P$\gamma$ clone C had a 1.6-fold increased azide sensitivity compared with its respective WT/WT$\gamma$ cell line after 2 months of culturing, consistent with what is seen at 2 weeks of culturing. L262P/L262P$\gamma$ clone C becomes more sensitive to azide than WT/L262P$\gamma$ over time.
In AnTat1.1 90:13 clones, the pattern was also similar, however there was variation between the azide sensitivity of WT/L262Pγ and L262P/L262Pγ clones (Fig. 2.18C).

2.3.14 The effect of L262Pγ and kDNA loss on Δψm

Δψm was assessed by a fluorescent TMRE assay. This cell-permeant cationic dye is sequestered into the negatively charged mitochondrial matrix due to Δψm. In cells with a higher Δψm, more dye is accumulated and this causes an increase in fluorescent signal. Cells were treated with TMRE in the presence of absence of the proton ionophore FCCP, which effectively dissipates the Δψm. The median value of fluorescence was used as a measure of relative Δψm. Data was normalised to the median fluorescence of the respective WT/WTγ cell lines, allowing comparison between related cell lines. These experiments could only be performed once before submission of the thesis, and so require repeating to give an estimate of the error in the data.

In 427SM cell lines, a trend is evident between independently generated clones of the same genotype. Δψm decreased from the level recorded in WT/WTγ cell lines for WT/L262Pγ and decreased again for acriflavine-treated WT/L262Pγ cell lines, but then increased from this level for L262P/L262Pγ cell lines (Fig. 2.19A). This agrees with the decrease in Δψm seen for mammalian ρ0 (lacking mtDNA) compared to ρ+ cells (mtDNA+) (Appleby et al., 1999). All WT/L262Pγ clones had approximately the same Δψm, at around 90% of the WT/WTγ cell lines. Δψm for WT/L262Pγ (AK) cell lines was between 60%-80% of the level of respective WT/WTγ clones. Of L262P/L262Pγ cell lines, Δψm varied from between a comparable Δψm to WT/WTγ for clone A, whereas clone C displayed a Δψm around 65% of that of WT/WTγ C.
This general pattern of relative $\Delta\psi_m$ was maintained in AnTat1.1 90:13 cell lines, although at 2 weeks of *in vitro* growth, WT/L262P$\gamma$ cell lines had a 30% higher $\Delta\psi_m$ than the WT/WT$\gamma$ cell lines (Fig. 2.19B). WT/L262P$\gamma$ (AK) cell lines seemed to have a considerably lower $\Delta\psi_m$, approximately 75% lower than WT/WT$\gamma$ cell lines. The $\Delta\psi_m$ of L262P/L262P$\gamma$ cell lines varied from a level 50% of WT/WT$\gamma$ levels, to a $\Delta\psi_m$ equivalent to that of WT/WT$\gamma$ cell lines.

These experiments have only been carried out once, so do need to be repeated. However, the data currently suggests that when $\Delta\psi_m$ is generated via the alternative method through the AAC, the $\Delta\psi_m$ production seems to be lower than when the classical F$_{o}$-dependent method is used. This can be justified, as $\Delta\psi_m$ production is 3 times more efficient via F$_{o}$ than via solely the AAC, per ATP hydrolysed. Hence in general WT/L262P$\gamma$ (AK) cells appear to have a lower $\Delta\psi_m$ than WT/L262P$\gamma$ and L262P/L262P$\gamma$ cell lines (Fig. 2.19). It is not immediately clear why L262P/L262P$\gamma$ expressing cell lines with functionally uncoupled F$_{i}$F$_{o}$ complexes generally have higher $\Delta\psi_m$ than their AK counterparts, as they use the less efficient alternative method of $\Delta\psi_m$ production (Fig. 2.20). $\Delta\psi_m$ is slightly lower in 427SM WT/L262P$\gamma$ cell lines than WT/WT$\gamma$ cell lines (Fig. 2.19). This could be due to a proton leak caused by the L262P$\gamma$-F$_{i}$F$_{o}$ complexes pairing with fully edited A6.

After a period of culture adaptation, AnTat1.1 90:13 WT/L262P$\gamma$ cell lines showed a decrease in their $\Delta\psi_m$ to around 70% of WT/WT$\gamma$ levels (Fig. 2.19C). WT/L262P$\gamma$ (AK) cell lines showed an increase in their $\Delta\psi_m$, with the $\Delta\psi_m$ of cell line WT/L262P$\gamma$ (AK) 1 being 20% higher than that of WT/WT$\gamma$ cell lines, and WT/L262P$\gamma$ AK 2 being 30% lower than WT/WT$\gamma$ cell lines. L262P/L262P$\gamma$ clones 1 and 2 also showed approximately a 30% lower $\Delta\psi_m$ than WT/WT$\gamma$ cell lines. L262P/L262P$\gamma$ clone 3 had approximately the same $\Delta\psi_m$ as the WT/WT$\gamma$ cell line.
2.4 Discussion

It is understood that point mutations in the C-terminus of ATPase γ provide at least some isolates of naturally occurring Dk subspecies of *T. b. brucei* with their ability to survive without kDNA (Dean et al., 2013). From structural studies in yeast expressing subunits with similar mutations we can infer that these mutations are able to alter the axis of rotation of ATPase γ within the F₁-α/β core, preventing F₁ fuelling the movement of proton through F₀ (Wang et al., 2007, Arsenieva et al., 2010). In the presence of a functionally uncoupled F₁F₀ ATPase, the proton pore F₀ is no longer used to generate Δѱm. The electrogenic action of AAC is used, with F₁-ATPase acting to maintain the ATP/ADP concentration gradient between the matrix and the cytosol.

The aim of this work was to further understand the molecular mechanism of the compensatory L262Pγ mutation. Does functional uncoupling of the F₁F₀ complex take place in the presence of the L262Pγ mutation? Can the compensatory mutation cause F₁F₀ structural disruption?

F₀ is a proton pore through the inner mitochondrial membrane. If the F₁F₀ complex is functionally uncoupled, F₁ no longer controls the direction of proton movement by driving the generation of Δѱm. Hence F₀ causes a proton leak, uncontrollably dissipating the proton accumulated in the IMS (Mueller, 2000). In yeast, the proton leak forces selection for cells with partial or complete deletions in mtDNA (Chen and Clark-Walker, 1993, Lai-Zhang et al., 1999), and thus removing the intact proton pore F₀ from the inner mitochondrial membrane. *T. brucei* however seems to retain its kDNA in the presence of an uncoupling ATPase γ mutation. How then does *T. brucei* tolerate generation of a proton leak?
To understand the implications of i) the L262P\textgamma mutation and ii) the loss of kDNA on the structure and function of the F\textsubscript{1}F\textsubscript{0} ATPase, we analysed the subunit composition and the structure of the F\textsubscript{1}F\textsubscript{0} complex in multiple clones expressing the mutation before and after acriflavine treatment. The effect of the mutation on kDNA and expression of pre-edited and edited transcripts was assessed by amplification of maxicircle genes from gDNA and cDNA. The level of F\textsubscript{1}F\textsubscript{0} functional uncoupling and dependency on F\textsubscript{1}-ATPase activity was examined by oligomycin and azide sensitivity assays, respectively. We also investigated the impact of the L262P\textgamma mutation and status of kDNA on the relative level of $\Delta\psi$m. Table 2.4 summarises the data presented in this chapter.
Table 2.4 Summary of data from Chapter 2

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Fully Edited A6(^1)</th>
<th>Pre-edited A6 level(^2)</th>
<th>Oligomycin sensitivity compared to WT(^3)</th>
<th>Azide sensitivity compared to WT(^4)</th>
<th>F, F(_o) coupled?(^5)</th>
<th>Level of F, F(_o) complex(^6)</th>
<th>F(_{1}) (\pm) x (^7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT/WT A</td>
<td>Y</td>
<td>WT</td>
<td>100%</td>
<td>10%</td>
<td>Y</td>
<td>WT</td>
<td>-</td>
</tr>
<tr>
<td>WT/L262P A</td>
<td>Y</td>
<td>WT</td>
<td>0%</td>
<td>10%</td>
<td>Y</td>
<td>WT</td>
<td>-</td>
</tr>
<tr>
<td>L262P/L262P A</td>
<td>N</td>
<td>Slightly increased</td>
<td>0%</td>
<td>50%</td>
<td>N</td>
<td>-</td>
<td>Y</td>
</tr>
<tr>
<td>L262P/L262P B</td>
<td>N</td>
<td>Slightly increased</td>
<td>0%</td>
<td>70%</td>
<td>N</td>
<td>-</td>
<td>Y</td>
</tr>
<tr>
<td>L262P/L262P C</td>
<td>Y</td>
<td>Slightly increased</td>
<td>0%</td>
<td>0%</td>
<td>Y</td>
<td>WT</td>
<td>Y</td>
</tr>
<tr>
<td>L262P/L262P 1</td>
<td>Y</td>
<td>Decreased</td>
<td>ND</td>
<td>50%</td>
<td>Y</td>
<td>Decreased</td>
<td>-</td>
</tr>
<tr>
<td>L262P/L262P 2</td>
<td>Y</td>
<td>Decreased</td>
<td>ND</td>
<td>20%</td>
<td>Y</td>
<td>Decreased</td>
<td>-</td>
</tr>
<tr>
<td>L262P/L262P 3</td>
<td>N</td>
<td>x</td>
<td>ND</td>
<td>85%</td>
<td>N</td>
<td>x</td>
<td>-</td>
</tr>
<tr>
<td>WT/L262P (AK)</td>
<td>N</td>
<td>x</td>
<td>0%</td>
<td>100%</td>
<td>N</td>
<td>x</td>
<td>-</td>
</tr>
<tr>
<td>L262P/L262P (AK)</td>
<td>N</td>
<td>x</td>
<td>0%</td>
<td>90%</td>
<td>N</td>
<td>x</td>
<td>-</td>
</tr>
</tbody>
</table>

\(^1\) At time of submission, the presence of fully edited A6 was assessed by size as judged by gel electrophoresis, not by sequencing  
\(^2\) Assessed by qRT-PCR. \(x\) = not detected  
\(^3\) After 2 weeks of growth. ND = not yet determined. 100% = average sensitivity levels in WT cells, 0% = average sensitivity levels in AK cells  
\(^4\) After 2 weeks of growth. 10% = average approximate sensitivity levels in WT cells  
\(^5\) Assessed by the presence of bands on hrCNE gels able to be detected by both antibodies specific to F\(_1\) and F\(_o\) subunits  
\(^6\) The density of the bands in \(^5\) assessed by eye. \(x\) = not detected  
\(^7\) Assessed by the presence of a band detectable by antibodies specific to F\(_1\) on hrCNE gels larger than 720 kDa
From Table 2.4 we can see that the results agree with our hypotheses to a certain extent. As cells expressing L262Pγ are as oligomycin resistant as AK cell lines, the L262Pγ mutation functionally uncouples the enzyme. The data agrees with the idea that a proton leak is generated in the presence of this compensatory ATPase γ mutation as A6 mRNA levels are affected in the presence of L262Pγ in most cell lines. Some L262Pγ expressing cell lines have been selected for that seem to alter A6 levels by decreasing the editing capacity of A6 (L262P/L262Pγ clone A and B). Alternatively some cell lines have decreased levels of pre-edited and edited A6 (L262P/L262Pγ clones 1 and 2), and one clone, L262P/L262Pγ clone 3 had selected for the loss of the full maxicircle. However, in disagreement with the hypothesis, some cell lines expressing L262Pγ do retain edited A6 and still have a detectable F₀ complex, as discussed in detail below.

2.4.1 kDNA loss causes disruption of the F₀ moiety

In the absence of kDNA, a stable F₀ moiety is not present. In 427SM WT/L262Pγ (AK) cell lines we see an F₁ band and an F₁+x band, whereas F₁F₀ monomer and multimers are seen in WT/WTγ and WT/L262Pγ cell lines (Fig. 2.14). Our data suggests that, like in yeast, bacteria and mammalian cells (Hadikusumo et al., 1988, Tzagoloff et al., 2004, Hermolin and Fillingame, 1995, Wittig et al., 2010b), subunit A6 is required to complete the stable assembly of the F₁F₀ complex. This is in agreement with data presented in (Hashimi et al., 2010), where indirect A6 knockdown was seen to cause F₁F₀ structural disruption in PCF T. b. brucei. The proton pore of F₀ is generated by the subunit c/a interface; A6 is thought to be added to the complex last to complete the pore in order to prevent dissipation of Δψm (Tzagoloff et al., 2004).
Additionally in cell lines lacking A6 (AK cell lines and L262P/L262Pγ clone A and B) loss of A6 causes a loss of Fo-Tb1 and stator-Tb2 subunits (Fig. 2.13). Disruption of the Fo moiety therefore either inhibits expression of Tb1 and Tb2 subunits via feedback loop or causes an increase in degradation of these unnecessary or unassociated subunits. The latter is more likely. Incomplete assembly of the F1Fo complex due to the deletion of subunits in yeast causes an increase in susceptibility to the degradation of other subunits at the protein level (Lefebvre-Legendre et al., 2005). This has not been tested in T. b. brucei, but upon RNAi of T. brucei ATPase subunits, a decrease in other subunits is seen at the protein level (Schnaufer et al., 2005, Brown et al., 2006).

2.4.2 The L262Pγ mutation causes functional uncoupling of the F1Fo complex

Oligomycin sensitivity assays verify that, in 427SM cell lines, in the presence of at least one L262Pγ allele there is functional uncoupling of the F1Fo complex (Fig. 2.17). This means that the ATP hydrolysis activity of F1 no longer drives the movement of protons through Fo into the IMS to manufacture Δψm. It is the presence of L262Pγ that causes independence from Fo, not the loss of kDNA. Even in the presence of kDNA, and therefore Fo, cells expressing L262Pγ can utilise the AAC to generate Δψm instead of using the Fo proton channel. The EC50 values for WTγ and L262Pγ expressing cells obtained are in the same order of magnitude as the cell lines assessed in Dean et al., 2013.
2.4.3 The L262Pγ mutation also causes a proton leak

Structural uncoupling, that is the physical separation between the $F_1$ and $F_o$ moieties, seems to be selected for as a consequence of the production of a proton leak in the presence of L262Pγ. As L262Pγ functionally uncouples the $F_1F_o$ complex, ATP hydrolysis no longer controls the direction of proton movement through $F_o$. Protons therefore are able to flow unchecked through $F_o$ from the IMS into the negatively charged matrix, dissipating the essential $\Delta \psi_m$. There appears to be a selection for cells that are able to close this proton leak. L262P/L262Pγ clones A and B do not express fully edited A6, and with the complex incomplete without A6, the $F_o$ moiety is not detected. In the lack of acriflavine treatment, these cells may have been selected as they may be missing certain minicircle classes that encode A6 gRNAs, preventing the production of edited A6.

In WT/L262Pγ cell lines, a mixture of complexes are present, one containing WTγ and one containing L262Pγ. The mutated complex will be able to generate a proton leak, as L262Pγ is present in the same complex as A6. There seems to be no loss of kDNA from these cell lines (which could close the proton leak), hence the proton leak generated by L262Pγ-containing complexes in these cells must be compensated for by the manufacture of $\Delta \psi_m$ via the WTγ-containing complexes. The WTγ-containing $F_1F_o$ complex must generate sufficient $\Delta \psi_m$ by the more efficient classical $F_1F_o$ method to compensate for the proton leak. In the presence of oligomycin, there is inhibition of the $\Delta \psi_m$ generated by WTγ-containing complexes, but also inhibition of the proton leak generated by mutated complexes, allowing these cell lines to manufacture $\Delta \psi_m$ via the alternative method.

Unlike yeast, where the proton leak causes selection for cells with a partial or complete loss of mitochondrial DNA in order to prevent $F_o$ forming, the presence of RNA editing provides T. b. brucei with more flexibility for the creation of cells that can be selected for their ability to tolerate the proton leak; there are therefore more ways in which cells can deal with the
proton leak. Full deletion of the maxicircle is not required, but can occur (AnTat 1.1 90:13 L262P/L262ϕ clone 3, Figure 2.4). The requirement for gRNAs to edit maxicircle transcripts provides an added level of regulation of mitochondrial DNA expression, hence allowing selection for cells that have lost or perhaps mutated A6 gRNAs. Selection for alteration in RNA editing has been implicated in maintaining cellular viability in other organisms. In primates the action of adenosine deaminase catalyses deimination of adenosine to inosine (George et al., 2014). This affects the coding of RNA, as I is read as G by ribosomes, spliceosomes and polymerases, and the stability of the RNA structure. Editing thus can affect transcript level, translation efficiency and protein structure and function. Mediated by editing, increased expression of certain oncogenes and decreased expression of certain tumour suppressor genes are thought to have a role in human cancer cell evolution (Paz et al., 2007, Chen et al., 2013). In plants, the chloroplast genome is post transcriptionally regulated by RNA editing of C to U bases (Shikanai, 2006). Inactivation of chloroplast NADH dehydrogenase by inhibiting RNA editing occurs upon challenge with fungal pathogens, and seems to have a role in innate immunity in plant cells (Garcia-Andrade et al., 2013).

427SM L262P/L262ϕ clone C is an exception. The F₁F₀ complex in this cell line is functionally uncoupled (Fig. 2.17). However Tb1, Tb2 and A6 are all expressed (Fig. 2.13, 2.15, 2.16) and seem to be incorporated into complex V (Fig. 2.14), generating F₁F₀ complexes. This suggests that it is the loss of subunit A6 that causes structural uncoupling of the F₁F₀ complex, and this causes loss of other subunits that are no longer required by or associated with the complex. F₁F₀ structural uncoupling can therefore be a secondary consequence of L262ϕ, depending on the status of A6. How L262P/L262ϕ clone C cells are dealing with the proton leak is not certain. This cell line does not have a slow growth phenotype (Fig. 2.6C), or loss of kDNA (Fig. 2.3) hence the lethal proton leak is not present. One option is that A6 is present, but is mutated, preventing it functioning in proton transfer.
In this situation, the proton leak could be abolished, or hindered. Sequencing the fully edited sequence of A6 from cDNA will uncover whether it is mutated. The c ring, the other component of the Fo proton pore, could also be mutated, preventing proton conductivity through Fo. This also needs to be analysed by sequencing.

Multiple L262P/L262Py transfectants were readily obtained using our transfection protocol, suggesting that there is a high frequency in which parasites emerge that can tolerate the proton leak. As defects in nuclearly-encoded proteins could also lead to the loss of Fo, for example the loss of subunit c, the fact that we have not yet observed nuclear defects in any of the L262P/L262Py clones analysed could imply that the likelihood for genetic changes in kDNA and its transcripts is higher than in nuclear DNA.

2.4.4 How does L262Py impact Δψm generation?

The ‘classical’ method of generating Δψm uses a coupled F1Fo ATPase complex. This involves the translocation of 3 protons through Fo for every ATP molecule hydrolysed by F1. The ‘alternative’ method functioning in the presence of uncoupled F1Fo complexes only translocates the equivalent of one net positive charge through the AAC for every ATP hydrolysed by F1, hence is less efficient in producing Δψm (Figure 1.8). Hence cells are more azide sensitive in the absence of kDNA and A6 (Fig. 2.18). In accordance with this, Δψm measurements suggest that when generated via the alternative method, the Δψm may be lower than when the classical Fo-dependent method can be used (Fig. 2.19). We are limited in the strength of the conclusions we can draw from this data as these measurements have only been performed once. It should also be stated that Δψm generation also depends on ATP/ADP ratios in the cytosol/IMS and matrix, and the kinetics of the AAC, parameters that have not yet been studied.
427SM L262P/L262Pγ cell lines are more azide resistant than their equivalent AK cell lines (Fig 2.18), despite the fact that they have uncoupled F_{1}F_{0} complexes (Fig. 2.17). Equally, Δψ\textsubscript{m} also seems higher in these L262P/L262Pγ cell lines compared with in AK cell lines (Fig. 2.19). This presents a conundrum: does the presence of at least some kDNA contribute an additional method of generating Δψ\textsubscript{m}, making these cells less dependent on F\textsubscript{1} than AK cells? Complex I is a possibility as complex I may be present in BSF (Surve et al., 2012) and ND7 continues to be edited in L262P/L262Pγ cell lines (Fig. 2.15A). However, this would mean that these cells should be less dependent on the AAC than L262P/L262Pγ (AK) cell lines. Assays to determine sensitivity to bongkrekic acid (a specific inhibitor of the AAC) were not performed here, but in Dean et al., 2013, cells expressing only L262Pγ are as bongkrekic acid sensitive as their AK equivalents; kDNA would therefore not be expected to contribute to Δψ\textsubscript{m} in these cell lines.

Another possibility is that in these cell lines, there are some complexes still tethered to the membrane. Increasing the proximity of F\textsubscript{1}-ATPase to the AAC in the inner mitochondrial membrane could prove important to increase the efficiency of Δψ\textsubscript{m} generation (Dean et al., 2013, Subrtova et al., 2015). Supporting this, L262P/L262Pγ clone C cells still retain F_{1}F_{0} complexes, meaning that F\textsubscript{1} is tethered to the membrane via an intact F\textsubscript{0} moiety. In L262P/L262Pγ clones A and B, where edited A6 is low or absent, we only see an F_{1}+x band (Fig. 2.14, 2.15). A band of comparable size has been seen in both WT BSF and WT PCF (Subrtova et al., 2015), and was hypothesized to be F_{1}+ c ring (Zikova et al., 2009), as in other systems (Meyer et al., 2007, Appleby et al., 1999). A hydrophobic c ring would also allow membrane tethering of the F\textsubscript{1} complex (Appleby et al., 1999). Azide resistance is higher in L262P/L262Pγ clone C than in clones A and B (Fig. 2.18), possibly reflecting a higher stability of this membrane tethering in clone C cells or a difference in the amount of complex tethered to the membranes, thus increasing the efficiency of Δψ\textsubscript{m} generation in this clone. The presence of a slightly lower Δψ\textsubscript{m} in L262P/L262Pγ clone C compared with
clones A and B (Fig. 2.19) may reveal that, despite a possibly mutated inefficient A6 subunit, clone C cells could be still suffering with some slight proton leak, causing some dissipation of the $\Delta \Psi m$ generated. The $\Delta \Psi m$ analysis does require repeating to see if the lower $\Delta \Psi m$ of clone C is significant, however this hypothesis could be verified by proton conductivity assays to see if a proton leak is present in clone C cells (Serviddio and Sastre, 2010, Affourtit et al., 2012).

2.4.5 Do the same conclusions apply to pleomorphic cell lines?

In AnTat1.1 90:13 L262P/L262P$\gamma$ clone 3, cells have been selected for that have lost the maxicircle (Figure 2.4). In accordance with 427SM AK cell lines, no F$_1$F$_0$ complexes can be detected. This is consistent with the idea that a proton leak is caused by the uncoupling of the F$_1$F$_0$ complex by L262P$\gamma$. It seems that the loss of the maxicircle in the presence of homozygous L262P$\gamma$ provides a benefit to the cell in terms of $\Delta \Psi m$. Compared to the low $\Delta \Psi m$ of WT/L262P$\gamma$ (AK) cells, the $\Delta \Psi m$ of L262P/L262P$\gamma$ clone 3 is maintained at a level comparable to WT/WT$\gamma$ expressing cells (Fig. 2.19A).

AnTat1.1 90:13 L262P/L262P$\gamma$ clones 1 and 2 seem to have normal levels of kDNA (Figure 2.4). They also have detectable F$_1$F$_0$ complexes (Figure 2.14B). There is a slight noticeable decrease in the levels of F$_1$F$_0$ detected by hrCNE compared with WT/WT$\gamma$ and WT/L262P$\gamma$ cell lines, however without a loading control this observation cannot be verified. We do not have verification that the L262P$\gamma$ mutation functionally uncoupled the F$_1$F$_0$ complexes; the slower growth of these cell lines means that the Alamar Blue assay is not suitable to assess if a drug sensitivity change is due to the mutation itself. Hence our hypothesis that the L262P$\gamma$ mutation uncouples the F$_1$F$_0$ complex and generates proton leak remains to be investigated.
There is no obvious identification of the method in which the cells selected for deal with the proton leak, if they do. In L262P/L262Pγ clone 1 and 2 cells, the levels of pre-edited A6 maxicircle transcripts are decreased compared to WT/WTγ cell lines (Fig. 2.15B). In order to combat the proton leak, it could be postulated that cells have been selected that have less stable A6 transcripts, causing their premature degradation. The low level of edited A6 expression could be limiting the presence of coupled F₁Fₒ complex. Clone 1 seems to have lower edited A6 levels than clone 2 (Fig. 2.15B). This could explain why azide sensitivity is higher for L262P/L262Pγ clone 1 (Fig. 2.18): lower fully edited A6 levels could cause less F₁Fₒ complexes to be present than in clone 2. If our hypothesis that membrane attachment increases the efficiency of Δψm generation is correct, this would mean that a greater number of F₁-ATPase moieties are less efficiently tethered to the inner mitochondrial membrane than in clone 2, hence the alternative method of Δψm generation would be less efficient in clone 1 cells. The presence of potentially more F₁Fₒ complexes in clone 2 could explain why the Δψm of this cell line is lower compared to clone 1 (Fig. 2.19B): a proton leak could be more apparent in this cell line.

In the instance where the manufacture of F₁Fₒ complexes is being limited, an accumulation of F₁ moiety would be expected, as the F₁ moiety is assembled independently of the Fₒ moiety (Rak et al., 2011). The levels of F₁ cannot yet be verified; at the time of submission anti-β had not yet been used to probe hrCNE gels to analyse F₁-containing complexes in AnTat1.1 90:13 cell lines. Hence this hypothesis remains to be tested.
2.4.6 The effect of prolonged culture on the compensatory mechanism

For most clones, sensitivity to ATPase inhibitors and magnitude of $\Delta \Psi m$ changed after a longer period of time in culture (Figs. 2.17, 2.18, 2.19). The phenotypes discussed above are therefore not stable, with further adaptations occurring over time. Other assays, analyzing complex structure, kDNA editing capacity and kDNA content, were carried out with cells grown between 2-4 weeks in culture, hence results from these assays do not necessarily represent the status of the cells after 2 months of culturing. Some of these results therefore cannot be explained without further study. For example after 2 months of adaptation to culture, the levels of oligomycin resistance of most L262P$\gamma$-expressing cell lines has increased >20 fold, whereas the sensitivity of WT/WT$\gamma$ cells has not changed. Azide resistance has in general decreased in all cell lines, suggesting that the efficient generation of $\Delta \Psi m$ is more dependent on $F_1$-ATPase activity over time. Some cell lines show a greater increase in sensitivity than others.

One clear anomaly to this pattern is L262P/L262P$\gamma$ clone C. The oligomycin ED$_{50}$ of this cell line stayed approximately the same during adaptation. For some reason after 2 months in culture, this cell line is no longer as resistant to oligomycin as other L262P$\gamma$-expressing clones. As this clone has $F_1F_o$ complexes, and fully edited A6, this could be limiting the ability of this clone to become more oligomycin resistant. This cell line has a higher resistance to azide at an earlier time point (Fig. 2.18A). This could suggest that more $F_1F_o$ complexes existed at an earlier time point in this cell line, possibly tethering $F_1$ to the membrane for efficiency, whereas at a later time point there may be less $F_1F_o$ complexes present due to structural instability of the complex in the presence of L262P$\gamma$ with A6 present. We do see $F_1^{+x}$ complexes in this cell line by approximately one month in culture (Fig. 2.14) hence perhaps a higher proportion of $F_1^{+x}$ complexes are present by 2 months in
culture. These may be less efficiently tethered to the membrane, causing increased $F_1$ dependence for $\Delta\psi_m$ generation. This theory requires experimental verification by performing complex structural assays with this cell line and other related cell lines at 2 weeks and 2 months of culturing.

By 2 months in culture, WT/L262Pγ B cell line has a reproducibly higher level of resistance to oligomycin than the AK version of the same cell line (Fig. 2.17A). This cell line is not dependent on $F_o$ to generate $\Delta\psi_m$, and also has a much lower azide resistance than the equivalent WT/WTγ cell line by 2 months, compared to after just 2 weeks of culturing (Fig. 2.18A, B). This could mean that by 2 months, this cell line may have lower levels of coupled $F_1F_o$ complexes, due to a decrease in WTγ expression. A higher level of proton leak caused by a higher proportion of uncoupled complexes may have selected for cells with kDNA loss. The structural uncoupling that follows could have caused a loss of stable tethering of $F_1$ complexes to the membrane, causing less efficient generation of $\Delta\psi_m$ using the alternative method. This theory also requires experimental validation by performing kDNA expression, kDNA editing and complex structure assays with this cell line and other related cell lines at 2 weeks and 2 months of culturing.

### 2.4.7 What else affects $\Delta\psi_m$ generation?

It would be useful to assess the extent to which oligomycin and azide inhibit generation of $\Delta\psi_m$ in these cell lines. For example, the AAC function in WT/WTγ cells is also electrogenic, contributing to $\Delta\psi_m$ to some extent. We also do not know the extent to which azide affects the ATP/ADP ratio inside the mitochondrion. There could for example be other mitochondrial ATPases insensitive to azide that are able to reestablish the ATP/ADP concentrations across the inner mitochondrial membrane that drives the AAC. There could
also be proteins producing ATP that fuel F$_1$-ATPase activity: for example mitochondrial ASCT is thought to contribute to acetate formation in BSF (Mazet et al., 2013) and, coupled to SCoAS, can produce ATP. These caveats could explain why the EC$_{50}$ values between clones of the same genotype are different. Equally we do not know the rates of uptake of these compounds in these adapted cell lines, or the rate of resazurin uptake or metabolism.

Culture adaptation evidently does affect Δψm generation. TMRE assays show that AnTat1.1 90:13 cell lines seem to stabilise their Δψm at a level around 75% of the WT/WTγ cell line in the presence of L262Pγ (Fig. 2.19Aii). We do not have enough information to understand why the Δψm of both WT/L262Pγ clones was higher than WT/WTγ cell lines initially, or why this Δψm decreased over time. Equally, WT/L262Pγ (AK) cell lines seem to increase their Δψm over time to become more comparable to WT/WTγ cell lines (Fig. 2.19B, C). Unlike the petite mutants of yeast which adapt to survival with low Δψm (Dunn and Jensen, 2003), AK T. brucei cells seem to adapt to produce a Δψm closer to WT. Indeed T. b. evansi and T. b. equiperdum strains have been shown to have comparable Δψm to a T. b. brucei strain (Lai et al., 2008). The molecular mechanism for this is not understood, but can be hypothesised. For example, an increase in AAC expression at the transcriptional or translational stage could allow Δψm to increase over time. Mutation of F$_1$ has also been shown to hyperactivate the moiety, altering the ATP/ADP ratio in the mitochondrial matrix, increasing Δψm (Francis et al., 2007). Additionally, the Δψm of yeast cells lacking mtDNA can be increased by the deletion of certain mitochondrial phosphatases, with a mitochondrial kinase found to be vital for cell proliferation (Garipler et al., 2014). This suggests that the phosphorylation state of mitochondrial ATPases, AAC or import machinery can also influence their efficiency or activity, in turn affecting the Δψm.
2.4.8 Does $\Delta \psi_m$ affect the growth of cells?

These results suggest that $\Delta \psi_m$ can limit cellular growth rate. $\Delta \psi_m$ is essential for the import of proteins into the mitochondrion to allow mitochondrial functions to occur (Gasser et al., 1982, Schleyer et al., 1982, Pfanner and Neupert, 1985, Pfanner and Neupert, 1990, Eilers and Schatz, 1988). Despite not being considered a site of net ATP production, the mitochondrion functions in many processes in BSF cells (Verner et al., 2015). We also know that maintenance of $\Delta \psi_m$ is essential in Dk cells (Schnaufer et al., 2005). A $\Delta \psi_m$ lower than that of WT/WT$\gamma$ cells can sustain growth at the same level as WT/WT$\gamma$ cells (Fig. 2.6, 2.19B), but it seems reasonable to assume that a threshold level of $\Delta \psi_m$ is required for protein import, and therefore for cell growth. Indeed, from studies in yeast, it seems that production of a negative mitochondrial matrix by the $\Delta \psi_m$ is the primary requirement of protein import (Martin et al., 1991), explaining why the electrogenic AAC can fuel protein import. A $\Delta \psi_m$ of 40-60 mV is thought to be required for mitochondrial protein import (Pfanner and Neupert, 1985, Martin et al., 1991). The $\Delta \psi_m$ has been estimated to be 130 mV in BSF cells (Vercesi et al., 1992). If these estimates are consistent in our strains, the 70% decrease in $\Delta \psi_m$ seen in WT/L262P$\gamma$ (AK) cell lines (Fig. 2.19B) would only just reach the lower limit of the threshold $\Delta \psi_m$ required for mitochondrial protein import, agreeing with this hypothesis. This could be the reason why these AK cell lines have a slightly lower growth rate than other cell lines (Fig. 2.6, 2.7).

Equally, Antat1.1 90:13 WT/L262P (AK) cell lines do grow slower than other cell lines after they are initially thawed and take a few weeks of in vitro growth to establish the same growth rate as other cell lines (data not shown). This is unlike petite mutants of yeast, which despite adaptation to life without mtDNA, retain a lower growth rate (Ephrussi et al., 1949).
2.4.9 Suggestions for follow up studies

There are several experiments needing to be done to verify and supplement these conclusions and hypotheses. Analysis of ATPase complexes in AnTat1.1 90:13 cells by hrCNE has been performed only once and requires repeating, and analysis with anti-β is required to see which additional complexes contain F₁. An F₁-x complex is present in all 427SM L262P/L262Pγ cell lines analysed so far (Fig. 2.14), and would be expected for AnTat1.1 90:13 L262P/L262Pγ cell lines.

Oligomycin sensitivity of the AnTat1.1 based cell lines remains to be determined. Measuring sensitivity to slow acting drugs such as oligomycin with the Alamar Blue assay is not possible for these cells. They are very sensitive to cell density and die above 1x10⁶ cells/ml. A seeding density of 5x10³/ml and growth for 96 hrs in the drug will mean that the cells will reach this density. It was also noted that Antat1.1 90:13 require 24 hrs to produce a robust fluorescent signal, compared to 4 hrs for 427SM, suggesting that these cells take up or metabolise resazurin more slowly. Azide sensitivity could be tested (Fig. 2.18C), probably due to the compound working more quickly than oligomycin.

The assay could be optimised by using a lower seeding density, or another method to determine whole cell oligomycin and azide sensitivity will have to be used. Previously sensitivity to these drugs have been performed by growth assays over several days (Schnaufer et al., 2005). This illustrates qualitative differences in drug sensitivity, but not quantitative differences. A propidium iodide (PI) assay may be usable to calculate EC₅₀ (Gould et al., 2008). PI is fluorescent upon binding to DNA and cannot cross membranes, therefore is an indicator of dead cells. EC₅₀ values are comparable to those generated by the Alamar Blue assay. The PI assay requires less incubation time, hence with optimisation of seeding densities to avoid reaching a density of >1x10⁶ cells/ml during the course of the experiment, this assay could be more suitable.
2.4.9.1 Kinetic analysis on F₁Fₒ-ATPase containing L262Pγ

A piece of information missing from this analysis is the Kₘ for ATP of *T. brucei* F₁Fₒ-ATPase with the L262Pγ mutation. It has been shown previously that when *K. lactis*, a petite negative yeast, expresses C-terminal mutated γ subunits, Kₘ is decreased, thought to be an adaptation for growth during O₂ starvation (Clark-Walker, 2003). When expressed in *K. lactis*, the equivalent of the L262Pγ mutation halved the Kₘ for ATP compared to WT cells (Schnaufer et al., 2005), resulting in a greater affinity for ATP at low concentrations. Therefore a lower Kₘ in L262Pγ expressing *T. brucei* cells is expected, which could allow sufficient hydrolysis of ATP to generate ADP to fuel Δψₘ by the electrogenic AAC in DK trypanosomes.

To investigate this possibility, Kₘ should be measured. Kₘ measurements require an assessment of F₁-ATPase activity in the presence of varying concentrations of ATP substrate. ATPase activity can be measured in a multitude of ways, but requires extremely large numbers of BSF cells due to the lower expression levels of F₁Fₒ-ATPase in BSF mitochondria. The activity of F₁-ATPase in cells can be investigated via the Sumner assay, allowing measurement of the release of phosphate during the ATP hydrolysis reaction (Schnaufer et al., 2005). Treatment with sodium azide would allow an assessment on what proportion of mitochondrial ATPase activity was F₁-specific.

More purified samples are required to provide a quantitative analysis of F₁-ATPase Kₘ. The Zikova lab has a protocol for purification of F₁-ATPase by chloroform extraction from hypotonically-lysed PCF cells (Gahura et al., unpublished). F₁-ATPase activity can then be measured by the ATP regeneration assay (Pullman et al., 1960). This protocol still requires adaptation for use on BSF cells.

ATPase activity can be qualitatively assessed by native gel fractionation and in-gel staining with lead nitrate (Zikova et al., 2009). This assay was attempted in this work (data not
shown), but the technique was not sensitive enough to detect signal from mitochondrial extracts of 2x10^8 BSF cells in hrCNE. 2x10^9 cells have been shown to produce a signal from BSF cells (Subrtova et al., 2015), hence more cells are required for this analysis.

### 2.4.9.2 Additional bioenergetic parameters

Safranine O could be used to quantify the Δψ in the presence of L262Pγ and in the absence of kDNA (Figueira et al., 2012). Safranine is a membrane permeant cationic dye that has a characteristic absorption spectra. When taken up into the matrix of energized mitochondria, the high concentration causes stacking of the safranine planar rings and quenching of the fluorescence signal. This technique measured the Δψm to be 130 mV in BSF *T. brucei* (Vercesi et al., 1992).

The effect of azide, bongkrekic acid and oligomycin on Δψ should also be quantified using this method. This will allow a full inspection of how affected Δψ is by the introduction of these drugs and consequently how Δψ is maintained in each cell line. It is evident this needs to be done on a clone by clone basis, as clones of the same genotype have adapted in different ways to the introduction of L262Pγ alleles. Culture adaptation has been seen to affect azide and oligomycin sensitivity and the level of Δψ generated (see Fig 2.17, 2.18, 2.19), hence an investigation on the effect of drug sensitivity on Δψ over time would also be useful.

### 2.4.9.3 Further subunit analysis of F₅-containing complexes

F₁F₅-ATPase complexes can be purified via TAP-tagged subunits and the bands detected by hrCNE would be analysed by SDS-PAGE and mass spectrometry. Examination of the subunit composition of each band would detail the effect of the L262Pγ mutation and loss of kDNA on the structure of F₁F₅-ATPase. Potential problems include the purification of incomplete complexes due to genuine low affinity interactions, the hydrophobicity of some
subunits, their membrane association and the lack of tryptic sites required for cleavage for mass spectrometry. For example, subunit \( a \) has not yet been confirmed as being part of \( T. brucei \) F1F\(_0\)-ATP synthase by mass spectrometry, and subunit \( c \) has only been detected in one TAP-tagged complex, possibly due to its small size (Zikova et al., 2009). This is a common problem (Wittig et al., 2010b).

In particular, the constituents of the \( F_1+x \) band require identification to understand the composition of the complex maintaining the \( \Delta \psi m \) in cells expressing homozygous L262P\(_{\gamma} \) and in AK cells. One promising candidate is the \( c \) ring, as discussed. A \( F_1-c \) ring band above the \( F_1 \) monomer is seen in bovine mitochondrial ATP synthase when separated using BNE (Meyer et al., 2007). The \( c \) ring is the first \( F_0 \) subunit to stably associate with \( F_1 \) during the assembly of the ATP synthase in yeast and \( E. coli \) (Hermolin and Fillingame, 1995, Tzagoloff et al., 2004). Interestingly, in human \( p^0 \) cells an active \( F_1-c \) ring complex is seen as the largest stably-assembled complex (Carrozzo et al., 2006). This analysis was performed under similar conditions (BNE) to the analysis here. In subsequent work, using the gentler separation technique CNE (Wittig and Schagger, 2005, Wittig and Schagger, 2008a), this \( F_1-c \) band was shown to be a breakdown product of a larger active ATPase complex that contained other \( F_0 \) subunits, despite the absence of \( A6 \) and \( A6L \) (Wittig et al., 2010b). Using gentler native electrophoresis conditions could yield the same result for the ATPase complex in L262P/L262P\(_{\gamma} \) and AK cells; however, of the \( T. brucei \) \( F_0 \) moiety, only subunits \( c, a \) and \( Tb1 \) have been identified (Hashimi et al., 2010, Zikova et al., 2009, Gulde et al., 2013) (Subrtova et al., unpublished). Only anti-\( Tb1 \) exists for analysis of bands. Numerous \( T. brucei \)-specific proteins are found associated with the complex but require characterisation to be proved functional subunits of the \( F_1F_0 \) complex (Zikova et al., 2009).

Subunit \( c \) antibodies have been published (Gulde et al., 2013) but are no longer available, hence hrCNE gels cannot be probed for the presence of \( c \) subunits in \( F_1F_0 \)-ATPase bands. A tagged \( c \) subunit could aid detection via western blot. The presence of three \( c \) subunit genes
of uncertain functional significance makes tagging this protein difficult. The significance of having three $c$ subunits is not understood, and the stoichiometry of the $c$ ring is not known in the BSF (Gulde et al., 2013). Additionally, adding a tag to part of the $F_o$ proton pore could alter the ability of the $c$ ring to rotate, which could affect the activity of the complex in an unpredictable manner. If this $F_{1}+x$ band is $F_{1}-c$ ring, $c$ subunit RNAi would reveal this by reducing the molecular size of this band. As the three $c$ subunits share regions of homology (Gulde et al., 2013), a general RNAi construct could be made to target all three subunits simultaneously.

A separate $F_1$ moiety was not detected in homozygous L262P$\gamma$ cells, and instead seemed to be preferentially found in an $F_{1}+x$ complex with other (currently unidentified) subunits (Fig. 2.14). Is there a functional requirement for an $F_{1}+x$ complex in cell lines that do not have coupled $F_1F_o$ complexes? The proton pore is incomplete in the absence of fully edited subunit $a$, therefore the $c$ ring could be a method of anchoring the $F_1$ to the inner mitochondrial membrane via the central stalk. In mammalian cells lacking mtDNA, the $F_{1}+c$ ring complex does seem to be membrane associated (Appleby et al., 1999, Norais et al., 1991). $c$ subunit knockdown would reveal if, in the presence of L262P$\gamma$, the subunit is required for ATPase activity, more efficient $\Delta\psi_m$ generation and cell viability.

A faint Tb2 band can be detected in 427SM L262P/L262P$\gamma$ cell lines (Fig. 2.13) and some DK strains (Subrtova et al., 2015), and seems to be associated with the $F_{1}+x$ band (Fig. 2.14). Therefore some complexes could involve the $c$-ring being attached to $F_1$ additionally via the stator. Tb2 seems to be important for normal growth of DK cells, and was hypothesised to prevent the still active $F_1$ from separating from the inner mitochondrial membrane, allowing increased AAC efficiency in the absence of mitochondrial cristae (Subrtova et al., 2015). Having $c$ ring attachment via both the central stalk and stator could increase the stability of the complex in the membrane and promote more efficient ATPase activity. Knocking out Tb2 in cell lines WT/WT$\gamma$ and L262P/L262P$\gamma$ could highlight
whether membrane attachment of F$_1$+x via the stator is required for $\Delta \psi_{m}$ generation and normal growth in the presence of L262P$_\gamma$.

A possible consequence of the L262P$_\gamma$ mutation could be stabilisation of the F$_1$+x complex. In WT/L262P$_\gamma$ (AK) cells, both free F$_1$ and F$_1$+x complexes are seen. This could be explained by a lack of A6 causing degradation of F$_1$F$_o$ complexes into different subcomplexes depending on the $\gamma$ subunit present, with WT$_\gamma$ being present in the free F$_1$ complex and L262P$_\gamma$ causing instead the formation of F$_1$+x. One could investigate the structure of the F$_1$F$_o$ complex during EtBr treatment of WT/WT$_\gamma$ cells. If a stable F$_1$+x ring complex is formed during kDNA loss in WT/WT$_\gamma$ cells, it is the loss of A6 that promotes this complex forming, rather than the presence of L262P$_\gamma$.

### 2.4.9.4 The effect of the L262P$_\gamma$ mutation on kDNA gene expression

Deep sequencing of kDNA and the kDNA transcriptomes in these cell lines should provide further evidence for the hypothesis that expression of L262P$_\gamma$ selects against expression of functional A6 in order to combat the proton leak. A bioinformatics pipeline has been established in our lab that allows the determination of the minicircle repertoire of a cell line and the gRNAs encoded on them. Applied to clones A and B of the 427SM L262P/L262P$_\gamma$ cell lines, we would expect to find a loss of particular gRNAs required for full editing of A6 and a build-up of A6 editing intermediates terminating at those editing sites. If the loss of fully edited A6 is due to a decrease in stability of the transcript, one would still expect to see full editing coverage of the A6 transcript in the gRNA repertoire. As cells adapt to prevent the proton leak, one could also expect to find other mutations helping to prevent A6 expression, for example RPS12 mutations impairing mitoribosome activity.
2.4.10 Outlook

A number of additional questions arise from this study. In addition, this section discusses several potential applications of the kDNA independent and AK cell lines generated in this study.

2.4.10.1 Time course of L262P expression

We currently assume that the selection for loss of functional A6 occurs during the selection of homozygous L262Pγ clones, but we do not know how quickly this occurs, or at what frequency. In order to resolve the order in which events occur, a time course could be performed in a heterozygous L262Pγ cell line. The WTγ allele could be replaced by a conditional knockout (cKO) WTγ construct. Before the time course begins, the cell line should be analysed for the presence of coupled F₁Fₒ by hrCNE. Removal of the WTγ allele would occur when the cKO construct is induced, leaving just L262Pγ. We would expect this to remove the selective pressure to retain A6. The effect of a proton leak through A6 would be evident by measuring the ΔѰm via safranine O fluorescence. The depletion of edited A6 over time should be evident in cDNA samples. If cells are selected for their ability to not edit A6, a decrease in growth could be expected at this point. The lack of A6 in turn would be expected to induce the structural alterations in coupling of the F₁Fₒ complex, detectable by hrCNE. The time over which these changes occur is not certain, the proton leak due to having an Fₒ moiety in the presence of only L262Pγ should manifest within hours, whereas the selection for cells lacking A6 and structural changes would likely occur over a longer time period.

2.4.10.2 Other γ mutations remain to be understood more fully

So far, of the four subunit γ mutations identified, two (L262P and A273P) have been confirmed as fully sufficient to compensate for the loss of kDNA (Dean et al., 2013). The
significance of a mutation found in a Kenyan isolate of *T. b. evansi*, M282L, still remains to be understood (Dean et al., 2013). Introduction of an M282Lγ allele into *T. brucei* cells lacking one or both endogenous subunit γ alleles was not compensatory for loss of kDNA, and hence it is not certain whether the mutation has any significance towards the survival of this isolate. If the cell line contains uncoupled F₁F₀, studies from yeast indicate that there are other ways to generate kDNA independence. Mutations in F₁ subunits α and β are known to be compensatory for loss of mtDNA in yeast (Chen and Clark-Walker, 1995, Chen and Clark-Walker, 1996). Additionally, loss of subunits δ or ε in yeast is known to cause deletion of mtDNA by uncoupling the ATP synthase (Xiao et al., 2000, Duvezin-Caubet et al., 2003). Whether these subunits are present and unmutated in this *T. b. evansi* strain therefore requires probing.

The significance of the A281-γ mutation also requires elucidation. This mutation is the most common γ mutation found in naturally occurring DK subspecies (Dean et al., 2013, Carnes et al., 2015). It seemed that A281-γ required secondary mutations in order to function as a compensatory mutation (Dean et al., 2013). Unpublished work in our lab has subsequently shown that a heterozygous or homozygous A281-γ mutation allows acriflavine and oligomycin resistance, but does not structurally uncouple the F₁F₀ ATPase (C. Dewar and L. Woodcock, unpublished, data not shown). How these A281-γ-expressing cells deal with the proton leak is therefore not understood. Sequencing of *T. b. evansi* STIB 805 genome has shown that there are mutations in F₁ subunits α and β in similar regions to those found in yeast (Wang et al., 2007, Arsenieva et al., 2010, Carnes et al., 2015). The kinetic and structural consequences of the A281-γ mutation need to be probed in the presence and absence of these additional α and β mutations to see whether they can cause a structural uncoupling effect in combination.
2.4.10.3 Uses of kDNA independent cell lines

This work indicates that some molecular consequences of mtDNA loss are conserved between yeast, human and trypanosome cells. Dysfunctional mitochondria have been linked to many human diseases, including cancer and diabetes (Wallace, 2010). As such, discoveries in the mechanism of how T. brucei tolerates kDNA loss could be used to inform researchers about the molecular switches involved and their consequences. There is an advantage in studying this phenomenon in T. brucei that contains just one mitochondrion per cell: heteroplasmy is not present, hence the effect of mutations on Δψm are undiluted in a clonal population.

What are the cellular consequences of mutant ATPase γ expression and kDNA loss? How do cells tolerate these changes?

In yeast, loss of mtDNA is linked to activation of retrograde signalling, alternative metabolic pathways and the iron starvation pathway (Veatch et al., 2009, Epstein et al., 2001). These are not caused by the absence of respiration, but by a decrease in Δψm. In human cells, there is decreased expression of the mitochondrial calcium transporter and cellular calcium homeostasis is altered in the absence of mtDNA (Chevallet et al., 2006, Sherer et al., 2000).

What could we expect in L262Pγ-expressing AK T. brucei cells? A systems approach to these questions is required, involving transcriptomics, proteomics, metabolomics and flux measurements to investigate whether other mitochondrial pathways are affected by a decrease in Δψm. To link genotype to phenotype, changes in ATP/ADP concentrations of different cellular compartments and Δψm as a result of the rates of the processes forming and consuming them will need to be investigated. BSF cells are restricted as to the metabolism they carry out. They are adapted for the non-mitochondrial production of ATP, so a complete reconfiguration of metabolism would be unexpected in AK cells. However, some mitochondrial pathways are essential in BSF. Mitochondrial acetate production is
required for fatty acid biosynthesis (Mazet et al., 2013). Interestingly, this pathway should be able to produce mitochondrial ATP through the action of ASCT, hence a decrease in flux through this pathway could affect $\Delta \psi m$. The mitochondrial Ca$^{2+}$ transporter is also essential in BSF, presumably due to the Ca$^{2+}$ dependent activity of pyruvate dehydrogenase (when threonine is depleted) for production of acetate (Huang et al., 2013) (Fig 1.11). In addition, the mitochondrion of T. brucei may have an important role in buffering cytosolic Ca$^{2+}$ (Xiong et al., 1997). Thus, due to the number of Ca$^{2+}$ binding proteins within the T. brucei genome, a perturbation of Ca$^{2+}$ signalling could have wide-reaching consequences in cellular bioenergetics, intracellular signalling and acidocalcisome function (Docampo and Huang, 2015). Indeed, knockout of the Ca$^{2+}$ transporter decreases virulence in BSF (Huang et al., 2013). Fe/S protein biogenesis also occurs mitochondrially in BSF (Kovarova et al., 2014), the products of which could include mitochondrial complex I and II and cytosolic proteins essential for DNA replication and translation.

As $\Delta \psi m$ is essential for mitochondrial protein import, flux through pathways involving these proteins could be affected by a decreased $\Delta \psi m$ in AK cells. Results in this chapter suggest that compensation for low $\Delta \psi m$ does occur after adaptation to growth in culture (Fig. 2.19), which could suggest another mechanism for increasing $\Delta \psi m$ is activated in AK cells over time.

**Which genes become dispensable when cells are kDNA-independent?**

With the loss of kDNA, it would be expected that genes required for expression, replication and maintenance of kDNA and its gene products would become dispensable. These genes should be able to be knocked down in cell lines expressing L262Pγ with no growth effect, but be essential in cells expressing WT/WTγ. This approach has been useful in validating the function of a TAC component, TAC40, as being essential for kDNA maintenance (Schnarwiler et al., 2014). A RIT-seq screen using a genome-wide RNAi library induced in
WTγ and L262Pγ (AK) cells will provide a high-throughput method to search for candidates (Alsford et al., 2011).

Additionally, this work illustrates that although F_o subunits would be thought to be a good positive control for validating this method, as AK cells generate their Δψm independently of F_o subunit c, Tb2 and perhaps other subunits may in fact have an important role in AK cell lines in tethering F_1 to the inner mitochondrial membrane. One could systematically knockdown each F_1F_o ATPase gene in AK cell lines in order to identify which subunits are dispensable in the absence of kDNA, and which ones are not.
2.5 Figures

A

B

Ribosomal spacer

Tet inducible

Subunit gene

TAP

Aldolase 3' UTR

T7 promoter

Aldolase 5' UTR

Acini 5' UTR

BL6
Figure 2.1 The introduction of TAP-tagged ATPase subunits into 427SM cells

A) pLew79-MH-TAP plasmid (Jensen et al., 2007) showing the Not1 site used for linearisation internal of the ribosomal spacer sequence. Versions of this plasmid containing either subunit β or p18 of F$_1$F$_0$-ATPase cloned between the HindIII and BamHI sites (Zikova et al., 2009) were used. Transgenic cell lines with stable integration of the construct are selected using phleomycin. B) Schematic of the homologous recombination occurring upon nucleofection of this linearised plasmid. A ribosomal spacer sequence is disrupted by the pLew79-MH-TAP construct, colour coded as above. Expression of the TAP-tagged ATPase subunit is under control of a tetracycline-inducible promoter. C+D) Confirmation of inducibility of the tagged subunits. Clones transfected with either pLew79-MH-TAP construct were induced with 1 µg/ml tetracycline (+), C) β-TAP cell line, D) p18-TAP cell line. Western blots developed with anti-PAP (left panel, detecting the tag), anti-β (right upper panel) and anti-p18 (right lower panel). Induced proteins highlighted in red box, untagged subunit indicated by arrow. The TAP tag adds ~25 kDa to the size of the subunit.
Figure 2.2  Approach taken to generate kDNA-independent cell lines in 427SM and AnTat1.1 90:13 background

(A) pEnT6-γ-PURO plasmid (Kelly et al., 2007) showing the Not1 site used for linearisation between γ CDS and γ 3’ UTR. Alternative versions of this plasmid contained either the Lister 427 WT ATPase γ CDS or the EATRO164 DK γ CDS containing the L262Pγ mutation (Stuart, 1971), with either puromycin or blasticydin resistance markers. (B) Schematic of the homologous recombination occurring upon nucleofection of this linearised plasmid. The endogenous ATPase γ locus on one copy of chromosome 10 is replaced with the pEnT6-γ-PURO construct, colour coded as above.
Acriflavine treated 427SM cell lines expressing WT/L262P do not retain kDNA

Fragments of maxicircle genes were amplified from gDNA from various 427SM derived cell lines with the following genotypes: WT/WT, WT/L262P pre-acriflavine treatment, WT/L262P (AK) post-acriflavine treatment and L262P/L262P pre-acriflavine treatment. Genetic background: A) 427SM with a TAP tagged copy of ATPase β subunit, B) 427SM with a TAP tagged copy of ATPase p18 subunit, C) 427SM. Amplicons from left to right: A6, ND4, ND7, ND5, MoA (minicircle type A-like), LipDH (nuclearly encoded gene).
Figure 2.4 Confirmation of kDNA loss in acriflavine treated AnTat1.1 90:13 cell lines expressing WT/L262Pγ
Fragments of maxicircle genes were amplified from gDNA from various AnTat1.1 90:13 derived cell lines as labelled. Amplicons from left to right: A6, ND4, ND7, ND5, MoA (minicircle type A), LipDH (nuclearly encoded gene).
Figure 2.5A  **Acriflavine treatment induces the loss of kDNA**
Images are representative of all clones of that genotype with respect to the presence of kDNA. Far left: 427SM WT/WTγ clone A, left: 427SM WT/L262Pγ clone A pre-acriflavine treatment, right: 427SM WT/L262Pγ clone A (AK), far right: 427SM L262P/L262Pγ clone A pre-acriflavine treatment. Top panel: Phase contrast image, Bottom panel: DAPI stained image.

Figure 2.5B  **Clone L262P/L262Pγ 3 retains visible kDNA**
Top panel: Phase contrast image, Bottom panel: DAPI stained image.
Figure 2.6 Verification of kDNA-independent 427SM cell lines by cumulative in vitro growth analysis +/- 10 nM EtBr

Cell density was assessed by taking cell counts every 24 hours. Cells were diluted down to 1x10^5/ml after cell density measurement. Cell background: (A) 427SM cells transfected with ATPase β-TAP construct (non-induced), (B) 427SM cells transfected with ATPase p18-TAP construct (non-induced), (C) 427SM cells.
Figure 2.7 Verification of kDNA-independent AnTat1.1 90:13 cell lines by cumulative in vitro growth analysis +/- 10 nM EtBr

(A) Growth in HMI-9, 10% FCS, (B) Growth in HMI-9, 10% FCS +/- 10 nM EtBr. Cell density was assessed by taking cell counts every 24 or 48 hours. Cells were diluted down to 1x10^6/ml after cell density measurement.
A) The region of interest is amplified via PCR, with the reverse strand amplified with a biotinylated primer. The sequencing primer anneals to this biotinylated template, allowing DNA polymerisation to begin.

B) A dNTP is added, and if this dNTP is complementary to the template it is able to be incorporated onto the 3’ end of the sequencing primer. Nucleotide addition results in release of a pyrophosphate moiety.

C) ATP sulfurylase catalyses the conversion of PPi to ATP, and the action of luciferase causes light to be produced proportional to the amount of ATP present. Light is detected and recorded as a peak proportional to the number of that type of dNTP incorporated.

D) Unincorporated nucleotides are degraded via apyrase, and another dNTP is then added.

E) Sequentially the dNTPs are incorporated. The sequence of the elongating strand can be determined by reading the pyrogram peaks of nucleotides detected.

Figure 2.8  Schematic detailing how pyrosequencing works as a technique for quantitative sequencing
Adapted from Qiagen, Pyrosequencing - the synergy of sequencing and quantification.
A

CTT/CTT (WT/WT)

CCT/CCT (L262P/L262P)

B

WT/L262Pγ clone 1

WT/L262Pγ clone C

C

D

<table>
<thead>
<tr>
<th></th>
<th>DNA</th>
<th>RNA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>WTγ allele</td>
<td>L262Pγ allele</td>
</tr>
<tr>
<td>WT/WTγ</td>
<td>98.1</td>
<td>1.9</td>
</tr>
<tr>
<td>WT/L262Pγ1</td>
<td>46.4</td>
<td>53.6</td>
</tr>
<tr>
<td>WT/L262Pγ2</td>
<td>49.0</td>
<td>51.0</td>
</tr>
<tr>
<td>L262P/L262Pγ1</td>
<td>0.1</td>
<td>99.9</td>
</tr>
<tr>
<td>WT/L262PγA</td>
<td>42.1</td>
<td>57.9</td>
</tr>
<tr>
<td>WT/L262PγB</td>
<td>46.9</td>
<td>53.1</td>
</tr>
<tr>
<td>WT/L262PγC</td>
<td>49.3</td>
<td>50.7</td>
</tr>
<tr>
<td>50:50</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 2.9  Quantification of WT and L262P γ alleles in heterozygous cell lines by pyrosequencing
A) Schematic to show the section of ATPase γ containing the mutation that was amplified and the primers used. The mutation at nucleotide 785 is highlighted in blue. Codon CUU is wt (Leu), CUC is L262P (Pro). B) Results for homozygous controls for the pyrosequencing reaction, with amplification from gDNA from cell lines WT/WTγ (upper) and L262P/L262Pγ (lower). The blue box contains the quantification between WTγ (T) and L262Pγ (C) allele expression. The G bases are added by the program as a control to ensure no signal is detected when a nucleotide not complementary to the next base in the sequence is added. There is no C in the template sequence following on from the sequencing primer annealing site, hence no signal should be picked up when G nucleotide is dispensed during this sequence. Peaks of double the height are seen on a pyrogram when two of the same bases are positioned next to each other in the sequence, meaning that the light intensity produced is twice that produced by one base. For example, the second A in the dispersion order is double the peak height of the first A in the order. As nucleotide #785 is preceded by a C and followed by TT (see A), the analysis is not as simple as taking the ratio between C and T peaks. The C peak is contributed to by one definite C nucleotide (#764) unaffected by the mutation and the T peak is contributed to by two definite T nucleotides (#766 and #767) unaffected by the mutation. The sequence detected for a WT/WTγ cell line is therefore CTTTACAACAA, with a pyrogram of 0G 1C 3T 0G 1A 1C 2A 1C 2A. The C peak is 1/3 of the height of the T peak, as one C is detected followed by three T nucleotides (upper panel). The sequence detected for a L262P/L262Pγ cell line is CCTTACAACAA, with a pyrogram of 0G 2C 2T 0G 1A 1C 2A 1C 2A. The C peak height is equal to the T peak as two C nucleotides are detected followed by two Ts (lower panel). C) Example for results for cDNA from AnTat1.1 90:13 WT/L262Pγ clone 1 and 427SM WT/L262Pγ clone C. E = injection of enzyme into the reaction, S = substrate injection. For cell line expressing an equal amount of both WTγ and L262Pγ alleles, the ratio between the two peaks would be 3:5, amounting to 37.5% C, 62.5% T. 25% of the C peak height is contributed to by the definite C nucleotide #784, and 12.5% is contributed by the SNP. 50% of the T base height would be contributed to by two T nucleotides #786 and #767, with 12.5% is contributed by the SNP. As the contribution to nucleotide #785 is equal between T and C nucleotides at 12.5%, the ratio of the two bases is 1:1, meaning an equal amount of each allele is expressed. Variations in this 1:1 ratio are quantified by the software from the relative T and C peak heights. D) Table showing the expression levels of the L262Pγ allele, shown as % of total expression, n=3. 50:50 is a 1:1 mix of cDNA from WT/WTγ and L262P/L262Pγ expressing cell lines.
Figure 2.10  Schematic detailing an abridged “mini” TAP tag purification
This purification allows pull-down of a tagged bait protein and any associated proteins. The TAP tag
has three components. A calmodulin binding domain (CBP), a tobacco etch virus (TEV) protease
cleavage site and protein A. Protein A is able to bind IgG. Lysates of cells containing TAP tagged
‘bait’ protein within protein complexes are incubated with IgG coated columns. An affinity
purification is carried out; the protein A part of the TAP tag binds the IgG beads, pulling the bait
protein down onto the beads. The beads are washed and TEV protease is added. This cleaves protein
A from the tag, leaving protein A bound to the column and CBP-tagged bait protein plus associated
complex is eluted. Adapted from (Huber, 2003)
Figure 2.11  **Tb1 and 2 are not present in homozygous L262Pγ or AK cell lines**

Cell lines analysed are 427SM βTAP. The βTAP construct was induced with 1 µg/ml tetracycline for 2 days. 2x10⁸ cells were harvested per cell line. After cell lysis with Triton X100, cleared lysate (CL) was incubated with the IgG-conjugated magnetic beads. The beads were pulled down with flow through (FT) being the solution that did not bind to the beads. TEV protease cleaved protein A from the TAP tag, eluting the bait protein and associated subunits from the beads (E). B fraction is the beads plus any uneluted protein. Fractions were denatured by being heated to 100°C in 2x SDS buffer, and run on a denaturing protein gel. Western blotting was performed with A) anti-Tb1, B) anti-Tb2, C) anti-β, D) anti-CBP (shown just for WT/WTγ cell line), E) anti-α (top), anti-p18 (bottom). Bands: 80kDa = ATPase β-TAP (red asterisk), 55 kDa = untagged β subunit (purple asterisk), 65 kDa = ATPase β-CBP (grey asterisk), 40 kDa = Tb1 (blue asterisk) and Tb2 (green asterisk). Black asterisk = cross reacting band.
A

Cell pellet + digitonin

Centrifuge

Organellar fraction P1 + DDM S1 Cytoplasmic fraction

Centrifuge

P2 S2 Non-permeabilised organellar fraction Crude mitochondrial lysate

hrCNE

B

<table>
<thead>
<tr>
<th>% digitonin</th>
<th>0</th>
<th>0.025</th>
<th>0.05</th>
<th>0.075</th>
<th>0.1</th>
</tr>
</thead>
<tbody>
<tr>
<td>kDa</td>
<td>WCL</td>
<td>P</td>
<td>S</td>
<td>P</td>
<td>S</td>
</tr>
<tr>
<td>60</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>50</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

anti-β (mt)

anti-EF1 (cyt)

C

Fresh

<table>
<thead>
<tr>
<th>kDa</th>
<th>WCL</th>
<th>P1</th>
<th>S1</th>
<th>P2</th>
<th>S2</th>
</tr>
</thead>
<tbody>
<tr>
<td>60</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>50</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

anti-β (mt)

anti-EF1 (cyt)

Frozen

<table>
<thead>
<tr>
<th>kDa</th>
<th>WCL</th>
<th>P1</th>
<th>S1</th>
<th>P2</th>
<th>S2</th>
</tr>
</thead>
<tbody>
<tr>
<td>60</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>50</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

anti-β (mt)

anti-EF1 (cyt)
Figure 2.12  Optimisation of crude mitochondrial extractions
A) Flow chart showing crude mitochondrial extraction protocol. B) Digitonin titration to optimise selective solubilisation of the cell membranes. $2 \times 10^6$ cells/sample were harvested from culture, washed and resuspended in SHE and HBSS buffer. Digitonin was added to the final concentration indicated. After 5 minute incubation, the organellar pellet (P) was collected by centrifugation, and the supernatant (S) and pellet were assessed for mitochondrial and cytoplasmic content via western blot. Top panel: anti-β (mt, mitochondrial), bottom panel: anti-EF1 (cyt, cytoplasmic). The equivalent of $2 \times 10^6$ cells were loaded for P and S fractions. C) Preparation of crude mitochondrial preparation from fresh and frozen cell pellets. P1= pellet after digitonin extraction, S1= supernatant after digitonin incubation, P2= final pellet after DDM incubation of P1, S2= final supernatant after DDM incubation of P1. Top panel: anti-β, bottom panel: anti-EF1. Equivalents of $2 \times 10^6$ cells were loaded.
Figure 2.13  Homozygous L262Pγ mutation can cause loss of ATPase subunits Tb1 and Tb2
Mitochondrial extractions were performed from 2x10⁸ cells. Gels were blotted and probed with antibodies for Tb1 (upper panel) and Tb2 (lower panel). * = cross reacting band acting as loading control. Cell background: A) left panel: 427SM cells transfected with ATPase β-TAP construct (non-induced), centre panel: 427SM cells transfected with ATPase p18-TAP construct (non-induced), right panel: 427SM cells, B) AnTat1.1 90:13 cells. Antibodies were kind gift from the Zikova lab.
Figure 2.14 The presence of L262Pγ alleles has clone-specific effects on the structure of F₁Fₒ complex

Mitochondrial extractions were performed from 2x10⁶ cells. The mitochondrial extracts from Figure 2.13 were used for this figure. A) 427SM cell lines. Left panel: cells transfected with ATPase β-TAP construct (non-induced), Centre panel: cells transfected with ATPase p18-TAP construct (non-induced), Right panel: 427SM cells. Probed with antibodies as shown. The T. b. evansi strain used was STIB 805. The data shown are representative for three separate experiments with highly reproducible results. B) AnTat1.1 90:13 cell lines. Probed with antibodies as shown. This experiment has only been performed once.
Edited and pre-edited maxicircle gene transcripts were amplified from cDNA from various 427SM derived cell lines using qPCR. Amplification of 18S rRNA was used as an endogenous control. Results are shown relative to respective transcript levels in cells expressing WT/WTγ. * = no transcript detected (colour coded).

**Figure 2.15A  427SM L262P/L262Pγ clone A and B have decreased levels of edited A6 transcripts**

Edited and pre-edited maxicircle gene transcripts were amplified from cDNA from various 427SM derived cell lines using qPCR. Amplification of 18S rRNA was used as an endogenous control. Results are shown relative to respective transcript levels in cells expressing WT/WTγ. * = no transcript detected (colour coded).
Edited and preedited maxicircle gene transcripts were amplified from cDNA from various AnTat1.1 90:13 derived cell lines using qPCR. Amplification of 18S rRNA was used as an endogenous control. Results are shown relative to respective transcript levels in cells expressing WT/WTγ. * = no transcript detected (colour coded).

Figure 2.15B  AnTat1.1 90:13 L262P/L262Pγ clones have decreased levels of maxicircle transcripts
Figure 2.16  Fully edited A6 is not detectable in 427SM L262P/L262Pγ clones A and B

A6 transcripts were amplified with primers that annealed to the 5’ and 3’ ends of A6, thus able to amplify all A6 species. Pre-edited A6 (blue arrow, 400 bp), editing intermediates (between 400-800 bp) and fully edited A6 (red arrow, 800 bp) are products from this reaction.
Figure 2.17  Oligomycin resistance in heterozygous and homozygous L262Pγ transfectants is initially highly similar but becomes more variable over time
An Alamar Blue protocol was used to assess oligomycin sensitivity of 427SM T. brucei cells expressing WT/WTγ, WT/L262Pγ or L262P/L262Pγ with or without acriflavine treatment. Cells were incubated with varying concentrations of oligomycin for 72 hours. Resazurin sodium salt was then added for 4 hours. Measuring fluorescence at λ_{em}=590 nm provides a measure of cellular viability. Average values for ED_{50} are given from two independent experiments, each containing two technical replicates, with error bars representing SEM. The assay was performed on cells A) grown for 2 weeks after initial selection of transfectants, B) continuously grown for >2 months.
Figure 2.18  
Acriflavine treated AK 427SM cell lines have increased sodium azide sensitivity

An Alamar Blue protocol was used to assess sodium azide sensitivity of 427SM *T. brucei* cells expressing WT/WTγ, WT/L262Pγ or L262P/L262Pγ with or without acriflavine treatment. Cells were incubated with varying concentrations of sodium azide for 72 hours. Resazurin sodium salt was then added for 4 hours. Measuring fluorescence at $\lambda_{em}=590$ nm provides a measure of cellular viability in the presence of concentrations of sodium azide. Average values for $EC_{50}$ are given from two independent experiments, each containing two technical replicates, with error bars representing SEM. Assay was performed on cells A) grown for 2 weeks after initial selection of transfectants, B) continuously grown for >2 months.
Figure 2.18C  Acriflavine treated AK AnTat1.1 90:13 cell lines have increased sodium azide sensitivity

An Alamar Blue protocol was used to assess sodium azide sensitivity of AnTat1.1 90:13 T. brucei cells expressing WT/WTγ, WT/L262Pγ or L262P/L262Pγ +/-kDNA. Cells were incubated with varying concentrations of sodium azide for 72 hours. Resazurin sodium salt was then added for 24 hours. Measuring fluorescence at λ\text{em}=590 nm provides a measure of cellular viability in the presence of concentrations of sodium azide. Average values for EC\textsubscript{50} are given from two independent experiments, each containing two technical replicates, with error bars representing SEM.
Figure 2.19  ΔΨm measurements of cell lines generated in this Chapter

2x10^6 cells were incubated with 100 nM TMRE +/- FCCP, cells were washed and measurements were made on a BD LSRII flow cytometer, with λ<sub>em</sub>=575 nm. Intact, single cells were gated as shown in Appendix B. Experiments were performed once. Median measurements here are shown relative to WT/WT γ cell line, with ΔΨm of WT/WT γ set to 100%. 427SM cell lines (A) were analysed after having been grown for 2 weeks after initial selection of transfectants. For Anttl.1 90:13 cell lines, the experiment was carried out cells grown for 2 weeks (B) or > 2 months (C) after initial selection of transfectants.
In these cell lines, the ‘classical’ method of generating $\Delta \psi$ in BSF *T. brucei* functions. Protons are translocated across the inner mitochondrial membrane, into the IMS, through $F_o$ by the energy of the ATP hydrolysis reaction from the coupled $F_1$ moiety.

In these cell lines, two versions of complex V are present. Complexes containing WTγ (left) perform the ‘classical’ method of $\Delta \psi$ generation. Complexes containing L262Pγ (right, mutation symbolised by yellow star) are functionally uncoupled, hence $F_1$ no longer powers the translocation of protons through $F_o$. This uncoupled complex thus generates a proton leak (dashed line). This proton leak can be inhibited by oligomycin. As the cells still express functional A6 and have a substantial $\Delta \psi$, the proton leak can evidently be compensated for by the generation of sufficient $\Delta \psi$ by the coupled $F_1F_o$ complexes.

As in WT/L262Pγ cells, these acriflavine treated cell lines contain two versions of complex V. Complexes containing WTγ (left) cannot perform the ‘classical’ method of $\Delta \psi$ generation, as kDNA-encoded A6 is not present. Complexes containing L262Pγ (right, mutation symbolised by yellow star) are functionally uncoupled, and generate no proton leak due to the absence of A6. $\Delta \psi$ is generated by the electrogenic action of AAC, with $F_1$ moieties providing the AAC with substrate. $F_1^{+x}$ complexes are present, these are hypothesised to be $F_1^{+c}$ ring, as depicted. The c ring may maintain membrane association of $F_1$ moiety, allowing $F_1$ to stay in close proximity to the AAC for optimal $\Delta \psi$ generation. Aiding this, the L262Pγ mutation may stabilise this $F_1^{+x}$ subcomplex. The WTγ-containing complexes are expected to be less efficient in ATPase activity than mutated complexes (Clark-Walker, 2003, Schnaufer et al., 2005). $F_1$ may even be completely disassociated from the inner mitochondrial membrane as depicted (left), reducing $\Delta \psi$ generation even further.
These acriflavine treated cell lines contain only functionally uncoupled complexes. There is no generation of a proton leak due to the absence of A6 editing. $\Delta \psi m$ is generated by the electrogenic action of AAC, with $F_1$ moieties providing the AAC with substrate. $F_1+x$ complexes are present, these are hypothesised to be $F_1+c$ ring, as depicted. It is not certain why these cell lines are less azide sensitive than L262P/L262Py (AK) cell lines, it could be due to increased association of $F_1$ complexes with the inner mitochondrial membrane, but this remains to be investigated.

This cell line appears to contain structurally intact $F_1F_o$ complexes, A6 being expressed. However, these complexes appear to be functionally uncoupled, indicated by oligomycin insensitivity. It is not understood if $F_o$ in these cells forms a proton leak and, if so, how these cells tolerate it. They may express a non-functional or inefficient A6 subunit. $\Delta \psi m$ is generated by the electrogenic action of AAC, with $F_1$ moieties providing the AAC with substrate and ensuring low local ATP concentration. It is not certain why this cell line is less azide sensitive than its equivalent L262P/L262Py (AK) cell lines, it could be due to $F_1F_o$ complexes being present, maintaining close association between $F_1$ and AAC at the inner mitochondrial membrane, but this remains to be investigated.

These acriflavine treated cell lines contain only functionally uncoupled complexes. There is no generation of a proton leak due to the absence of A6. $\Delta \psi m$ is generated by the electrogenic action of AAC, with $F_1$ moieties providing the AAC with substrate. This cell line is less azide sensitive than WT/L262Py (AK) cell lines as there is approximately twice the amount of efficient $L262P\gamma-F_1$-ATPase complex present.
Figure 2.20  Models depicting current hypotheses for $\Delta \psi_m$ generation in 427SM clones based on data shown in Chapter 2
Note: complex V is likely to operate as a dimer, but for simplicity the complexes produced from the two alleles are shown as monomers. Also for simplicity, the AAC is only shown for those genotypes where it is thought to have a direct role in generating the $\Delta \psi$. 

179
3. The requirement for kDNA in stumpy forms
3.1 Introduction

Naturally occurring AK and DK strains of *T. b. brucei*, *T. b. evansi* and *T. b. equiperdum*, are widely reported to be monomorphic (Section 1.10.2). It has been inferred that the inability of *T. b. evansi* to survive within the fly may be a consequence of the lack of kDNA (Borst et al., 1987). As the biological purpose of the stumpy form is for preadaptation for life as PCF within the fly midgut, and as the DK subspecies of *T. b. brucei* are mechanically transmitted and do not transform into PCF (Zweygarth and Kaminsky, 1989), the absence of a functional DK stumpy form would be expected. Stumpy forms have however occasionally been seen in field-harvested DK strains (Section 1.10.2).

Is kDNA required for stumpy formation and/or viability? It has not been possible previously to reliably study the requirement for kDNA in stumpy form *T. brucei* prior to having kDNA-independent pleomorphic cell lines. The L262Pγ mutation was originally identified in the DK EATRO164 cell line that was generated via continuous passage through mice that were repeatedly injected with acriflavine. This DK *T. b. brucei* line had been labelled pleomorphic (Stuart, 1971); it was reported to produce infections with peak parasitaemia consisting of 12% stumpy forms, compared to the parental cell line which produced a peak infection with 84% stumpy forms. This could suggest that the lack of kDNA either limited the ability of the cells to go stumpy, or that repeated acriflavine exposure over 102 days caused secondary mutations preventing pleomorphism. In the hands of another lab group at a later date, the parental and the DK cell lines were both found unable to differentiate (Timms et al., 2002); evidently after this time period the cell lines were now monomorphic. It has not proved possible to spontaneously produce another clonal pleomorphic DK cell line via repeating the protocol performed in Stuart, 1971. Timms *et al.*, 2002, could not produce a stable clonal
population of DK *T. brucei* despite many DK cells being produced by this treatment. DK slender forms generated by acriflavine treatment were able to differentiate into stumpy forms, allowing them to conclude that kDNA was not required for the differentiation of slender forms to stumpy forms. However as these DK slender cells were not able to proliferate *in vitro*, these slender forms had been still kDNA-dependent, and so these cells could not be used to accurately investigate the viability of stumpy cells minus kDNA. Additionally, as acriflavine treatment only occurred 24 hrs before morphology scoring took place, kDNA-encoded mRNAs and proteins in the cells were most probably still present during stumpy formation. We do not know how quickly these products are turned over. Therefore, as these kDNA-encoded products could possibly still be utilised by the cells, the slender ‘DK’ cells that differentiated to the stumpy form were not actually functioning in the absence of kDNA. Whether kDNA is required for slender to stumpy formation is therefore still not known.

### 3.1.1 Hypothesis

Based on previous results, there is a limited ability to differentiate into the stumpy life cycle stage in the absence of kDNA. Hence a lower proportion of stumpy forms would be generated during an *in vivo* infection compared with a strain retaining its kDNA (kDNA⁺).

### 3.1.2 Study design

Here, the L262Pγ mutation that allows kDNA-independence was for the first time expressed in pleomorphic AnTat1.1 90:13 cells, and clonal AK cells were generated by acriflavine treatment. Mouse infections using trypanosomes expressing L262Pγ with and without kDNA showed that kDNA-independent and AK *T. brucei* are capable of differentiating to the
stumpy form *in vivo*. The generation of stumpy forms was assessed based on the expression of biochemical markers and morphology. A time-course of slender to stumpy differentiation was performed to observe any potential differences in parasitaemia generated by parasites with kDNA present or absent. ΔΨm of these cell lines was also measured in the stumpy form. Additionally the infection parameters of kDNA\(^+\) and AK cells were compared using mathematical modelling, allowing a predictive quantitative analysis of the characteristics of an infection with AK *T. brucei*.

### 3.2 Materials and methods

#### 3.2.1 Growth analysis of *T. brucei* cell lines *in vitro*

Cell lines were grown over a period of a week to measure the growth effect of the L262P\(γ\) allele and the effect of an absence of kDNA *in vitro*. Cells were grown in HMI-9 media (Hirumi and Hirumi, 1989) containing 10% FCS supplemented with 2.5 \(\mu\)g/ml G418, 5 \(\mu\)g/ml hygromycin and 0.1 \(\mu\)g/ml puromycin, +/- 10 nM EtBr (Sigma). Cell counts were performed daily using a Beckmann Z2 Coulter counter, and cells were split to a concentration of 1x10\(^5\)/ml after counting.

#### 3.2.2 Animal handling

Mouse strain MF1 was used throughout this study. All stock mice care was carried out by the staff of the March Building, University of Edinburgh. All mouse handling was carried
out by Caroline Dewar, working under the project licence of Professor Keith Matthews, in accordance with conditions of Home Office personal and project licenses.

3.2.3 In vivo mouse infection with *T. brucei*

Around 1000 *T. brucei* cells of cell lines WT/WTγ (kDNA−), WT/L262Pγ (kDNA+) and WT/L262Pγ (AK) clones 1 and 2 were each injected into mice via an intraperitoneal (IP) injection in 200 µl HMI-9 (see Table 2.3 for cell line table). No immunosuppressant was used. Parasitaemia was monitored daily with a tail snip being performed, and cells were viewed from a drop of blood compressed under a cover slip on a microscope slide at 40x objective. Cell numbers per field of view were estimated by viewing 5 fields of view from these wet smears based on the Rapid Matching method (Herbert and Lumsden, 1976). Morphology was also scored qualitatively from these wet smear slides, judged via the length of the flagella, motility of the cells, the length and breadth of the cells and the roundness of the posterior end of the cells.

For the timecourse experiment, 4 mice were infected with each cell line. To monitor the parasitaemia more closely once a trypanosome density of score 16 (a density of 6x10⁷ trypanosomes/ml blood) had been reached, tail snips were carried out every 6 hours. 5 µl blood was also taken for an immunofluorescence assay (IFA) (Section 3.2.5). The experiment was terminated at day 15 post infection. Cell counts were performed straight after a tail snip, from a wet blood smear as detailed above. For morphology counts, dry blood smear slides were prepared by spreading a bead of blood thinly across a slide and leaving to air dry. These slides were methanol fixed and DAPI staining was carried out by mounting the slides using 50 µl Prolong Gold Antifade with DAPI (Life Tech.). This allowed visualisation of the position of the nucleus with respect to the kDNA. In a slender cell, the nucleus is positioned away from the posterior of the cell and the kDNA, towards the
centre. In an intermediate cell, the nucleus has begun to migrate towards the posterior of the cell, whereas it is found next to the kDNA in stumpy cells. Dry blood smear slides were blinded by a colleague with respect to cell line, day and time point to prevent bias. Morphology was scored at each time point from these slides by two individuals, Caroline Dewar and Dr Paula MacGregor, independently on separate occasions.

The parasitaemia was judged by eye, based on the Rapid Matching method (Herbert and Lumsden, 1976). This involves looking at a blood smear and estimating the number of parasites in the field of view by matching the similarity of the density against images of set densities. The technique allows rapid good estimation of parasite density from multiple fields of view and multiple samples. This was limited by an upper limit of 64 parasites per field of view, correlating to a density of 2.5x10^8 cells/ml, as, from experience, above a count of 64 trypanosomes per field of view, often with differing morphologies, plus blood cells, the field is extremely motile, dense and heterogeneous, and it becomes difficult to estimate counts accurately. This upper limit can be seen as a plateau in the parasitaemia density (see Figure 3.8). In the thesis of P. MacGregor, 2011, parasitaemia judged by this Rapid Matching method was compared to parasitaemia judged by qRT-PCR of a housekeeping transcript, ZFP3. The pattern of parasitaemia seen via these two methods correlated well, however at peak parasitaemia, microscopy underestimated the cell counts compared with ZFP3 qRT-PCR. Counting parasite density via qRT-PCR of ZFP3 was not possible for this study due to equipment limitations.

Morphology was judged based on qualitative morphology scoring. This is generally seen as a subjective analysis (Macgregor and Matthews, 2010), due to the range of morphology differences seen during slender to stumpy transition (see Figure 3.6). Generally, slender cells are longer, thinner, have a long detached flagellum at the anterior end and have a larger distance between the nucleus and kinetoplast. Replicating cells are always scored as slender. Stumpy cells are shorter, wider, with an extremely short attached flagellum, and a small
nucleus-kinetoplast distance. Intermediates are classed as the cells well in between these classifications, with some possible variability in the scoring of late slender forms and early stumpy forms without staining for molecular markers. From the proportion of cells that are one of the three morphological forms, the density of this form within the total parasitaemia can be plotted (Figure 3.12-3.15, purple and pink panels). For this analysis, intermediate forms are combined with stumpy forms, to create a ‘differentiated’ cell class, for ease named stumpy.

Morphology was judged based on the following parameters (Macgregor et al., 2012, Vickerman, 1985):

- the presence of dividing cells

As stumpy forms are cell cycle arrested, the only dividing population within a trypanosome BSF infection are slender.

- the length of the flagellum

In the slender form, the flagellum is extended unattached past the end of the cell body, whereas in the stumpy form, the flagellum is shortened and is attached to the cell body throughout its entire length.

- rounding of the posterior of the cell

Stumpy forms have a more rounded posterior end of the cell.

- shortening and widening of the cell

Stumpy forms are shorter and fatter than slender forms.

- the position of the nucleus with respect to the kDNA at the posterior of the cell
Stumpy cells have their nucleus positioned close to the posterior of the cell, whereas in the slender and intermediate form, the nucleus is positioned closer to the centre of the cell. This occurs at a late stage in intermediate to stumpy differentiation. For representative images, see Figure 3.6.

### 3.2.4 In vitro slender to stumpy differentiation of *T. brucei*

**AnTat1.1 90:13 WT/WTγ, WT/L262Pγ, WT/L262Pγ (AK) cell lines**

AnTat1.1 90:13 cell lines WT/WTγ, WT/L262Pγ (kDNA−) and WT/L262Pγ (AK) clone 1 and 2 were grown for 48 hours in HMI-9, 10% FCS supplemented with 100 μM 8-(4-chlorophenylthio)adenosine 3′,5′-cyclic monophosphate sodium salt (8pCPT-cAMP, Sigma). Cells were seeded at 3x10⁵/ml, and cell counts were performed every 24 hours using Beckmann Z2 Coulter counter.

### 3.2.5 Anti-PAD1 IFA protocol using cells from *in vivo* mouse infections

Tail snips were performed on mice, and each 5 μl blood sample was pipetted into an 1.5 ml microcentrifuge tube on ice. Up to 40 minutes later 100 μl ice cold vPBS (8 g/l NaCl; 0.22 g/l KCl; 2.27 g/l Na₂HPO₄; 0.41 g/l KH₂PO₄; 15.7 g/l sucrose; 1.8 g/l glucose; pH 7.4) was added to the 1.5 ml microcentrifuge tube, and the 1.5 ml microcentrifuge tube was flicked several times in order to mix thoroughly. The cells were centrifuged at 10,000 x g for 5 minutes and the pellet resuspended in 50 μl ice cold vPBS. 150 μl ice cold 4% paraformaldehyde (PFA) (Fisher) was then added, making a final concentration of 3%. After
10 minutes on ice 1 ml ice cold vPBS was added and cells were pelleted. 130 µl 0.2 M glycine was then added allowing cells to be left for an overnight step if necessary. Cells were pelleted, resuspended in 100 µl PBS, added to poly-L-lysine slides (Gerhard Menzel) and allowed to adhere for 30 minutes in a humidity chamber. The slides were washed once in PBS and permeabilised in 0.05% Triton X-100 in PBS for 10 minutes. Slides were washed three times in PBS, and blocked with 200 µl 20% FCS in vPBS for at least 30 minutes. Slides were washed once in PBS, and 200 µl 1/500 anti-PAD1 primary antibody (Table 3.1; courtesy of Professor Keith Matthews) in 20% FCS in vPBS was added and left for 1 hour, and the washed three times in PBS. 200 µl 1/500 anti-Rabbit-FITC secondary antibody (Sigma) in 20% FCS in vPBS was added and slides were left for 1 hour. Slides were washed three times in PBS, incubated with 50 µl 1 µg/ml DAPI in PBS for 2 minutes in the dark and mounted using 40 µl Mowiol (CalBiochem) containing 4 µl PDA (Sigma) per slide.

3.2.6 Assessing life cycle stage markers PAD1 and COX IV via western blot

Stumpy form trypanosomes were harvested via a cardiac puncture to collect the infected blood. Blood was collected in a 2 ml syringe containing 200 µl 2% sodium citrate (anti-coagulant). Stumpy form trypanosomes were purified from blood using DEAE-cellulose DE52 (Whatman) anionic exchange columns (Lanham, 1968) that were preincubated with PSG solution (44 mM NaCl, 57 mM Na₂HPO₄, 3 mM KH₂PO₄, 55 mM glucose, pH 7.8) warmed to 37°C. Blood was loaded onto the column, and the column was continuously topped up with warm PSG, and eluate was collected. The density of the cells was measured using a Beckmann Z2 Coulter counter, and 2x10⁶ cells per sample were washed in PBS, pelleted and lysed in 10 µl 2xSDS buffer (4% SDS, 20% glycerol, 120 mM Tris-HCl, pH 6.8, 0.2% bromophenol blue, 200 mM DTT).
Trypanosomes were harvested from culture by centrifuging $2 \times 10^6$ cells, washing and resuspending the cell pellet in $10 \mu$l 2xSDS solution.

Samples were either incubated for 10 minutes at 95°C or sonicated for 3 minutes in ice cold water depending on the primary antibody used (Table 3.1). Denatured samples were loaded onto a Novex NuPAGE Bis-Tris 10% precast gel (Invitrogen), which was run at 150 V with 500 ml ice cold 1x MOPS SDS running buffer (Life Tech.). The gel was blotted onto a methanol-equilibrated PVDF membrane (Millipore) using 1 L ice cold transfer buffer (100 ml 10x transfer buffer (390 mM glycine, 480 mM Tris, 0.36% SDS); 200 ml MeOH; 700 ml dH$_2$O) and a Biorad Criterion blotter at 90V for 45 minutes, and blocked overnight in 1x TBST (10 mM Tris-HCl, 150 mM NaCl and 0.05% Tween-20) with 10% milk powder at 4°C. Antibody incubations were performed in 1x TBST with 5% milk powder, unless stated otherwise, and washes were performed with 1x TBST. For detection, Enhanced Chemiluminescence reagents 1 and 2 (Amersham) were mixed in equal volumes and incubated with the membrane. The membrane was placed into an X-ray film cassette and exposed using Kodak BioMax light film, with exposure time depending on intensity of signal produced. Developing was performed by a SRX-101A X-ray developer (Konica Minolta).
### Table 3.1 Antibodies used for western blotting in this chapter

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Concentration</th>
<th>Sample preparation</th>
<th>Incubation conditions</th>
<th>Secondary</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-PAD1</td>
<td>1:1000</td>
<td>Sonication</td>
<td>1x TBST, 1% BSA</td>
<td>Anti-rabbit 1:5000</td>
<td>(Dean et al., 2009)</td>
</tr>
<tr>
<td>Anti-COX IV</td>
<td>1:1000</td>
<td>Boiling</td>
<td>1x TBST, 5% milk</td>
<td>Anti-rabbit 1:2000</td>
<td>(Maslov et al., 2002)</td>
</tr>
<tr>
<td>Anti-EF1α</td>
<td>1:7000</td>
<td>Boiling</td>
<td>1x TBST, 5% milk</td>
<td>Anti-mouse 1:2000</td>
<td>Millipore</td>
</tr>
</tbody>
</table>

#### 3.2.7 Mathematical model for *T. brucei* infection dynamics

The mathematical model used in MacGregor *et al.*, 2011, was utilised in order to quantitate the differences observed in *in vivo* mouse infections with *T. brucei* AnTat1.1 90:13 of genotypes WT/WTγ, WT/L262Pγ and WT/L262Pγ (AK). The model was modified to include a parameter for SIF-independent slender to stumpy differentiation. The model was designed and fitted to the observed parasitaemia and the morphological data produced from each infection by Dr Nick Savill, University of Edinburgh. Mathematical derivation for the model is described in Appendix D.

#### 3.2.8 Cell cycle analysis via flow cytometry

2x10⁶ cells per sample were harvested from *in vitro* culture by centrifugation at 2000 x g for 5 minutes in 5 ml polystyrene round bottom tubes (BD Falcon 352052) and washed three times. Supernatant was removed and cells were resuspended and fixed by the addition of 500 µl fixing solution (2% formaldehyde, 0.05% glutaraldehyde in PBS), and left at 4°C for 30
minutes minimum. Cells were pelleted and washed three times in PBS, and resuspended in 500 µl PBS with 10 µl 1 µg/ml DAPI.

From an *in vivo* infection, cells were prepared as described in Section 3.2.5 up to the glycine step, which allowed storage until the infection had been completed. From here, cells were pelleted by centrifugation, washed three times in PBS, and resuspended in 500 µl PBS with 10 µl 1 µg/ml DAPI.

DAPI stained cells were analysed by flow cytometry (peak excitation 358 nm, peak emission 461 nm) using a Beckton Dickinson LSRII machine with BD FACSDiva software. Machine settings were set by comparing positively and negatively stained samples with the assistance of Martin Waterfall, University of Edinburgh. 2.5x10⁴ events per sample were measured. Results were analysed with FlowJo software.

### 3.2.9 Measurement of relative Δψm using flow cytometry

The TMRE Mitochondrial Membrane Potential kit (Abcam) was used. After the harvest of cells from a mouse infection during peak parasitaemia, whilst the population was approximately 90% stumpy, cells were purified from blood as detailed above. Cells from each sample were resuspended in HMI-9, 10% FCS, at a concentration of 2x10⁶/ml. An aliquot from each sample (2x10⁶ cells) was supplemented with 20 µM FCCP to ablate the Δψm (+FCCP controls). All samples were incubated for 10 minutes at 37°C. All samples were then supplemented with 100 nM TMRE and left at 37°C for 20 minutes. Cells were pelleted at 2000 x g for 5 minutes in 5 ml polystyrene round bottom tubes, and washed three times in 5 ml 0.2% BSA in PBS. Cell pellets were resuspended in 500 µl 0.2% BSA in PBS containing 5 µg/ml Hoechst 33342 DNA staining dye (Life Tech.) and left for 1 hour in foil
before analysis on BD LSRII instrument as detailed above, with peak excitation at 549 nm and peak emission at 575 nm for TMRE. 2\times 10^4 events per sample were measured.

### 3.3 Results

#### 3.3.1 Pleomorphic slender cells independent of kDNA are viable in \textit{in vitro} culture

The growth of pleomorphic AnTat1.1 90:13 cell lines expressing WT/WT\gamma, WT/L262P\gamma and WT/L262P\gamma (AK) (see Table 2.3) in HMI-9 was assessed by performing cell counts of cultures maintained in the logarithmic phase over a period of a week. Figure 3.1A shows cell lines expressing one L262P\gamma allele and AK cells grew at the same rate as the WT/WT\gamma cell line, showing that both modifications had no effect on the \textit{in vitro} growth of the cells. As expected (Gould and Schnaufer, 2014), cells expressing an L262P\gamma allele +/- kDNA are resistant to EtBr treatment, unlike cells expressing solely WT\gamma (Figure 3.1B).

#### 3.3.2 Pleomorphic cells independent of kDNA are able to differentiate to morphologically stumpy forms \textit{in vivo}

In order to investigate whether kDNA-independent and AK \textit{T. brucei} were able to differentiate to the stumpy life cycle form, a set of preliminary \textit{in vivo} infections were performed. Mice of strain MF1 were infected with AnTat1.1 90:13 cells expressing WT/WT\gamma, WT/L262P\gamma and WT/L262P\gamma (AK) via IP injection. The infection status of these mice was monitored daily via tail snips, with blood smears being visualised to score both the
density and the morphology of cells within the parasitaemia. In all cell lines investigated, by around day 5 post infection the parasites had reached a density score of 64 and began to differentiate to the intermediate form. By around day 7, parasites that were morphologically stumpy characterised as in Section 3.2.3 began to emerge in all infections (Figure 3.2Ai + ii). In multiple repeat infections, stumpy form AK *T. brucei* seemed to be cleared faster than stumpy cells retaining kDNA (this initial observation was investigated in more detail in subsequent studies, see below).

### 3.3.3 AK cells that are morphologically stumpy express PAD1

Stumpy cells express PAD1 on their surface, a carboxylate transporter that receives the CA signal for efficient PCF differentiation (Dean et al., 2009). In order to assess whether AK stumpy cells were able to express PAD1, stumpy form trypanosomes were harvested from mice via cardiac puncture, and purified on a DE52 anionic exchange column. They were analysed by western blotting for the expression of proteins that are considered as specific markers for different life cycle stages (Figure 3.2B). Cells expressing WT/WTγ, WT/L262Pγ and WT/L262Pγ (AK) that were morphologically stumpy were found to express the stumpy specific protein PAD1 (Dean et al., 2009), unlike cells morphologically staged as slender, or PCF cells included as control. None of the stumpy form cells (or the slender cells) expressed COX subunit VI, a PCF specific protein that is only expressed at the RNA level in the stumpy form (Priest and Hajduk, 1994).
3.3.4 AK pleomorphic slender cells are able to differentiate to a stumpy-like form in *in vitro* culture

As the faster clearance of AK stumpy cells could signify that AK stumpy forms have a shorter lifespan than stumpy cells with the full kDNA repertoire, we wished to experimentally verify this observation. The clearance of stumpy cells from the blood of a mouse is the result of both cell senescence and the actions of the immune system, hence it would prove difficult to quantify the lifespan of a stumpy cell *in vivo*. *In vitro* alternatives were therefore first pursued.

Analogs of cAMP are able to induce the partial differentiation of slender form *T. brucei* to the stumpy-like form in both monomorphic and pleomorphic cell lines (Vassella et al., 1997) (Breidbach et al., 2002). This stumpy-like form is cell cycle arrested, but it has been reported that cells do not reliably look morphologically stumpy (Macgregor et al., 2014). In order to investigate whether this was true for AnTat1.1 90:13 derived cell lines, and therefore whether we could quantify the lifespan of stumpy cells by using this stumpy-like form, cells expressing WT/WT, WT/L262P and WT/L262P (AK) were grown in the presence of 100 µM 8-(4-chlorophenylthio)adenosine 3',5'-cyclic monophosphate (8pCPT-cAMP, Sigma) in *in vitro* culture.

A comparison of the growth of these cell lines *in vitro* in the presence and absence of 8pCPT-cAMP shows that all cell lines entered growth arrest between 0 and 20 hrs of treatment with the cAMP analogue (Figure 3.3A). In agreement, preliminary analysis of the cell cycle status within these populations of cells via DAPI-staining and flow cytometry suggested that, by 24 hrs of treatment, there seems to be a decrease in the % of WT/WTγ, WT/L262Pγ and AK clone 1 intact cells in G2 stage (Figure 3.3B). As growth-arrested cells are quiescent in G1 of the cell cycle, a decrease in % cells in G2 in a population is characteristic of cells entering growth arrest. This decrease in % intact cells in G2 was only
visible in AK clone 2 cells after 48 hrs of treatment. This may have occurred earlier, between 24hr and 48hr of treatment, however cells were only analysed every 24 hrs. After 48 hrs in the presence of 8pCPT-cAMP, the stumpy specific cell surface marker PAD1 was detectable in all cell lines investigated (Figure 3.3C). However, analysis of cell morphology by microscopy did not support differentiation into genuine stumpy cells (Section 3.2.3)(Figure 3.4): in all cell types investigated (WT/WTγ, WT/L262Pγ and WT/L262Pγ (AK)) a long flagellum was visible, dividing cells were apparent in the culture, and the nucleus had not moved towards the posterior of the cell, as would expected for true stumpy cells (Macgregor et al., 2012, Robertson, 1912). Moreover, there were many cells in each population with more than two nuclei that had an extremely aberrant morphology (Figure 3.4, right hand side panel). As this treatment did not induce a convincing stumpy morphology and instead produced abnormal looking cells in all cell lines investigated, it was decided that analysis of stumpy longevity should not be done via induction of the stumpy like phenotype in vitro using the cAMP analogue 8pCPT-cAMP.

3.3.5 A lack of kDNA does not affect the dynamics of slender to stumpy differentiation in vivo

As an in vitro assessment of stumpy viability was therefore not going to be possible, a time course of infection was then carried out, where an accurate measure of parasitaemia level and morphology was recorded over time for each cell line. This would allow a detailed comparison between cell lines in terms of the efficiency of the slender to stumpy transition, and the length of time that the parasitaemia was maintained, in order to judge the effect of kDNA loss on the in vivo dynamics of a mouse infection.
In order to investigate whether the absence of kDNA affects the dynamics of a *T. brucei* infection, four mice were infected with either cells expressing WT/WTγ, or WT/L262Pγ clone 2 or WT/L262Pγ (AK) clones 1 or 2. After a trypanosome density of score 16 had been reached, parasitaemia was monitored every 6 hours. Trypanosome density was scored directly from wet blood smears. Morphology scoring was performed by two individuals (C. Dewar and P. MacGregor) from DAPI-stained dry blood smear slides that had been methanol fixed. Slides were blinded and scoring was performed independently as per Section 3.2.3. 5 µl blood was harvested at each time point to allow for PAD1 IFA analysis if the two morphology scores were extremely different.

The dynamics of a *T. brucei* infection in the first peak of parasitaemia would be expected to follow a characteristic pattern (Rico et al., 2013)(Fig. 1.6). The differentiation of *T. brucei* is expected to be predominantly synchronous at this stage of the infection. After initial proliferation of slender form cells, by around day 4 post infection, cells begin to differentiate. The proportion of slender cells in the population begin to decrease from 100% to around 10% within 2 or 3 days. The proportion of cells in the population that are slender remains low for the rest of the infection but are still present. As slender proportion falls, the proportion of cells with an intermediate morphology increases to a peak at around 6 days post infection. By now the population is formed of predominantly non-proliferating parasites, and the density of parasitaemia plateaus. The peak in intermediate cells then begins to fall, and stumpy forms begin to emerge around day 6 post infection. By day 8, stumpy forms dominate the infection, making up around 80% of the population. At this point, parasitaemia begins to fall, with parasites reaching the end of their limited life span in the stumpy form and with the immune system acting to clear stumpy forms. The low number of slender forms still present in the parasitaemia persist. This allows a second wave of parasitaemia, which follows with a shift in the expression of new VSG surface proteins.
allowing immune system evasion. The population thus expresses a heterogeneous set of VSG proteins (Mugnier et al., 2015).

This pattern of slender to stumpy differentiation is seen not only in parasites expressing WT/WTγ, but also WT/L262Pγ and in both AK clones of *T. brucei* (Figure 3.5, Figure 3.6), allowing us to conclude that kDNA is not required for the differentiation of *T. brucei* from slender to stumpy forms. There is a high reproducibility in these results, with error bars showing little variation between the four mice infected with each cell line.

There is also high reproducibility between the morphology scoring of individual 1 and individual 2. Figure 3.7 shows that when comparing these scoring results, the gradient of the line of best fit is 1. Hence if the morphology scoring is erroneous, the scoring is still consistent. The scoring was performed blinded and independently meaning the consistency seen is a good indication of accuracy. As the relative position of the nucleus and kDNA is one of the criteria for scoring cellular morphology (Section 3.2.3), the lack of kDNA could affect the morphology scoring of AK cells. The results for all cell lines sit on this line of best fit (Figure 3.7); reassuringly the absence of kDNA again does not affect the ability to score AK cells for morphology.

### 3.3.6 AK *T. brucei* have an altered first peak of parasitaemia

Interestingly, there is no difference in the density of peak parasitaemia reached between all 4 cell lines (Figure 3.8), with a peak density of score 64, a density of $2.5 \times 10^8$ cells per ml blood, being recorded in all cases. Hence, the lack of kDNA does not affect the ability of the cells to grow to the same peak parasitaemia as cells with kDNA. In terms of cell counts, parasites expressing WT/WTγ and WT/L262Pγ have identical first peaks of parasitaemia (Figure 3.8A), with the rise and fall in parasite numbers indistinguishable. In both cases,
plateaus in cell numbers occur at day 5 post infection, and last until day 7. This plateau is an artefact of the scoring system. Above a score of 64 it is difficult to accurately count the parasites within the field of view due to their high density and motility, hence all scores above a count of 64 were recorded as 64. In reality, the cell density would have a more distinguishable peak during the plateau that we see in Figure 3.8C.

Conversely, AK *T. brucei* clones 1 and 2 show differing patterns in cell number across the first peak of parasitaemia. AK clone 1 shows a delayed rise in cell numbers, with parasite numbers beginning to increase at day 5, and reaching their maximum during day 6 (Figure 3.8B). This is in contrast to kDNA\(^+\) parasites, where parasite numbers start to increase at day 4, and reach a maximum at day 5. The plateau in the parasitaemia peak for this AK clone 1 is less well defined, with cell numbers appearing to be less static than kDNA\(^+\) cells where the density scores do not go lower than 64. The plateau length for AK clone 1 also appears shorter than kDNA\(^+\) cells, at 30 hrs compared with 42 hours. The fall occurs at a slightly slower rate in this AK clone 1, but the infection is cleared by the same time, with parasites being undetectable in the blood by day 9 in kDNA\(^+\) parasites and in this AK clone 1.

The AK clone 2 contrasts with clone 1 and kDNA\(^+\) cells in the pattern of its first peak of parasitaemia (Figure 3.8C). This AK clone, like clone 1, shows a delay in rise of parasitaemia over time, with cells first detected at day 4, but cell numbers only peak during day 6. The plateau in cell numbers for this clone 2 is not present, instead there is a single time point during day 6 at which cells were detected as being at a maximum parasitaemia of density score 64. From here, cell numbers begin to fall, at a gradual but slightly lower rate to that of kDNA\(^+\) cells, but with cells clearing faster than kDNA\(^+\) cells, becoming undetectable in the blood by day 8.
The differences seen within the first peak of parasitaemia between *T. brucei* kDNA+ and AK parasites suggested that a lack of kDNA affects the parasite’s ability to either proliferate or persist during the transitions they undergo through a mouse infection.

### 3.3.7 Normalisation of morphology scoring by PAD1 staining

Scoring progression through the life cycle by morphology is known to be subjective (Macgregor and Matthews, 2010). Both a lack of consistency between different scorers and between different cell lines are of potential concern. PAD1 protein is expressed on the cell surface solely during the stumpy stage (Figure 3.2B), so can be used as a molecular marker for the stumpy form. Comparison of morphology scores and PAD1 positive counts performed by two individuals would therefore provide a measure of morphology scoring error, as illustrated in Appendix C.

An optimised PAD1 IFA protocol was established, allowing cells to be taken directly from 5 μl blood, to be fixed, purified from blood cells and then stored for up to a week before cells were adhered to slides, permeabilised, and IFA was performed (Section 3.2.5). Storage in glycine allowed cells to be stored after fixation, allowing collection of all time point samples before all samples were stained simultaneously, and also caused the blood cells to lyse. A concentration of 0.2 M glycine was chosen as this allowed cells to maintain their morphology, however after 3 weeks of storage staining was not accurate, with even dividing cells stained as PAD1 positive (data not shown). Staining intensity was also variable between slides (data not shown). This therefore was not an accurate technique to measure the number of PAD1 positive cells in a population, and so could not be used during the time course as a quantitative marker for stumpy cells in this experiment. By extrapolation, as a previous pilot experiment had shown that morphology scoring had in fact been extremely
rigorous (Appendix C), it was assumed that the morphology scoring in Figure 3.5 was accurate.

3.3.8 Mathematical modelling of an in vivo infection with kDNA\(^+\) and AK \textit{T. brucei}

Mathematical models allow complex biological systems to be represented in a simplified form. They allow the testing of hypotheses and quantification of parameters that are not immediately obvious from observations of the system. The dynamics of slender to stumpy transition has been modelled mathematically for example (Macgregor et al., 2011). These models come with caveats however, as model predictions do still require experimental testing. Although guided by biological grounds, to some extent finding a good model is based on trial and error. A measurement of goodness of fit, parameter significance and confidence limits are essential. Accuracy is limited by the data to which the model is fitted, with most investigations using subjective morphological data and the original parameter priors upon which the model is based. These original parameter priors describe the information already known about the system, for example, for a trypanosome \textit{in vivo} infection, the estimated length of slender cell cycle is known (Vassella et al., 1997, Savill and Seed, 2004, Seed and Black, 1997), and so can be used to build the model. An estimate of error is incorporated into these parameter priors to show uncertainty. There are some parameters of an \textit{in vivo} trypanosome infection we do not know. For example, due to the lack of knowledge of the impact of the immune system on a trypanosome \textit{in vivo} infection. The immune system is either regularly omitted from models, or models are applied to data generated in immunocompromised mice.
Differentiation has previously been modelled as cell density dependent (Seed and Black, 1997, Seed and Black, 1999, Turner et al., 1995, Tyler et al., 2001a). Since experiments have suggested that a trypanosome-derived factor, SIF, drives the density-sensing mechanism to trigger differentiation (Seed and Sechelski, 1989, Hesse et al., 1995, Vassella et al., 1997), this factor has been incorporated into some models. Savill and Seed (2004) used a model with (1) differentiation driven by SIF, and therefore at a rate proportional to SIF concentration, and (2) a constant background SIF-independent differentiation rate that had been included due to the presence of a low number of stumpy cells at all time points (Savill and Seed, 2004). Intermediate forms and stumpy forms were classed together as differentiated cells due to the difficulty of distinguishing between them. The model fit well to the observed data, suggesting that SIF concentration is proportional to differentiation rate and that slender cells, but not stumpy forms, produce SIF. Parameter estimates, for example doubling time, agreed with previous investigations (Seed and Black, 1997). Savill and Seed also tested models based on the slender to stumpy transition being proportional to the concentration of slender cells with heterogeneous differentiation rates, as in Turner et al., 1995, and found that this model gave a poor fit to the data.

A previous attempt to model SIF impact on differentiation utilised slender concentration instead of SIF concentration, based on the assumption that slender concentration is proportional to SIF concentration (Tyler et al., 2001a). However this does not take into account SIF secretion rate, SIF breakdown rate or the sensitivity of the cells to SIF. The model was applied to diaphorase assay data as a proxy for mitochondrial activity, in lieu of morphological data, with an unsatisfactory fit.

The MacGregor model for the slender to stumpy transition was adapted from the Savill and Seed model, but only used SIF-dependent differentiation and included a term for the differential action of immune system on slender and stumpy forms (Macgregor et al., 2011). This model was applied to PAD1 mRNA qRT-PCR data, using this as an early indicator of
differentiation, and as a consequence grouping together intermediate and stumpy forms, with parasite density measured by qRT-PCR of the constitutively expressed ZFP3 mRNA. This model fit the data well based on residual analysis, unlike a model based on cell density dependent differentiation. Parameter estimates again agreed with previous investigations.

In order to more specifically define the differences observed between a kDNA$^+$ and AK *T. brucei* infection, the mathematical model for *T. brucei* infection dynamics used in MacGregor *et al.*, 2011 was fit to the microscopy data presented here, observing the change in parasitaemia and morphology over time (Figure 3.5, 3.8).

### 3.3.8.1 The mathematical model

The model was constructed and fit to the parasitaemia and morphological data from each infection by Dr Nick Savill, University of Edinburgh. The model is described in full by Dr Nick Savill in Appendix D. The model takes the form of differential equations that represent the infection dynamics of *T. b. brucei*. Certain aspects regarding *in vivo* trypanosome infections are well known and were used to inform the model as parameter priors, as detailed in Appendix D. Other parameters, for example SIF production rate, were unknown. The model was fitted to the data for each mouse and the parameters were varied, until a best fit model was produced. The most likely parameter values plus error range for each mouse were then estimated.

The cell types in this model are (i) non-committed slender cells, (ii) committed replicating slender cells, (iii) non-proliferating stumpy forms. They are related to each other in an *in vivo* infection as described in Figure 3.9. The infection begins with an injection of around 1000 non-committed slender cells into the mouse, with SIF concentration at zero and no immune response. The concentration of each cell type depends on the proliferation rate (applicable to slender forms only), the immune clearance rate, the lifespan of that cell type and the differentiation rate (applicable to slender forms only).
The model includes SIF concentration. SIF is produced by non-committed and committed slender cells, and acts on slender cells, inducing them to commit to differentiation (Vassella et al., 1997). SIF is not measured, only estimated by the model. A slender cell is modelled to produce 1 unit of SIF per hour. SIF is removed from the system by clearance by the host by turnover at a fixed and high rate. In this model, intermediate forms are classed as stumpsies. The concentration of stumpy forms is determined by their formation rate, cell senescence and immune system clearance.

The action of the immune system during a trypanosome infection is not fully understood, and expected to be complex, hence a detailed model is difficult to construct. Separate immune responses are activated by the presence of either slender or stumpy cells (Engstler et al., 2007), with the initial immune response occurring after a time lag. The immune responses are self-enhancing.

**3.3.8.2 A model including a term for SIF-independent differentiation better fits our infection dynamics data**

Two versions of the model depicted in Figure 3.9 were produced. The way in which they differ is in the control of differentiation from slender to stumpy form. In one model the differentiation rate is proportional to SIF concentration. A model similar to this was used in MacGregor et al., 2011. A second model has differentiation influenced by two terms: SIF dependent as in model 1, and also a SIF independent differentiation term. A model similar to this was used in Savill and Seed, 2004. This term is a constant background level of slender form differentiation, independent of the concentration of SIF. Hence each slender cell has a fixed probability of differentiating independently of SIF per cell cycle. This was originally added into the Savill and Seed model due to an observation of a low number of stumpy cells during exponential growth. The two terms are summed, meaning that the SIF dependent term only has a significant effect at high slender form concentrations.
Residual analysis was performed to assess the adequacy of the models fit to the experimentally determined data from all 16 mice (Figure 3.10). In this analysis, the position of the mean of standardised residuals are compared to the 95% predictive interval of the mean. The standardised residuals of the data points for each mouse at each time point are depicted by crosses, this is the difference between the data point and the model prediction, with the red line being the average across all mice. A more adequately fitting model has i) its crosses lying between ±3 standard deviations from zero and ii) the average line fluctuating around zero in a random pattern, lying within the 95% predictive interval 95% of time. At any time point where these two prerequisites are not true, the model does not explain this time point adequately. If the average of the standardised residuals is greater than the upper limit of the 95% predictive interval, this suggests the model underestimates the data, whereas the average lying below the lower limit of the 95% predictive interval suggests the model overestimates the data. In model A containing SIF independent differentiation (Figure 3.10A), there is a lower number of outlying residuals (blue crosses), and the mean of the residuals lay within the 95% predictive interval (dashed lines) more of the time than in the model containing only SIF dependent differentiation (model B) (Figure 3.10B). The difference is most apparent when looking at day 4 in the slender proportion, where the SIF-dependent model B overestimates the slender proportion (Figure 3.10B). There is also slender proportion overestimation during day 6. Additionally, SIF-dependent model B underestimates the slender proportion during days 5 and 7. Looking at the fit of the SIF dependent model to the slender density data (Figure 3.12B-3.15B, purple panel), the underestimation of the model B at day 5 is obvious for all 8 mice infected with kDNA+ cells, but not as evident for mice infected with AK cells where only two of the 8 mice have an underestimation of the model at day 5, and two mice have an overestimation at day 5. This model seems to fit the slender density of AK cells better than kDNA+ cells at peak slender density. The other inaccuracies are all evident but less extreme.
Looking the accuracy of model A that includes SIF independent differentiation (Figure 3.10B), similarly with model B there is an underestimation of the slender proportion at days 5 and day 7, although this is less extreme. These are visible in Figure 3.12A-3.15A, green panel. There is also a slight overestimation of the slender proportion just before day 5, but the other inaccuracies of the SIF dependent model are not present. There is a slight underestimation of the parasitaemia during day 5, which is visible in the fits of the model to the data (Figures 3.12A-3.15A, red panel), and an overestimation of the parasitaemia at day 9, which is not visible in the model fit.

Interestingly the underestimation of the parasitaemia on day 5 is also present in the SIF dependent model fit to the MacGregor data (Macgregor et al., 2011), although here, due to the faster onset of parasitaemia, it is seen in day 4. Hence the underestimation of slender cell growth at early infection time points seems to be an artefact of the model. This shows that this model is not a completely accurate representation of the biological system, some relevant biological parameters are not yet included in the model.

Both models were assessed by the Akaike information criterion (AIC). This is a method used to select a model by measuring the quality of a fit of mathematical model to a set of data, and takes into account the goodness of fit and the number of parameters estimated in the model. As increasing the number of parameters improves the goodness of fit, AIC penalizes models with more estimated parameters to discourage overfitting. Hence the model with the lowest AIC, i.e. the model with the lowest number of parameters to prevent overfitting, is preferred.

<table>
<thead>
<tr>
<th>Model</th>
<th>AIC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Without SIF independent differentiation</td>
<td>3033</td>
</tr>
<tr>
<td>With SIF independent differentiation</td>
<td>2659</td>
</tr>
</tbody>
</table>
The model with SIF independent differentiation included (model A) is preferred (Table 3.2).

Looking at the data, this model fits the data better because without SIF dependent differentiation the model tends to overestimate the slender form fraction at day 4 (Figures 3.12B-3.15B), meaning these data points are often lower than the median fit line of the model. The model including SIF independent differentiation allows for a small drop in slender concentration at early time points demonstrative of earlier differentiation of slender forms at a low density, and therefore at a low SIF concentration, allowing the model median fit line to pass closer to these data points (Figure 3.12A-3.15A).

For the model that includes SIF independent differentiation, the four AnTat1.1 90:13 cell lines, WT/WTγ, WT/L262Pγ, WT/L262Pγ (AK) 1 and WT/L262Pγ (AK) 2 have a similar probability of differentiating via this pathway per cell cycle, at ~5% of cells per cell cycle (Table 3.4A, Figure 3.15). The products of SIF-independent differentiation can be seen in the data, as differentiated forms are seen during day 4 in the data set presented here (Figure 3.12A-3.15A, blue).

In MacGregor et al., 2011, parasite density was measured by quantitative RT-PCR (qRT-PCR) of TbZFP3, while PAD1 expression by qRT-PCR was used as a measure of differentiated cells. The fit of the SIF-dependent model to the data was good, however the model line of best fit sits above some parasitaemia data points, i.e. there was a slight overestimation of parasite density, and the model line of best fit sits below some PAD1 expression data points, i.e. there was underestimation of PAD1 expression (Figure 3.10C). When the data from MacGregor et al., 2011 was reanalysed including an additional SIF independent term however, it was shown to have a better fit to the data (Figure 3.10D). AnTat1.1 parasites have a 1-2% probability of differentiating per cell cycle (data not shown), less than the AnTat1.1 90:13 cell line used in this work (Table 3.4A). SIF-independent differentiated forms appear slightly later in the MacGregor data set, by day 5 (Macgregor et
al., 2011). This could be due to strain differences and culture adaptation of the AnTat1.1 90:13 cell line in the slender form. The SIF-independent model was also the preferred model when mathematically assessed by the AIC (Table 3.3).

### Table 3.3 AIC analysis for comparison of models used in MacGregor et al., 2011

<table>
<thead>
<tr>
<th>Model</th>
<th>AIC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Without SIF independent differentiation</td>
<td>449.5</td>
</tr>
<tr>
<td>With SIF independent differentiation</td>
<td>419.1</td>
</tr>
</tbody>
</table>

**3.3.8.3 Fitting alternative models to the data**

Several alternative models were also fit to the data to see if they offered a different interpretation of the data.

A model with a SIF independent term included was retained, but intermediate forms were classed as slender forms. This model C does not fit the data well (Figure 3.11A). Regarding both parasitaemia data and morphology scoring, the majority of the residual means are outside of the 95% predictive intervals than are inside. The trend is also non-random. This results in model C underestimating the slender density at multiple time points, apart from during day 6, when slender density would be overestimated. The converse is true for stumpy density. Parasitaemia is underestimated at the start of the infection, and begins to be overestimated by day 6 once cell cycle-arrested intermediate forms have peaked, and then is underestimated again by day 7 once the stumpy forms have emerged and plateaued.

The generation of mathematical models for biological phenomenon needs to guided by biological fact, and it is quite difficult to justify the categorisation of intermediate forms as slender undifferentiated forms, as intermediates have begun to differentiate to stumpy forms, showing both morphological and biochemical alterations, for example they have begun to
transcribe stumpy specific PAD1 mRNA and are cell cycle arrested. Hence model C was not analysed further; it was deemed unrepresentative of the data and the biological situation.

A model similar to the model used in MacGregor et al., 2011 was also tried, i.e. with intermediates classed as themselves, but again we included a term for SIF independent differentiation. This is labelled model D. In this publication, qRT-PCR of PAD1 was included in the model as a readout of differentiation, i.e. intermediate and stumpy forms were grouped together as differentiated cells. The data did not include separate intermediate and stumpy categories. As a consequence, the parameter estimates generated for intermediate form lifespan and intermediate form immune clearance were concluded to be inaccurate and disregarded as the model indicated there was insufficient information provided to inform reliably.

The measure of differentiation in the data set presented in this thesis is morphology, hence we do have intermediate form-specific data. The fit is better for model D (Figure 3.11B) than for model C detailed above (Figure 3.11A). The residual mean is more randomly distributed and keeps within the 95% predicted intervals more consistently. The residual analysis for the parasitaemia data and for the proportion of slender cells is very similar to the analysis of the SIF independent model (Figure 3.10B). However model D gives more of an underestimation of parasitaemia density at earlier time points. Additionally, although it still underestimates slender proportion at day 5, it does not underestimate slender proportion at day 7. Instead it overestimates slender forms at day 6. The main issues with model D are the intermediate and stumpy form proportions. There is overestimation of intermediate proportion at days 5 and 6, with underestimation at day 7. Stumpy forms are overestimated at early time points of the infection, until day 5, and are underestimated during day 6. For model D to be accurate, the data would need to show stumpy forms emerging by day 3, however intermediates emerge at this point, with stumpy forms not appearing until day 5.
Biologically speaking, the basis of model D is accurate as the intermediate form is morphologically and biochemically distinct from the slender and stumpy forms, for example the intermediate form expresses PAD1 mRNA, and the stumpy form expresses PAD1 protein. However, the inaccuracy of the fit of model D may come from the error incorporated into collection of the data. There is a wide spectrum of intermediate morphologies, and morphologically speaking a late slender form or an early stumpy form could have been classed as intermediates inconsistently. Taking this into account, it would be more accurate to apply a looser model to the data; the model where intermediates are classed as stumpies (as in Figure 3.10A, B, models A and B) lowers the error, as late stumpy forms will be classified in this sole category rather than one of the other two. There is also biological relevance in grouping these two categories together, as discussed previously.

For interest, another model has also been included, model E. This model has two subpopulations of slender cells: one subpopulation differentiates in a SIF dependent manner into intermediate and stumpy forms, whereas the second only differentiate at the background rate, and terminate at intermediate forms. This model was originally detailed in Savill and Seed, 2004, where it was found to have a very good fit to the data. Residual analysis on model E fitting to the data presented in this thesis is shown in Figure 3.11C, which reveals a similar fit to model D (Figure 3.11B). The only major difference is that the stumpy population is not overestimated at the early stages of the infection, unlike model D, meaning that there is a better fit of model E to the data than model D.

There is however no biological basis for this model as there is no experimental evidence for two subsets of slender form cells. Hence, although the fit of this model is an interesting observation, this does not definitively mean the model is correct in its prediction, and this model was not further used in the subsequent work. Savill and Seed (2004) concluded that the good fit of this model may be merely a consequence of having an intermediate class of cells.
3.3.9 Infection dynamics as predicted from models with and without SIF independent differentiation term

The fits of the models to the parasitaemia and morphology data are shown in Figures 3.12-3.15. The fits are generally good and, as discussed above, are significantly better for the model with a term for SIF independent differentiation.

The models’ lines of best fit are able to capture differences between kDNA$^+$ and AK cells lines. Firstly, a longer plateau in cell density at peak parasitaemia in seen and is recognised by the models in kDNA$^+$ cells (Figure 3.12-3.15, red panel). AK cells however have on average a shorter cell density plateau that is again recognised by both models. Secondly, regarding the slender density, kDNA$^+$ cells are seen to have a second peak in parasitaemia around day 7 (Figure 3.12-3.15, purple panel). This peak is seen in the data and is recognised by the models. This second peak is completely absent in AK cells. Thirdly, regarding the stumpy form density, the fall in stumpy density is faster in rate in kDNA$^+$ cells, but begins at a later time point, around between days 7-8. This is in comparison to AK cells, where the stumpy density falls at a slower rate, but occurs between days 6-7.

The models allow for SIF concentration to be estimated during the infection, as SIF induces differentiation of slender forms at a rate proportional to its concentration. SIF cannot be experimentally measured as its identity is unknown, so the SIF concentrations in Figure 3.12-3.15 (blue panel) are predictions. The pattern of SIF concentration follows the slender cell density: as slender forms proliferate, SIF begins to rise, which increases the rate of differentiation, and stumpy forms begin to emerge. As the number of slender forms thus decreases, SIF concentration then falls, causing the rate of differentiation to also fall. The models predict therefore that the drop in parasitaemia is due to SIF-induced differentiation to
the stumpy form. However in kDNA$^+$ cells, a second peak in parasitaemia emerges around day 7, when a reduced SIF concentration allows slender cells to proliferate again, causing SIF concentration to rise, and slender density to fall again due to the increased rate of differentiation. With AK cells this does not occur, this will be discussed subsequently. This second peak in parasite density has also been described by MacGregor et al., 2011.

3.3.10 Parameter estimation using models with and without SIF independent differentiation term

As discussed above, the mathematical models contain various parameters, some of which can be estimated based on prior knowledge, and some of which are unknown. Estimate for these unknown parameters can be obtained by applying the models to our data and varying these parameters until a parameter distribution is reached that best explains the data. This approach also allows a quantification of the differences between genotypes WT/WTγ, WT/L262Pγ, WT/L262Pγ (AK) 1, WT/L262Pγ (AK) 2, that are apparent by comparing Figures 3.12 – 3.15.
Table 3.4  A comparison between the average parameter estimates for all cell lines

The parameter estimates from (A) the model including SIF independent differentiation and (B) the model including only SIF dependent differentiation, for the 4 mice for each infection group were averaged from the mean parameter values for each mouse.

## A

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Mean +/- S.E.M. (+SIF INDEPENDENT DIFFERENTIATION)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>WT/WTγ</td>
</tr>
<tr>
<td></td>
<td>WT/L262Pγ</td>
</tr>
<tr>
<td></td>
<td>WT/L262Pγ (AK) 1</td>
</tr>
<tr>
<td></td>
<td>WT/L262Pγ (AK) 2</td>
</tr>
<tr>
<td>Slender doubling time (hr)</td>
<td>6.70 ± 0.49</td>
</tr>
<tr>
<td></td>
<td>6.77 ± 0.26</td>
</tr>
<tr>
<td></td>
<td>6.93 ± 0.32</td>
</tr>
<tr>
<td></td>
<td>4.85 ± 0.09</td>
</tr>
<tr>
<td>SIF dependent differentiation rate ((cells/ml)/hr)</td>
<td>2.12E-09 ± 1.42E-10</td>
</tr>
<tr>
<td></td>
<td>2.16E-09 ± 1.30E-10</td>
</tr>
<tr>
<td></td>
<td>5.59E-10 ± 8.07E-11</td>
</tr>
<tr>
<td></td>
<td>2.27E-09 ± 3.87E-10</td>
</tr>
<tr>
<td>Log₁₀ initial slender concentration at t=0 (cells/ml)</td>
<td>3.58 ± 0.42</td>
</tr>
<tr>
<td></td>
<td>3.29 ± 0.14</td>
</tr>
<tr>
<td></td>
<td>3.29 ± 0.20</td>
</tr>
<tr>
<td></td>
<td>1.57 ± 0.12</td>
</tr>
<tr>
<td>Start time of immune response (hr)</td>
<td>178.86 ± 4.32</td>
</tr>
<tr>
<td></td>
<td>179.83 ± 1.16</td>
</tr>
<tr>
<td></td>
<td>145.78 ± 4.41</td>
</tr>
<tr>
<td></td>
<td>143.75 ± 4.57</td>
</tr>
<tr>
<td>Duration of committed slender form (hr)</td>
<td>15.21 ± 1.27</td>
</tr>
<tr>
<td></td>
<td>15.18 ± 0.14</td>
</tr>
<tr>
<td></td>
<td>2.27 ± 1.33</td>
</tr>
<tr>
<td></td>
<td>5.73 ± 1.44</td>
</tr>
<tr>
<td>Duration of stumpy form (hr)</td>
<td>56.39 ± 4.67</td>
</tr>
<tr>
<td></td>
<td>62.00 ± 1.31</td>
</tr>
<tr>
<td></td>
<td>48.60 ± 1.15</td>
</tr>
<tr>
<td></td>
<td>35.75 ± 4.05</td>
</tr>
<tr>
<td>Total committed lifespan (hr)</td>
<td>71.58 ± 4.64</td>
</tr>
<tr>
<td></td>
<td>77.17 ± 1.34</td>
</tr>
<tr>
<td></td>
<td>50.93 ± 2.22</td>
</tr>
<tr>
<td></td>
<td>41.46 ± 3.55</td>
</tr>
<tr>
<td>Immune clearance rate of slender forms (/hr)</td>
<td>0.36 ± 0.11</td>
</tr>
<tr>
<td></td>
<td>0.24 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>0.16 ± 0.002</td>
</tr>
<tr>
<td></td>
<td>0.20 ± 0.01</td>
</tr>
<tr>
<td>Immune clearance rate of stumpy forms (/hr)</td>
<td>0.24 ± 0.08</td>
</tr>
<tr>
<td></td>
<td>0.17 ± 0.02</td>
</tr>
<tr>
<td>Committed slender replications</td>
<td>2.26 ± 0.05</td>
</tr>
<tr>
<td></td>
<td>2.25 ± 0.09</td>
</tr>
<tr>
<td></td>
<td>0.40 ± 0.20</td>
</tr>
<tr>
<td></td>
<td>1.17 ± 0.29</td>
</tr>
<tr>
<td>Probability of SIF independent differentiation per cell cycle (%)</td>
<td>4.94 ± 0.47</td>
</tr>
<tr>
<td></td>
<td>4.58 ± 0.59</td>
</tr>
<tr>
<td></td>
<td>5.78 ± 0.75</td>
</tr>
<tr>
<td></td>
<td>5.86 ± 0.94</td>
</tr>
</tbody>
</table>

## B

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Mean +/- S.E.M. (SIF DEPENDENT DIFFERENTIATION)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>WT/WTγ</td>
</tr>
<tr>
<td></td>
<td>WT/L262Pγ</td>
</tr>
<tr>
<td></td>
<td>WT/L262Pγ (AK) 1</td>
</tr>
<tr>
<td></td>
<td>WT/L262Pγ (AK) 2</td>
</tr>
<tr>
<td>Slender doubling time (hr)</td>
<td>8.39 ± 0.53</td>
</tr>
<tr>
<td></td>
<td>8.39 ± 0.17</td>
</tr>
<tr>
<td></td>
<td>8.22 ± 0.15</td>
</tr>
<tr>
<td></td>
<td>6.70 ± 0.40</td>
</tr>
<tr>
<td>SIF dependent differentiation rate ((cells/ml)/hr)</td>
<td>1.78E-09 ± 1.11E-10</td>
</tr>
<tr>
<td></td>
<td>1.79E-09 ± 9.07E-11</td>
</tr>
<tr>
<td></td>
<td>5.78E-10 ± 6.16E-11</td>
</tr>
<tr>
<td></td>
<td>1.13E-09 ± 3.82E-10</td>
</tr>
<tr>
<td>Log₁₀ initial slender concentration at t=0 (cells/ml)</td>
<td>4.27 ± 0.29</td>
</tr>
<tr>
<td></td>
<td>4.06 ± 0.10</td>
</tr>
<tr>
<td></td>
<td>3.74 ± 0.13</td>
</tr>
<tr>
<td></td>
<td>3.33 ± 0.34</td>
</tr>
<tr>
<td>Start time of immune response (hr)</td>
<td>180.79 ± 3.10</td>
</tr>
<tr>
<td></td>
<td>181.40 ± 0.33</td>
</tr>
<tr>
<td></td>
<td>146.84 ± 3.85</td>
</tr>
<tr>
<td></td>
<td>134.69 ± 4.24</td>
</tr>
<tr>
<td>Duration of committed slender form (hr)</td>
<td>17.72 ± 1.13</td>
</tr>
<tr>
<td></td>
<td>17.66 ± 0.57</td>
</tr>
<tr>
<td></td>
<td>0.46 ± 0.21</td>
</tr>
<tr>
<td></td>
<td>0.97 ± 0.80</td>
</tr>
<tr>
<td>Duration of stumpy form (hr)</td>
<td>57.80 ± 5.08</td>
</tr>
<tr>
<td></td>
<td>60.60 ± 3.54</td>
</tr>
<tr>
<td></td>
<td>50.83 ± 1.44</td>
</tr>
<tr>
<td></td>
<td>44.77 ± 2.7</td>
</tr>
<tr>
<td>Total committed lifespan (hr)</td>
<td>75.52 ± 4.97</td>
</tr>
<tr>
<td></td>
<td>78.25 ± 3.13</td>
</tr>
<tr>
<td></td>
<td>51.38 ± 1.51</td>
</tr>
<tr>
<td></td>
<td>45.83 ± 3.15</td>
</tr>
<tr>
<td>Immune clearance rate of slender forms (/hr)</td>
<td>0.37 ± 0.11</td>
</tr>
<tr>
<td></td>
<td>0.24 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>0.15 ± 0.003</td>
</tr>
<tr>
<td></td>
<td>0.18 ± 0.01</td>
</tr>
<tr>
<td>Immune clearance rate of stumpy forms (/hr)</td>
<td>0.25 ± 0.08</td>
</tr>
<tr>
<td></td>
<td>0.17 ± 0.02</td>
</tr>
<tr>
<td>Committed slender replications</td>
<td>2.11 ± 0.04</td>
</tr>
<tr>
<td></td>
<td>2.11 ± 0.07</td>
</tr>
<tr>
<td></td>
<td>0.07 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>0.16 ± 0.12</td>
</tr>
</tbody>
</table>
3.3.10.1 Differentiation

The average SIF dependent differentiation rates of cells expressing WT/WTγ, WT/L262Pγ and WT/L262Pγ (AK) 2 are predicted to be similar, at around 2.20E-9 cells/ml/hour, whereas AK clone 1 is less sensitive to SIF dependent differentiation, with a rate of 5.60E-10 cells/ml/hour (Table 3.4A, Figure 3.16A). This might explain the delayed differentiation of this clone (Figure 3.5).

For the model that only considers SIF dependent differentiation, the same general pattern is observed, but rates are on average lower (Table 3.4B). Data for individual mice within each cohort are usually in good agreement, although there is more variation in the median values for the mice infected by AK clone 2 than for other cell lines (Figures 3.16 and 3.17).

3.3.10.2 Cell cycle duration

The average doubling times predicted by the SIF independent model are consistent at around 6.8 hrs for cells expressing WT/WTγ, WT/L262Pγ and WT/L262Pγ (AK) 1, with some variation between mice of the same group (Table 3.4A, Figure 3.16I). However, the doubling time for the second AK clone is shorter, with this clone having a faster doubling time of 4.8 hrs. Predictions with the SIF-dependent model follow the same pattern, but have all increased by approximately 20%, to around 8.3 hrs and 6.7 hrs, respectively (Table 3.4B, Figure 3.17G).

The study by MacGregor et al., estimated cell cycle duration to be approximately 5 hours, lower than both estimates here, with peak parasitaemia being reached at an earlier time point, by day 4/5 post infection. In contrast to that study, the cell line used in this present work, AnTat1.1 90:13, is culture-adapted, which could explain the longer cell cycle duration in vivo. The estimates with the SIF independent model are in good agreement with other
estimates from previous models (Savill and Seed, 2004, Seed and Black, 1999), but it is important to remember that these studies all used different strains and models, so comparisons are difficult.

The initial slender concentration upon infection is predicted by the SIF independent model to be relatively consistent between mice infected with each cell line (Figure 3.16G), and correlates well with the predictions for slender doubling time, as would be expected (Table 3.4A, Figure 3.16I). Mice infected with AK clone 2 seemed to be infected with fewer cells than mice infected with other cell lines (Table 3.4A).

The SIF dependent model predicts more consistent initial cell numbers across all cell lines (Figure 3.16E). This model predicts a differing pattern of initial cell numbers infected into mice, with mice infected with a higher number of WT/WTγ / WT/L262Pγ cells than mice with AK clone 1, which has a higher level of initial infection than AK clone 2. This pattern could contribute towards the lower slender proliferation rate in both AK clones compared to kDNA+ cells (Figure 3.8B, C).

The variability between predicted initiating numbers of cells is unexpected, as all mice were infected with approximately 1000 cells, with all cultures counted by Coulter counter. One potential source of variability between mice is the IP injection, as there will be varying numbers of cells actually reaching the bloodstream from each of these injections. Differences between cell lines could be because the four mice infected with each cell line were infected with cells from the same culture. Additionally, the volume of blood in a mouse was estimated to be 1 ml for the purpose of this model; the volume of blood for each mouse or size of the mouse was not measured, and will have varied from this value hence the infecting concentration will vary from these predicted values.
3.3.10.3 Differentiated AK cells are predicted to have a shorter lifespan than kDNA$^+$ cells

The lifespan of the cells from differentiation commitment to cell death can be estimated from these models; this is broken down into duration of the committed slender form, the time taken between a committed slender cell receiving the SIF signal and entering cell cycle arrest as the intermediate form, and lifespan of the stumpy form. As intermediates and stumpies are combined for the purpose of this model the latter is the time between the cell entering cell cycle arrest in the intermediate form and stumpy cell death. Both models predict a significant difference between the total committed lifespan of kDNA$^+$ and AK cells (Figure 3.15L, 3.16J); kDNA$^+$ cells have a committed lifespan of approximately 71 - 78 hrs (in good agreement with MacGregor et al., (2011)), whereas AK cells are predicted to have a committed lifespan of 41 – 51 hrs, about 30 hrs less (Table 3.4A and B).

For cells expressing WT/WTγ and WT/L262Pγ, duration of committed slender forms is estimated at approximately 15 hrs by both models (Table 3.4A, B). The results for individual mice are similar (Figure 3.15J, 3.16H). The number of committed slender replications, that is the number of cell cycle divisions that a committed cell goes through before entering cell cycle arrest, is consistent between these two clones with both models predicting ~2 replications (Table 3.4A, B).

For AK clones 1 and 2, the duration of the committed slender form is much lower for both models, with the SIF independent model predicting a duration of 2 and 6 hrs, respectively (Table 3.4A), and the SIF dependent model predicting < 1 hr (Table 3.4B). However, the parameter distribution estimates by the SIF independent model of mice 1 and 4 infected by AK clone 1 include zero, indicating there is not enough information to determine this parameter accurately (Figure 3.16J). Disregarding these two replicates, the average committed slender life span becomes 4.05 ± 2.10 hrs. For the SIF dependent model, all but
one prediction includes the value zero for mice infected with AK clones (Figure 3.17H). This suggests that the model predicts these clones are not cleared by the immune system, and instead are cleared by differentiation to the intermediate form. The number of committed slender replications for AK clone 1 is on average < 1 replication, and for clone 2 is 1 replication for the SIF independent model (Table 3.4A, Figure 3.16H). With the SIF dependent model, both AK clones are predicted to not undergo a further cell cycle division before entering cell cycle arrest (Table 3.4B, Figure 3.17F).

For both of these parameters in both models, there is considerable variability between mice infected by the same AK strain. For example, for AK clone 2, using the SIF independent model the duration of the committed slender form is predicted to be between 3 and 9 hrs (Figure 3.16J). Nonetheless, we can conclude that slender kDNA+ cells are estimated to go through at least one further cell cycle division before entering cell cycle arrest as the intermediate form compared to AK cells.

There is a similar pattern for lifespan of the stumpy form. For cell lines WT/WTγ and WT/L262Pγ the models estimated an average lifespan of approximately 56 - 62 hrs (Table 3.4A, B). The median lifespan for kDNA+ cells calculated from the distribution per mouse are relatively consistent, however there is quite a large spread in the distributions (Figure 3.16K, 3.17I). For AK cells the predicted average stumpy lifespan is clearly lower, ranging from 35 – 51 hrs, depending on model and cell line (Table 3.4A, B). For both models, there is some variation in the predicted median values per mouse for these AK clones, however the distribution of predictions shows very little spread (Figure 3.16K, 3.17I). Predicted and experimental values of stumpy form lifespan have ranged between 24 hrs and 72 hrs previously (Black et al., 1982, Turner et al., 1995), with the model from MacGregor et al., (2011) predicting the total differentiated cell lifespan of AnTat1.1 cells, i.e. intermediate forms plus stumpy forms, to be 56 hrs, correlating well with the estimations of WT/WTγ cells from these models.
3.3.10.4 AK cells are predicted to activate the mouse immune system at an earlier time point

Both models include separate terms for the immune system action on slender and stumpy form cells. With the SIF independent model, the immune response is predicted to be activated in infections with either type of kDNA\(^+\) cells at approximately 179 hrs (7.5 days) post infection (Table 3.4A). This result is very consistent between mice, with the exception of mouse 2 from infection with WT/WT\(\gamma\) cells which has a lower activation time of 7 days (Figure 3.15D). However, both AK cell lines have an average estimated immune system activation time of 144 hrs (6 days) (Table 3.4A). The results are very similar for the model with SIF dependent differentiation (Table 3.4B), however AK clone 2 is predicted to activate on average slightly earlier, towards the end of day 5. There is greater variation between the median activation times in the mice for clones 1 and 2, however there is no overlap between values estimated for kDNA\(^+\) compared to AK cell lines for either model (Figure 3.15D, 3.16B). The earlier activation of the immune system in mice infected with AK cells could explain the lack of second peak in slender form density (Figure 3.12, purple panel). The models predict that AK slender cells are cleared by both SIF-induced differentiation and the immune system, preventing an increase in slender numbers once SIF concentration has fallen, whereas kDNA\(^+\) cells only activate the immune system during the second peak in parasite density, and as such are incompletely cleared by just SIF-induced differentiation in the first slender peak, but by both methods in the second peak. The model in MacGregor et al., 2011, estimated an immune system activation by day 6, however the parasitaemia data incorporated into that model showed a faster onset of the infection, meaning a plateau in cell numbers had been reached by day 5, whereas in the present study, the plateau was reached by day 6, with immune activation similarly a day later at day 7.
The immune clearance of slender cells can be estimated. Here, with both models there is an overlap between the range of the distributions predicted for kDNA$^+$ and AK cell lines (Table 3.4A, B). There are also different degrees of variability in the values from each group of mice (Figure 3.15E, 3.16C). For example, mice 1 and 3 infected with strain WT/WT$\gamma$ have a higher rate of clearance, at 55% per hour, compared to mice 2 and 4, at <20% per hour. This could indicate that for mice 1 and 3 immune clearance has a more significant impact on slender form lifespan than for mice 2 and 4, where clearance of slender forms may have been predominantly due to differentiation. Indeed, looking at the fit of the model to the parasitaemia data (Figure 3.12, red panel), it looks as if a steep fall in parasite density occurs upon activation of the immune system in these mice, whereas mice 2 and 4 the steep fall in parasite density occurs before or after day 7, i.e. independently of activation of the immune system.

3.3.10.5 AK stumpy cells clearance is predicted to be independent of the immune system

The immune clearance of stumpy cells does however show a clear difference between the response to kDNA$^+$ and AK cells (Table 3.4A, B). While both models predict a similar and consistent clearance rate for kDNA$^+$ stumpy cells, at around 20% per hour (Figure 3.15F, 3.16D), both models predict that immune clearance is not required to explain the data for AK clones 1 or 2, thus the immune system does not act to clear AK stumpy cells from the blood. This could explain the slower rate of clearance of AK clones from the mouse blood over time compared to kDNA$^+$ cell lines. As it is unlikely that these AK stumpy forms could be less immunogenic, and as the model has predicted a more limited lifespan compared to kDNA$^-$ cell lines, it suggests that AK stumpy forms are cleared solely via cell senescence, not through the actions of the immune system.
Investigating the rates of clearance per mouse for kDNA\(^+\) cells, the slender form of these cells are roughly twice as sensitive to immune clearance as stumpy forms (Table 3.4A, B).

### 3.3.11 AK cells do not have a second slender peak at day 7

A verification of the observation that infections with kDNA\(^-\) cells have a second peak in slender form parasitaemia at around day 7 post infection was carried out using cell cycle analysis. Cells that had been fixed and stored in glycine were washed and DAPI stained, and flow cytometry was performed on these samples. As stumpy cells are arrested in the cell cycle, they do not enter S or G\(_2\) phase, instead remaining with the DNA content found in G\(_1\). The percentage of intact alive cells in G\(_2\) phase of the cell cycle was quantified for each time point and these results were plotted, allowing a visualisation of the percentage of slender cells in the populations over time. Figure 3.17 shows the fall in % of slender cells until day 6 of the infections. From here, the percentage of slender cells in infections consisting of AK *T. brucei* continues to fall. In kDNA\(^+\) cells, however, the percentage of slender cells increases at day 7, and then falls again at day 8. The percentage of slender kDNA\(^-\) cells then settles at a level higher than that of AK cells.

### 3.3.12 Absence of kDNA can be associated with loss of \(\Delta\Psi_m\)

in stumpy forms

As the model predicted a reduced lifespan for AK stumpy forms, \(\Delta\Psi_m\) was measured using a TMRE assay. It is not clear how the \(\Delta\Psi_m\) is generated in stumpy forms, although it has been reported to be sensitive to complex I inhibitors but insensitive to the F\(_{1}\)F\(_{0}\) inhibitor oligomycin (Bienen et al., 1991). It is also unclear whether \(\Delta\Psi_m\) is required for stumpy
viability, although it is essential for viability of slender form *T. brucei* and other proliferating cells (Schnaufer et al., 2005). TMRE is positively charged dye that accumulates and fluoresces in active mitochondria in a concentration proportional to the negative charge present in the matrix due to the $\Delta \Psi m$. Thus, TMRE staining provides a quantitative but indirect measurement of $\Delta \Psi m$ that can be compared between cell lines. FCCP is an ionophore that uncouples the $\Delta \Psi m$ by transporting protons across the inner mitochondrial membrane independently of $\Delta \Psi m$. Hence, cells treated with FCCP and TMRE will allow a measurement of the baseline TMRE signal in the presence of an uncoupled $\Delta \Psi m$.

Cells were harvested from infected mice at maximum parasitaemia, with a proportion of approximately 90% stumpy cells. Figure 3.18A shows the TMRE staining of this population of cells, +/- FCCP. All cell lines treated with FCCP (red) display low fluorescence, close to the background level of cells not treated with TMRE (blue). The ‘$\Delta \Psi m$ negative’ gate was set to include all counts up to the maximum fluorescence detected for WT/WT $\gamma$ cells +FCCP. All counts to the right of this gate were defined as ‘$\Delta \Psi m$ positive’.

Across the cell lines, there is a differing pattern of TMRE staining (orange peak) in Figure 3.18A. WT/WT $\gamma$ cells have a single peak of $+\Delta \Psi m$ cells, WT/L262P $\gamma$ cells and WT/L262P $\gamma$ (AK) 1 cells have two peaks, only one of which is clearly in the $+\Delta \Psi m$ gate, and WT/L262P $\gamma$ (AK) 2 cells have one peak that superimposes onto the +FCCP peak (red). Figure 3.18C quantifies the number of intact cells from the –FCCP +TMRE populations scored as ‘$\Delta \psi m$ positive’. Around 90% of WT/WT $\gamma$ stumpy cells and around 60% of WT/L262P $\gamma$ stumpy cells fell into that category. For the AK stumpy cells, clone 1 had around 45% cells with $\Delta \Psi m$, whereas clone 2 had no cells with $\Delta \Psi m$. Figure 3.18D compares the median TMRE fluorescence values for ‘$\Delta \psi m$ positive’ intact cells. Here, the TMRE staining of WT/WT $\gamma$ cells is set to 100%, and the median TMRE staining value for other cell lines are compared to the median value for WT/WT $\gamma$ cells. This gives an
approximate comparison between the strength of the ΔΨm across the cell lines. Interestingly, cells with WT/L262Pγ and WT/L262Pγ (AK) clone 1 have approximately the same strength of ΔΨm.

Figure 3.18B shows cell cycle analysis of –FCCP +TMRE stained stumpy cells. In green, a slender cell control is shown for each cell line for comparison. These show clear G1, S and G2 stages of the cell cycle. Stumpy cells are arrested in the G1 phase of the cell cycle (G0) (Shapiro et al., 1984). The WT/L262Pγ and WT/L262Pγ (AK) 2 cells have clear, single G1 peaks representing cells that are either ‘ΔΨm positive’ (orange, WT/L262Pγ) or ‘ΔΨm negative’ (red, WT/L262Pγ and WT/L262Pγ (AK) 2). Although the WT/WTγ and WT/L262Pγ (AK) 1 cells do show some ‘ΔΨm positive’ cells (orange) in S and G2 phase, indicating a minor contamination with slender cells, which can be expected to result in a small overestimation of ‘ΔΨm positive’ cells in these populations. Figure 3.18B also shows that all cells stained with –FCCP +TMRE that have no ΔΨm (red) are arrested in G1, but as they are intact we can say that, if stumpy cells require ΔΨm for viability, these are probably dying stumpy cells within that population.

From this we can say that there is a uniform, high ΔΨm across 80% of stumpy WT/WTγ cell lines; the orange peak in Figure 3.18A is tight, showing very little variation in level of TMRE staining. There are two populations of stumpy WT/L262Pγ cells, one with a higher ΔΨm than the other (Figure 3.18A, orange peaks). The median ΔΨm is lower than that of WTγ cells (Figure 3.18D), and less WT/L262Pγ cells have a ΔΨm (Figure 3.18C). Whether this is a consistent consequence of the presence of an L262Pγ allele is uncertain as only one WT/L262Pγ clone was analysed here, and the analysis has only been performed once. In WT/L262Pγ (AK) clone 1 there are also two populations of cells, one with no ΔΨm, and one with a ΔΨm that has a median value similar to that of WT/L262Pγ cells (Figure 3.18A, orange peaks, D). More than 40% cells are stained positively for TMRE however (Figure
3.18C and above). Interestingly, in WT/L262Pγ (AK) 2 stumpy cells no ΔΨm could be
detected with this assay (Figure 3.18A).

3.4 Discussion

Due to the lack of a clonal AK pleomorphic strain of T. brucei, it has previously proved
difficult to investigate the requirement of kDNA in the differentiation of slender cells to the
stumpy form. Additionally, stumpy forms are not easy to study in vitro: they are non-
proliferative and have a limited strain-dependent lifespan of approximately 48-72 hrs
(Turner et al., 1995). Generation of stumpy forms is time consuming and requires either
mice infections with the purification of trypanosomes from the blood, or differentiation in
vitro via an involved protocol by HMI-9 medium containing methylcellulose. Stumpy forms
do survive for a few days in HMI-9 and SDM-79 media, but whether these conditions
maintain the specific physiological status of stumpy forms is not known, and the
proliferation of slender forms that contaminate purified stumpy cell populations causes
additional complications. These challenges have resulted in limited knowledge on the
mitochondrial biology of stumpy forms and their metabolism.

Previous attempts to investigate potential functions of kDNA in stumpy cell formation,
viability and further differentiation into PCF cells have used DK cells generated through
continuous exposure to acriflavine over the course of 15 repeated passages though mice
(Stuart, 1971, Timms et al., 2002). The one clonal line that was produced was reported to
generate stumpy forms, but at a proportion 7x lower than the parental line (Stuart, 1971). A
non-clonal line containing DK slender forms was able to differentiate to the stumpy form
(Timms et al., 2002), as the DK slender forms did not have a ΔΨm, these cells were not
viable. From these results it was concluded that the slender to stumpy transition could occur minus kDNA, but whether it could occur at the same rate or resulted in the same proportion of stumpy forms was not known. A caveat in that study was that, as the DK forms were non-viable, differentiation was studied in cells only 24 hrs after acriflavine dosage. Thus, kDNA-encoded products were most probably still present in the mitochondria of these cells. Furthermore, the continuous exposure to a mutagen like acriflavine over 100 days as in Stuart (1971) is likely to cause secondary mutations, hence the observation that DK cells differentiated less efficiently could have been a product of non kDNA-related mutagenesis.

Three factors allowed the production of a clonal DK pleomorphic cell line in the present study: the discovery of mutations in the mitochondrial F₁Fₒ-ATPase γ subunit that confer kDNA-independence in slender BSF form T. brucei (Schnaufer et al., 2005, Lai et al., 2008, Dean et al., 2013), the production of a protocol allowing transfection of pleomorphic cell line AnTat1.1 90:13 (Macgregor et al., 2013) and the adaptation of this strain to in vitro culture (Federico Rojas, Matthews lab).

Here, an AK pleomorphic cell line was generated by the replacement of an endogenous ATPase γ allele with a mutated L262P version, and the deletion of kDNA during a 3 day exposure to acriflavine. Deletion of kDNA was confirmed to have no effect on growth rate in vitro. Mouse infections were performed with cell lines with the genotypes WT/WTγ, WT/L262Pγ and WT/L262Pγ (AK). This confirmed that a clonal AK cell line could differentiate to the stumpy form, agreeing with the results of Stuart (1971) (to some extent, see below) and Timms et al., (2002). Comparison with the kDNA⁺ cell lines showed that differentiation was as efficient minus kDNA as with kDNA, which disagrees with the result from Stuart (1971). This also disproves our initial hypothesis. As the cell line used in Stuart (1971) was subsequently found to have lost its pleomorphic character (Timms et al., 2002), it could be that the pleomorphism of this cell line could have been already deteriorating at the stage of the original analysis. Off target effects of acriflavine also cannot be ruled out.
A time course of differentiation was then performed to compare the rate of stumpy production over 10 days, with analysis of parasitaemia density and morphology by microscopy. Mathematical modelling was applied to this data, allowing estimation of parameters of the infections that are difficult to dissect experimentally. The $\Delta \Psi m$ of the stumpy forms of each cell line was also measured to further probe the characteristics of AK stumpy forms.

3.4.1 The characteristics of in vivo mouse infections with AK trypanosomes

In order to investigate the impact that a loss of kDNA had on trypanosome mouse infection dynamics, we infected two *T. b. brucei* clones retaining their kDNA and two AK clones into mice and followed the course of infection. The two clones with kDNA had different genotypes with respect to the ATPase $\gamma$ subunit locus, WT/WT$\gamma$ and WT/L262P$\gamma$. We detected no differences between the courses of these infections, either in the observed parasitaemia and morphological changes, or in the predicted model parameters. There is therefore no difference between the courses of infection with cells that are kDNA dependent or kDNA independent, presumably as long as kDNA-independent cells have the ability to express the gene products of kDNA at a similar level to WT. Hence, despite the fact that in WT/L262P$\gamma$ cell lines approximately half of the ATPase complexes only contribute to $\Delta \Psi m$ indirectly by powering the AAC (chapter 2), which is more inefficient than via $F_o$, this has no impact on the in vivo dynamics of this cell line.

There were some differences between the data for the two AK clones. As both AK clones were originally genetically identical as they were generated initially from the same transfection, any observed differences between AK clones could been adaptations after these
two clones had been separated or as a consequence of mutagenic acriflavine treatment. In order to see the impact of loss of kDNA, we are more interested in the characteristics the two AK clones have that are similar, as both AK clones lack kDNA to an equal extent (Figure 2.3).

The morphology changes observed are similar for kDNA+ and AK cells (Figure 3.5). The differentiation of clone AK 1 slightly lags behind, and modelling suggested this was due to a slower rate of differentiation (Table 3.4). However, AK clone 2 has a differentiation rate at the levels of WT/WTγ cells. This lower differentiation rate of AK clone 1 could be the result of adaptations whilst grown in in vitro culture or due to acriflavine-induced mutagenesis. Additionally, there is no impact on the maximum proportions of slender, intermediate and stumpy forms (Figure 3.5), despite the lower differentiation rate of AK clone 1, hence we can say that the absence of kDNA has no effect on the efficiency of slender to stumpy differentiation. This suggests that SIF manufacture, release and the signal transduction involved in receiving and responding to the SIF signal are unaffected by the loss of kDNA.

The rate of fall in parasitaemia for both AK clones is slower than the rate for kDNA+ cells (Figure 3.8B, C). Modelling suggested that this was due to the lower lifespan of cells as stumpy forms (Table 3.4), hence AK stumpy forms die via earlier cell senescence, rather than due to the immune system action as in kDNA+ stumpy forms.

In the slender form in vitro, AK T. b. brucei is able to grow at an equivalent rate to kDNA+ cells (Figure 3.1A). However in vivo, this is not the case. Parasitaemia counts during mouse infections indicated that the parasitaemia from both AK clones increases slower in comparison to kDNA+ cells (Figure 3.8B, C). Modelling suggested that less AK clone 2 cells for this clone were originally causing infection, and AK clone 1 was suggested to have a slower differentiation rate, neither of which explain the slower rise in parasitaemia (Table 3.4). However modelling also predicted that the immune system activates earlier against AK
slender forms than against kDNA+ cells (Table 3.4), which could explain the slower rise in parasitaemia.

In relation to this, AK cells did not have an observed 2nd peak of slender parasitaemia at day 7 (Figure 3.12-9, 3.17). This second slender peak is predicted to have occurred in infections with kDNA+ cells due to SIF dependent differentiation causing slender density and therefore SIF density to fall, allowing remaining slender form cells to begin proliferation rather than entering cell cycle arrest. Modelling predicted that this second peak did not occur in infections with AK cells due to the earlier activation of the immune system by day 5 (Table 3.4), meaning any remaining slender cells do not begin proliferating. In infections with kDNA+ cells, the immune system is predicted to have activated at day 7, clearing this second slender peak. The conclusions from the model have to be taken with some caution, as the parameter estimations only allow the prediction of the impact of changes on the course of parasitaemia. Experimental verification of any conclusions from the modelling, as in Fig. 3.17, will be required.

One of the most curious conclusions of the mathematical modelling is the differential activation of the immune system in infections with cells with and without kDNA. Although the slender clearance rates of kDNA+ and AK cells are predicted to be the same (Table 3.4), the time of immune system activation is estimated to be 2 days earlier in AK cells. There could be several reasons for this. i) AK cells could be less efficient at VSG switching or production, allowing clearance of slender cells to begin at an earlier stage of the infection. This inefficiency could possibly be due to a lack of kDNA, or an indirect effect of acriflavine, a DNA mutagen, on nuclear DNA. There is nothing known on the switch rate of T. b. evansi or T. b. equiperdum in comparison to T. b. brucei, so we cannot surmise from the naturally occurring subspecies whether lack of kDNA could have an effect. For both AK clones to be affected by the same nonspecific acriflavine-induced mutation would be unlikely, but as the process of VSG switching is highly complex, mutations could have
occurred in one of many proteins to decrease the efficiency of switching. The switch rate of AK cells could be assessed to investigate this theory, as in (Aitcheson et al., 2005) or (Kim and Cross, 2010). ii) Alternatively, AK cells could swim more slowly or in a different way. There could have been a decrease in energy levels due to a lack of kDNA, or again, an acriflavine-induced mutation in the machinery required to swim could have occurred, preventing the efficient clearance of antibody that is mediated by swimming (Engstler et al., 2007). Additionally, motility of the stumpy form could be assessed to see if AK cells swim in a different way to kDNA+ cells as in (Engstler et al., 2007).

There is evidence to suggest that, as mitochondria evolved from α-proteobacteria, and some bacterial DNA is able to stimulate the innate immune response via pattern recognition receptors (PRRs), mitochondrial DNA is also able to stimulate an inflammatory response in humans and mice (Zhang et al., 2010a, Collins et al., 2004, Oka et al., 2012). Although not investigated in trypanosomes, we could postulate that kDNA from dying slender cells could prime the immune system to a higher state of vigilance, increasing the efficiency with which cells are then cleared. In agreement with this, kDNA− slender cells are predicted to be cleared by the immune system at a faster rate than AK cells (Table 3.4). Equally, although the model predicts that AK stumpy form cells die quicker than kDNA+ cells via only cell senescence, the lack of kDNA could be preventing the immune system efficiently activating against stumpy cells. However, the immune system is predicted to be activated against AK slender cells earlier in an infection than against kDNA+ cells, disagreeing with this hypothesis.

### 3.4.2 The generation of ΔΨm in stumpy forms

Although we do not know for sure, it is likely that ΔΨm is essential for the survival of stumpy forms. There are cases in biology where the mitochondrion does not express its mitochondrial DNA in an attempt to escape DNA mutation by reactive oxygen species
(ROS) (De Paula et al., 2013a); jellyfish oocyte mitochondria downregulate their mitochondrion to prevent generation of ΔΨm or ROS, theoretically allowing increasing the fidelity of the mtDNA inherited in the female germline (De Paula et al., 2013b). However, in the T. brucei stumpy form there is upregulation of mitochondrial structure and function in preparation for the PCF stage (Vickerman, 1965). This is unlikely to occur if proteins cannot be imported into the mitochondrion in a ΔΨm-dependent fashion. Hence it is probably correct to suggest that if intact stumpy cells do not have ΔΨm, their mitochondria are not functioning well. The build-up of polypeptides on the cytosolic side of the mitochondria could also be detrimental to the cell, and the expression of polypeptides that will not be imported into the mitochondria is also a drain on energy, further affecting the health of AK stumpy forms. However, we do not know whether the natural death of stumpy cells is the cause of loss of ΔΨm or is an effect of ΔΨm loss.

Interestingly, replacing one allele of the γ subunit with an L262P mutant version does seem to have an impact on ΔΨm: compared to WT/WTγ cells, WT/L262Pγ cells have a decreased ΔΨm and a decrease in the number of cells with a ΔΨm (Figure 3.18). Despite this, the infection profile of these cells is identical to WTγ cells (Figure 3.5, 3.8) and the model does not predict any impact of the L262Pγ allele on stumpy lifespan (Table 3.4). However, it must be taken into account that this analysis has only been carried out on one WT/L262Pγ clone. Differences indicated by this single analysis may be accurate, but the analysis needs to be repeated.

It is understandable why slender WT/L262Pγ cells would have a decreased ΔΨm compared to WTγ cells (Chapter 2): assuming equal levels of γ allele expression, approximately half of the ATPase complexes are not contributing to ΔΨm via proton movement through F₀, only indirectly by powering AAC, which contributes only 1 proton per hydrolysed ATP to the ΔΨm, compared to an intact F₁F₀, which contributes 3.3H⁺/ATP (Fig. 2.20). Slender cells
proliferate, however, and so can adapt via mutation or via feedback loops to generate a higher $\Delta \Psi m$ if required. Stumpy forms cannot adapt to the same extent as they are arrested in $G_1$, have in general a repression of translation (Brecht and Parsons, 1998) and have a limited lifespan. Hence, in correlation with what is occurring in slender forms, a lower $\Delta \Psi m$-generating capacity in stumpy WT/L262P$\gamma$ cells compared to stumpy WT$\gamma$ cells can be explained if stumpy cells are generating $\Delta \Psi m$ by a decreased level of ATPase proton pumping. Other possibilities are that the levels of kDNA in this cell line are not at WT levels, and kDNA gene products other than $F_0$ subunit A6 are contributing to a reduced $\Delta \Psi m$ generation in stumpy forms. Also, at the time of harvesting, the WT/L262P$\gamma$ stumpy forms could have been further on in their limited lifespan than the WT/WT$\gamma$ stumpy forms that they are being compared with, explaining why a higher percentage have no $\Delta \Psi m$ and are dying.

The mathematical model estimated that the lifespan of AK cells was decreased compared to the lifespan of cells retaining kDNA (Table 3.4). At this point, it is not known whether the loss of $\Delta \Psi m$ in AK stumpy forms is the cause of the decrease in lifespan, or an effect of it. The two AK clones have a difference in their $\Delta \Psi m$ as stumpy forms. AK clone 1 has $>40\%$ stumpy cells with a $\Delta \Psi m$ around 40\% lower than WT/WT$\gamma$ cells. This $\Delta \Psi m$ was approximately equal to the $\Delta \Psi m$ seen in 60\% WT/L262P$\gamma$ cells. The remaining stumpy cells had no $\Delta \Psi m$. Virtually no cells in the population of AK clone 2 had a detectable $\Delta \Psi m$. Two factors make explaining these observations difficult. Firstly, without knowing how the $\Delta \Psi m$ is generated in WT/WT$\gamma$ stumpy forms it is difficult to be able to explain why $\Delta \Psi m$ would be affected by lack of kDNA. Secondly, comparing the same WT/L262P$\gamma$ clone before and after acriflavine treatment is essential, to rule out innate differences in $\Delta \Psi m$ between clones. Thus cell lines WT/L262P$\gamma$ and AK clone 2, as can be compared, as AK clone 2 is the acriflavine descendant of the WT/L262P$\gamma$ cell line here, but the kDNA$^+$ cell line of AK clone 1 was not analysed here, hence no firm conclusion can be made about the impact of loss of
kDNA on the \( \Delta \Psi \text{m} \) of AK clone 1 stumpy form. This experiment needs to be repeated adding in this cell line. Thirdly, we do not know the proportion of established stumpy cells in AK clones 1 and 2 when \( \Delta \Psi \text{m} \) was measured. It is possible that at the time of harvesting, the AK clone 2 stumpy forms could have been further on in their limited lifespan than the AK clone 1 stumpy forms that they are being compared with, explaining why a higher percentage have no \( \Delta \Psi \text{m} \).

The divergent result when measuring \( \Delta \Psi \text{m} \) in AK stumpy cells suggests two possibilities. (1) kDNA has a role in maintaining \( \Delta \Psi \text{m} \) in stumpy forms. The result for AK clone 2 agrees with this hypothesis, particularly when compared to its non-acriflavine treated progenitor WT/L262P\( \gamma \), which does generate \( \Delta \Psi \text{m} \). In this case, how can AK clone 1 generate a \( \Delta \Psi \text{m} \) that appears comparable to kDNA\( ^+ \) stumpy forms expressing WT/L262P\( \gamma \)? AK clone 1 could have a higher proportion of slender and intermediate forms present, or have differentiated later, meaning that the stumpy forms are earlier in their limited lifespan. In future, the proportion of PAD1 positive stumpy forms in a population could be assessed by \( \alpha \)PAD1 flow cytometry analysis. (2) kDNA does not have a role in maintaining \( \Delta \Psi \text{m} \) in stumpy forms. The fact that AK clone 1 does have a \( \Delta \Psi \text{m} \) supports this. However, how can we explain why AK clone 2 does not? This could be due to acriflavine-related damage to nuclear DNA. Analysis of other non-acriflavine treated AK clones will be crucial in order to conclude whether kDNA-encoded proteins maintain \( \Delta \Psi \text{m} \) in stumpy forms.

We can only postulate on the possible role of kDNA encoded proteins in generating \( \Delta \Psi \text{m} \), of which there are a few possibilities (Section 1.9.4). Complexes III and IV are not present in the stumpy form and so cannot contribute to \( \Delta \Psi \text{m} \) generation (Priest and Hajduk, 1994). (i) Complex I has not been proved to participate in the generation of \( \Delta \Psi \text{m} \) in BSF or PCF (Verner et al., 2011, Surve et al., 2012). However, in stumpy forms, the \( \Delta \Psi \text{m} \) was sensitive to the complex I inhibitor rotenone (Bienen et al., 1991), implicating complex I in the
generation of $\Delta \Psi m$ in stumpy forms (Bienen et al., 1991). Hence it would be expected that stumpy forms require RPS12, and ND1, 2, 3, 4, 5, 7, 8 and 9 to be expressed and edited in order to remain viable (Fig. 1.3). To investigate whether complex I participates in $\Delta \Psi m$ generation in the stumpy form, nuclearly-encoded subunits of complex I could be knocked out in the AnTat1.1 90:13 cell line. Complex I is evidently not required in slender to stumpy differentiation, as AK cells can differentiate to the stumpy form. If complex I is required in the stumpy form, a decrease in $\Delta \Psi m$ and lifespan would be predicted in its absence. (ii) The functions of the proteins encoded by MURF 2 and 5 and cytosine-rich template (CR) 3 and 4 are unknown, hence may have a role in $\Delta \Psi m$ generation. (iii) Complex V may function as an ATPase to contribute to $\Delta \Psi m$ generation, at least in early stumpy forms. The loss of kDNA-encoded A6 from complex V would prevent the generation of $\Delta \Psi m$ directly through $F_o$, forcing the $F_o$-independent mode of $\Delta \Psi m$ generation that depends on the electrogenic action of AAC to act (Fig. 1.8). This may not be feasible in stumpy forms. There are changes to the metabolic network of the cell in the stumpy form (Section 1.9.3). If a shift in metabolism required ATP to be exported from the mitochondrion in the stumpy form, for example due to the ATP/ADP balance across the mitochondrial membrane altering, AAC function would be forced to reverse, effectively starving the $F_1F_o$-ATPase of substrate, using $\Delta \Psi m$ rather than contributing to it.

3.4.3 Complex V direction in stumpy forms

It is known that complex V must switch from the function of an ATPase to an ATP synthase during differentiation from slender to PCF form (Nolan and Voorheis, 1990, Nolan and Voorheis, 1992). Complex V is also thought to act as an ATP synthase in established stumpy forms (Bienen et al., 1991) like in PCF (Fig. 1.3B). Hence it is not currently known when this does take place. This switch could occur during the slender to stumpy differentiation,
with stumpy forms having an ATP synthase functioning to generate more ATP for the upregulation in mitochondrial function required in preparation for the PCF. Such a switch could not occur in AK cells as, firstly, without subunit A6, an ATP synthase would be incomplete and non-functional, and secondly, this scenario would require generation of the $\Delta \psi_m$ by respiratory complex I, which would also be absent in AK cells. In this case, it is unlikely that stumpy forms would occur at all, hence the data presented here argues against complex V transition occurring during slender to stumpy differentiation.

A shift in direction of complex V after stumpy formation would be more consistent with the results presented here. AK slender cells are able to differentiate to stumpy forms, presumably while utilising their alternative way of manufacturing the $\Delta \psi_m$ via AAC and ATPase-$F_1$. However, when they reach the stumpy form, the transition does begin. The $\Delta \psi_m$ in stumpy forms is unaffected by oligomycin, suggesting that complex V is present as an ATP synthase in the stumpy form, at least at late stages (Bienen et al., 1991). One hypothesis of how this occurs could be the expression of IF$_1$ during the stumpy form. This protein binds complex V, inhibiting ATPase activity, and forcing ATP synthesis activity in the presence of an alternative proton pumping $\Delta \psi_m$ source (Walker, 1994). This alternative $\Delta \psi_m$ source could be complex I in the stumpy life cycle stage (Bienen et al., 1991). IF1 is known to not be expressed in the T. b. brucei BSF, but expressed in PCF (A. Zivoka, unpublished). Its status in the stumpy form is unknown. Initially, an expression profile of IF$_1$ mRNA and protein through differentiation could be established in the cell lines presented here. If IF$_1$ is expressed earlier in AK clone 2 than in AK clone 1, AK clone 1 would generate $\Delta \psi_m$ via the ATPase-$F_1$ and AAC for a longer period of time. This could explain why AK clone 1 retains some $\Delta \psi_m$. If expression during the stumpy form is shown, proof would be required of its function in reversing the direction of complex V during the stumpy form. A conditional knockout of IF$_1$ could be produced in BSF, allowing an assessment on the ability of in vivo generated stumpy forms to differentiate into the PCF. An IF$_1$ knockout in the AK cell lines
could be predicted to lengthen the lifespan of these stumpy cells, prolonging the generation of $\Delta \Psi m$ using AAC and ATPase-$F_1$.

3.4.4 T. b. evansi is monomorphic not due to a loss of kDNA

As cells that have had their kDNA deleted are able to make the transition from slender to stumpy form as efficiently as cells that have their kDNA intact, we can conclude that the absence of kDNA is not the primary reason why strains of T. b. evansi and T. b. equiperdum are generally only found in the slender life cycle form.

An adaptation must have been selected for in naturally occurring AK T. brucei subspecies that prevents cells differentiating reliably to the stumpy life cycle form. This could have been a loss of components of the SIF secretion pathway or stumpy induction pathway. This is similar to monomorphic BSF forms that are reported to be unresponsive to SIF (Vassella et al., 1997). One strain of T. b. evansi, STIB805, has had its genome sequenced (Carnes et al., 2015), and so could be investigated to see if there is a defect in genes encoding proteins required for differentiation in the genome. However the full repertoire of proteins needed for slender to stumpy differentiation is not known: identity of SIF is unknown, and although some components of the stumpy induction pathway have recently been elucidated (Mony et al., 2014), the full pathway is not currently understood. Additionally, once the identity of SIF is elucidated, naturally occurring DK subspecies could be analysed to see if they do secrete SIF.

Understanding the primary molecular reason why T. b. evansi is unable to differentiate to the stumpy form could be compounded by the evolutionary time between the divergence of T. b. brucei and T. b. evansi. It is probable that further adaptations have occurred in now non-essential genes for life cycle progression, for example mutations, segment deletion and
reduced copy number in the procyclin loci have been found in *T. b. evansi* STIB805 (Carnes et al., 2015).

Additionally the fact that some AK strains have been reported to have some limited capacity to produce stumpy forms (for example Hoare, 1956) could be due to the currently hypothetical SIF independent background differentiation that we included in our model (discussed below).

We now know that AK cells are able to differentiate into stumpy forms. The stumpy form does represent a dead end if the parasite cannot progress to the fly life cycle stages, so biologically it does make sense that a proliferative slender form is retained, and that this is the form mechanically transmitted to the next mammalian host. What could have been the driving force that caused the loss of differentiation to the stumpy form in naturally occurring DK subspecies? SIF-dependent differentiation to the stumpy form places a limit on the maximum proliferating slender parasitaemia level, presumably to retain the viability of the host. Adjusting this maximum limit on parasitaemia by preventing slender to stumpy differentiation could increase the efficiency of mechanical transmission of parasites; it is thought that high prolonged parasitaemia could be required as this increases the density of parasites sticking to the mouthparts of the fly (Desquesnes et al., 2009). If loss of kDNA does not cause a decrease in differentiation, then what does?

The adoption of monomorphism in slender cells *in vitro* involves the faster growth of cells, causing outgrowth and selection for cells that do not respond to SIF (Vassella et al., 1997). *In vivo*, monomorphism that allows mechanical transmission must maintain some control element that prevents death of the host. The genome of *T. b. evansi* STIB805 offered no suggestion as to which adaptations are required for efficient mechanical transmission (Carnes et al., 2015). Cells could have a decreased sensitivity to SIF, allowing a higher level of parasitaemia to be achieved that is still controlled. This could explain the low levels of
stumpy forms that have been found in parasitaemia caused by either *T. b. evansi* or *T. b. equiperdum* (Section 1.10.2). The SIF-dependent differentiation pathway is being elucidated (Mony et al., 2014), potentially presenting candidates that could be mutated in these subspecies and therefore reducing SIF sensitivity. The process could also involve infecting specific hosts that are able to control the infection via the immune system at a higher sustained parasitaemia (Desquesnes et al., 2013).

Our conclusions drawn from mathematical modelling suggest that AK slender forms are initially more immunogenic than kDNA+ cells (Table 3.4), which contrasts with the idea of a prolonged infection being a characteristic of infections with naturally occurring AK forms. Perhaps this immunogenicity is specific to mice, which are not standard hosts of either WT or naturally occurring AK forms of *T. brucei*. Chronic infections with our AK cell lines have not yet been performed, but could be measured to see if a more lower-lying chronic type of infection does form compared with infections with kDNA+ cells.

Crucially, we do not understand how kDNA loss is linked to adoption of transmission via mechanical methods and, now, loss of slender to stumpy differentiation, in the evolution of naturally occurring AK parasites. Mechanical transmission is always linked to kDNA loss in *T. b. brucei*, as cyclical transmission requires kDNA (Chapter 4). Apparently this also true in *T. vivax* (Greif et al., 2015). However, can sustained mechanical transmission lead to loss of kDNA? The lack of cyclical transmission could streamline the kDNA, selecting for cells that lack genes necessary for survival within the fly. DK *T. b. brucei* subspecies have ATPase γ mutations (Lai et al., 2008), allowing complete loss of functional kDNA (Dean et al., 2013), unlike American strains of *T. vivax*, where the kDNA is streamlined, but retains A6 and RPS12 (Greif et al., 2015). The driving force of complete loss of kDNA in *T. b. brucei* is not known. Genetic drift could be responsible, with some cells already randomly having ATPase γ mutations, but this is unlikely to account for the fact that almost all investigated *T. b. evansi* and *T. b. equiperdum* strains have the mutation present. The loss of kDNA could be
seen as a clear selective advantage over cells maintaining some kDNA, as cells have to invest much energy into expressing and importing proteins for kDNA replication and maintenance (Schnaufer, 2010). However, naturally occurring DK cells do still generate these proteins (Lai et al., 2008), hence this replicative advantage does not seem to be present.

3.4.5 The mathematical model supports SIF concentration dependent differentiation

Previous models for stumpy formation have included a cell density-dependent differentiation term (Tyler et al., 2001a, Lythgoe et al., 2007). However, this is an incorrect approximation, as SIF has been shown to control the differentiation of slender to stumpy forms (Vassella et al., 1997), but has unfortunately not been identified. Having stumpy formation dependent on SIF concentration rather than total cell density will make a difference on the dynamics of the infection: SIF will remain in the system longer, and thus have a longer lasting effect on the rate of stumpy formation than if the density directly controlled the rate of stumpy formation. In the former case, when cell numbers fall, stumpy formation will also fall without delay. In the present study, we tested if a density-dependent model (data not shown) or a SIF concentration dependent model provided a better fit to our data (as SIF cannot be measured, slender cells were assumed to produce it at a set number of units per hour). Like an earlier study (Macgregor et al., 2011), the SIF concentration dependent differentiation model was found to fit our experimental infection data better than a cell density dependent model.
3.4.6 The model also supports that there is a constant level of background slender to stumpy differentiation in vivo

Here, additionally, we have also applied the data to a model that includes a term for SIF independent differentiation. This term was originally used in a mathematical model in Savill and Seed, 2004. In that paper, the model fit better to the data if this term was included. This improvement in fit also occurs here (Figure 3.10A, B). Additionally, reassessing the data from MacGregor et al., 2011, we found that this model also provides a better fit to the experimental data from that study than a model only including SIF-dependent differentiation (Figure 3.10C, D).

There is yet no biological evidence for this phenomenon in trypanosome infections. However, in other parasites, there is some emerging evidence for a background level of random differentiation. Plasmodium gametogenesis requires commitment to the sexual stages of the parasite in order for successful transmission of the parasite through mosquitoes. A member of the ApiAP2 family of transcription factors, PbAP2-G, has been found to be essential for gametogenesis (Sinha et al., 2014). Mutations in PbAP2-G were found in strains unable to undertake gametogenesis, and complementation of these strains with a WT copy of the gene restored gametogenesis. Interestingly, complete knockout of one ApiAP2 gene only abolished 95% of mature gametocytes, with some non-functional female gametocytes observed to still be produced, meaning that a few parasites were able to achieve commitment despite the disruption of the signalling pathway for commitment.

A model for stochastic differentiation has been put forward for Theileria annulata parasites (Shiels, 1999). The differentiation of macroschizont parasites to the merozoite stage can be induced by a change in temperature from 37°C to 41°C. However, at 37°C, after a certain time period that varies between cells, the parasite can also commit and differentiate (Shiels et al., 1992). This suggests that to some extent, differentiation in Theileria is cell-specific,
and as such is programmed within each cell, but occurs randomly and asynchronously within a population of cells. Because this differentiation is not synchronous, a signal transduction model does not support this observation, and search for an extracellular factor that could trigger differentiation did not yield any evidence for one (Shiels et al., 1998). A predetermined condition is required for this model of differentiation to function: for example, once a reversible and gradual build-up of regulators of gene expression reaches a particular threshold concentration, the parasite is committed to an upregulation of gene expression that is irreversible (Shiels, 1999), and thus committed to differentiation. Thus, in this example, an increase in temperature is known to increase protein synthesis in *Theileria*, which could alter the rate of factor production relative to cell division (Shiels et al., 1997). It could be hypothesised that, over time, each cell individually accumulates factors, i.e. regulators of gene expression.

This may not apply to all parasite differentiation steps, and molecular validation is required, both in *Theileria* and *Trypanosoma*. Importantly though, this stochastic model is not mutually exclusive to a signal transduction-type of differentiation also occurring, i.e. SIF dependent differentiation in *T. b. brucei* could occur at the same time as stochastic differentiation in the same population of cells.

### 3.4.7 Limitations of this study

#### 3.4.7.1 Does ethidium bromide or acriflavine treatment alter infection dynamics?

It has long been known that both acriflavine and EtBr are DNA mutagens (Lopes et al., 2011, Singer et al., 1999). Hence, although the AK cells generated in this study were subjected only to 3 days of acriflavine treatment, these drugs will likely have resulted in
mutations in nuclear DNA. The growth rate in vitro in the slender form was not affected (Figure 3.1A), but we cannot discount the fact that any difference we see between infections with kDNA-containing cells and AK cells could be in part due to the mutagenic effect of the drug. We looked at two independently-generated AK clones to try to eliminate clone-specific differences, but analysis of more independently-generated AK clones would be a more robust test of this.

In order to assess the impact of acriflavine treatment on these cells, we could perform the same time-course investigation on cells that have lost their kDNA naturally, as can occur in cells with mutated ATPase γ subunit (Schnaufer lab, unpublished observations; such cell lines are available). These data would need to be compared to the results from the same clone that has been acriflavine treated, in order to compensate for clonal differences.

3.4.7.2 Stumpy longevity in the absence of an immune system

The model prediction that stumpy form AK cells have a lower lifespan than their WT counterparts requires assessment experimentally. This would be difficult to assess in vivo, as the decline of stumpy forms in vivo involves a combination of the senescence of stumpy cells and the immune system. Immune system activity could be reduced by drug treatment, for example cyclophosphamide removes committed immune cell progenitors (Gordon et al., 1985, Kastan et al., 1990, Jones et al., 1995), but there would have to be an assessment made on the extent of immune system depletion over time for each mouse. Mice bred without an immune system could also be used, but the course and progression of the infection could be much altered.

To remove the influence of the immune system, the assessment could also be performed in vitro, with stumpy forms harvested and purified from blood at maximum stumpy proportion. The proportion of stumpy forms could be assessed by IFA or flow cytometry using PAD1 staining for stumpy forms. These cells could then be put in HMI-9 with FCS, and a time-
course could be performed using a fixable live-dead stain, for example ethidium homodimer, and PAD1 staining. Flow cytometry would show the proportion of live stumpy cells over time.

One complication would be the presence of slender forms in the harvested material. Although PAD1 will only stain for stumpy forms, proliferative slender forms would still deplete the media of any nutrients needed to sustain stumpy forms. The proportion of slender forms in each harvested sample will vary, and so the extent of the effect this has on the lifespan of stumpy forms would be difficult to measure. Some attempts at depleting slender forms from a mixed population were made in the course of this study (data not shown). At first, we tried to prevent slender forms proliferating by placing them in a growth media containing 25 mM α-ketoglutarate instead of glucose, as stumpy forms but not slender forms can utilise this as a carbon source for energy production (Bienen et al., 1993). Interestingly, in the presence of either glucose or α-ketoglutarate stumpy forms with WT/WT and WT/L262P ATPase γ genotypes are sustained for around 2-3 days, but AK cells die within 24 hours under α-ketoglutarate conditions. Whether this is due to a cytocidal effect of α-ketoglutarate on AK stumpy forms, or if AK stumpy forms for some reason cannot uptake or utilise α-ketoglutarate as a mitochondrial substrate is an important question that remains to be investigated. Secondly, we tried to prevent slender forms proliferating by treatment with DFMO, a trypanostatic compound (De Rycker et al., 2012). Although DFMO has been reported to cause slender forms to morphologically differentiate to stumpy forms (Feagin et al., 1986), it does not cause PAD1 expression (data not shown), suggesting that the stumpy forms generated in the former study were not genuine. Preliminary results suggest that 40 µM DFMO has an anti-proliferative effect on slender forms by day 2 of treatment, but no effect on the lifespan of stumpy forms (personal observations). Hence if incubated in HMI-9 with DFMO, the number of stumpy cells within a population can be measured over time by
cell counting and flow cytometry with αPAD1, with any slender cells within the population being induced to enter a PAD1 negative growth arrest.

3.4.7.3 Improvements to the mathematical model

Mathematical models often do incorporate factors that are known to influence the system, but that have not, or cannot, be measured. The model used here used simplified or arbitrary parameters for SIF concentration, the immune response of the host, and PAD1 expression, all of which would be useful pieces of information to have in order to assess more accurately the fit of the model to the data. Until the identity of SIF is known, this parameter cannot be measured, and its concentration can only be guessed. The immune response of the host to trypanosomes is not well understood, but predicted to be complex. Here a simple model of the immune system was used, as there is no quantitative information known about the clearance of trypanosomes. This allowed slender and stumpy cells to be cleared at separate rates, in accordance with Engstler et al., 2007, with this clearing mechanism activating at separate rates. This oversimplification will inevitably prove incorrect, but a more detailed model is currently not possible to construct.

An attempt was made to measure the number of PAD1 positive cells within the population by IFA, to provide a measure of error in the quantification of stumpy proportion by morphology. This did not prove successful. Preliminary results for the accuracy of morphology scoring (Appendix C) were not used in the final model as estimates of error as they were performed on separate sets of infections, and samples were only taken on 3 days during the infection. A normal distribution estimation of error was instead used. Using a parental strain of T. b. brucei that has PAD1 tagged with GFP for example could allow the counting of PAD1 positive parasites to occur while the parasites were live, on a fresh blood smear, rather than using an involved IFA protocol.
Similarly, an error in the parasitaemia counts was estimated, not measured. Multiple blood smears could have been generated to sample more blood, and cell number could have been actually counted to improve accuracy, rather than using the rapid sampling method. This method was used in order to speed up parasitaemia counting due to the number of slides generated at each time point, but is a method of estimation rather than actual counting.

3.4.8 Outlook

3.4.8.1 Pleomorphic AK trypanosomes as a tool

These cell lines can subsequently be used to investigate further outstanding questions about the requirement for kDNA in both BSF forms of the parasite.

How is stumpy metabolism altered in the absence of kDNA, and how is ΔΨm generated in stumpy forms? A detailed study of stumpy metabolism has not yet been performed. Metabolomic studies will enable us to determine the output of carbon metabolism during incubation with substrates like $^{13}$C-glucose or $^{13}$C-α-ketoglutarate, as in for example (Millerioux et al., 2013, Coustou et al., 2008), and the consequences of the loss of maxicircle gene products on carbon metabolism. Metabolic flux analyses would enable analysis on which pathways were still functional in the absence of kDNA, and perhaps answer why one AK cell line is able to generate ΔΨm, whereas the other AK clone cannot. Additionally, results may suggest why AK stumpy forms cannot survive using α-ketoglutarate as a substrate.

Do slender or stumpy forms require NDH2 when the classical complex I does not function? By knocking out the alternative complex in these AK pleomorphic cell lines, both complexes will not be present. This would show whether NDH2 is able to compensate for loss of Complex I NADH dehydrogenase activity, and whether there are other complexes present
that are able to regenerate NAD$^+$ in the mitochondria in the BSF that are able to compensate for the loss of both of these activities, for example PDH.

Mitochondrial remodelling occurs during the stumpy form, with the mitochondrial network expanding and cristae beginning to be formed (Bohringer and Hecker, 1975, Vickerman, 1965). In other organisms, mitochondrial cristae are thought to be formed by rows of ATP synthase dimers manipulating the inner mitochondrial membrane to bend (Davies et al., 2012). Whether this also occurs in trypanosomes is currently being investigated by the Schnaufer lab in collaboration with the Davies lab. The beginning of cristae formation during the stumpy form would suggest that fully formed ATP synthase dimers would be required. However, AK forms do not retain the full F$_1$F$_o$ structure of complex V in the absence of subunit A6 (Chapter 2), therefore this mitochondrial remodelling would be assumed not to occur in AK stumpy forms. This could be investigated with electron cryo-tomography on mitochondrial membranes. The lack of mitochondrial remodelling could contribute to the reduced lifespan of AK stumpy forms. For example, as cristae increase the surface area of the mitochondrial inner membrane across which a $\Delta\psi_m$ can be generated, no increase in surface area could limit the production of $\Delta\psi_m$, and affect the rates of processes dependent on it.

Does a lack of kDNA does impact the efficiency of mechanical transmission? These experiments would be hard to carry out and the parameters of mechanical transmission, and their relative importance, are not known. To start with, factors that could increase the likelihood of mechanical transmission could be investigated, for example survival rate of the trypanosome on the mouthparts of the fly. This could be measured over time by qRT-PCR. Experiments into the effectiveness of mechanical transmission with these AK strains could also be carried out, by disrupting fly feeding on live infected animals and forcing resumption of feeding on uninfected animals.
If the infections detailed here had continued over a longer time course, an addition to the output of the model could have been a prediction of the number of antigenic variants during the infection. Antigenic variation in DK subspecies of *T. brucei* has not yet been investigated, and could influence the ability of the infection to become chronic, which is predicted to increase the efficiency of mechanical transmission if the parasitaemia is sufficiently high (Desquesnes et al., 2009). In different mammalian systems, antigenic variation could influence the occurrence of a mammal acting as a host or a reservoir. It would be interesting to see if a lack of kDNA would be predicted to influence the rate of switching. Additionally, data on VSG switching could be obtained to enhance the accuracy of the model prediction, for example deep sequencing of parasites over time during *in vivo* infections, and enhancement of VSG coverage by use of active expression site primers (Mugnier et al., 2015).

### 3.4.8.2 Is SIF independent differentiation an actual phenomenon?

In order to investigate whether slender form trypanosomes are able to differentiate at a basal level independently to SIF concentrations, monomorphic cells that are unresponsive to SIF, but with other components of the differentiation pathway intact, could be generated. These would be predicted to retain a limited capacity to display stumpy morphology.

Additionally, as some proteins involved in the SIF dependent differentiation pathway have been discovered (Mony et al., 2014), and investigations on the rest of the pathway is ongoing, knocking out one of these proteins should reduce the level of stumpy form production to this basal level, as long as the protein target does not have a dual role in both pathways. A candidate for this would be the SIF receptor, if basal differentiation is indeed SIF independent.
3.5 Figures

**Figure 3.1**  Cumulative *in vitro* growth analysis of WTγ and kDNA-independent AnTat1.1 90:13 cell lines

(A) Growth in HMI-9, 10% FCS, (B) Growth in HMI-9, 10% FCS +/- 10 nM ethidium bromide. Cell density was assessed by taking cell counts every 24 or 48 hours. Cells were diluted down to 1x10^5/ml after cell density measurement. Independent clones are labelled as in Table 2.3.
Figure 3.2  AnTat1.1 90:13 cells with and without kDNA are able to differentiate to the stumpy form

A) Cells expressing either i) WT/L262Pγ kDNA⁺, or ii) WT/L262Pγ (AK) were infected into mice, and blood smears were taken daily. SL= slender form, ST= stumpy form. Dashed lines = + compound, full lines = - compound. Left panel= phase contrast image, Right panel= DAPI stained image. B) Immunoblot analysing expression of stumpy specific protein PAD1 and PCF protein COX VI. Cells were harvested at peak parasitaemia. 2x10⁶ cells of each cell line were analysed. Anti PAD1 was a kind gift from the Matthews lab.
Figure 3.3 Investigation into differentiation of kDNA-independent and AK cell lines to the stumpy-like form in the presence of 100 µM 8pCPT-cAMP

Cells were grown in HMI-9, 10% FCS +/−8pCPT-cAMP over 3 days. A) Growth analysis in the presence and absence of 8pCPT-cAMP. The cell density of cell lines were assessed daily. Dashed line = +8pCPT-cAMP. B) Analysis of the proportion of cells completing the cell cycle in the presence and absence of 8pCPT-cAMP using flow cytometry. 1x10⁶ cells were taken daily, fixed and DAPI stained allowing for cell cycle analysis. Stumpy forms are cell cycle arrested, and so do not enter G2 phase of the cell cycle. C) Immunoblot analysing the expression of stumpy specific protein PAD1 at 48 hrs after addition of 8pCPT-cAMP into the media 2x10⁶ cells were used per cell line. Anti PAD1 was a kind gift from the Matthews lab.
Figure 3.4  Representative phase contrast images showing the morphology of cells through induction to the stumpy-like form using 8pCPT-cAMP
Cell samples were taken daily, mounted on microscope slides and DAPI stained allowing positioning of the nucleus and kinetoplast to also be assessed.
Figure 3.5  The morphological changes occurring during *in vivo* mouse infection over time
Mice were infected with 1000 cells at day 0 with cells expressing either A) WT/WTγ, B) WT/L262Pγ +kDNA clone 2, C) WT/L262Pγ (AK) clone 1, D) WT/L262Pγ (AK) clone 2. 4 mice were infected per cell line. Tail snips were performed every 6 hours from day 4 to day 8 post infection. The population of cells were scored as having slender, intermediate or stumpy form morphology from DAPI-stained dry blood smears.
Figure 3.6  Representative phase contrast images showing the morphology of cells at days 4 to 8 of mouse infection
Blood smears were taken daily and DAPI stained allowing positioning of the nucleus and kinetoplast to also be assessed.
Figure 3.7    A comparison between the morphology scoring of individual 1 and 2
Mice were infected with 1000 cells at day 0 with cells expressing either WT/WTγ (red), WT/L262Pγ +kDNA clone 2 (blue), WT/L262Pγ (AK) clone 1 (green) and WT/L262Pγ (AK) clone 2 (black). 4 mice were infected per cell line. Tail snips were performed every 6 hours from day 4 to day 8 post infection. The population of cells were scored as having slender, intermediate or stumpy form morphology from DAPI-stained dry blood smears. The slides were scored by two individuals, and the scores of these two individuals for 3 time points per cell line were plotted against each other. The dashed line is a line of gradient 1. The solid black line is the line of best fit between the scores.
Figure 3.8  Parasitaemia within *in vivo* mouse infections over time

Mice were infected with 1000 cells at day 0 with cell lines WT/WTγ (red), WT/L262Pγ +kDNA clone 2 (blue, B), WT/L262Pγ (AK) clone 1 (green, C), WT/L262Pγ (AK) clone 2 (black, D). 4 mice were infected per cell line. Tail snips were performed every 6 hours from day 4 to day 8 post infection and cell counts were estimated from blood smears. Each parasitaemia is plotted in comparison with the parasitaemia for the WT/WTγ cell line.
There are three cell types: non-committed dividing slender forms, committed dividing slender forms and stumpy forms, the latter of which, for the purpose of this model, also includes intermediate forms. Here, there are 2 ways that cells can become committed to differentiation, a SIF dependent route, proportional to SIF concentration, and a SIF independent route. SIF is produced by both committed and non-committed slender forms, and is cleared over time. The model includes immune system action against slender and stumpy forms.
Figure 3.10  A comparison between models with and without SIF independent differentiation term
A+B) Measuring the goodness of fit of the mathematical models to the data presented in this thesis. Here, the blue crosses are the standardised residuals of each data point for each mouse. This is the difference between each data point and the model predictions. The thick red line shows the average, across all mice of the residuals at a particular time point. The dashed lines symbolise the 95% predicted intervals of the model. If a model fits the data well, the red line should fluctuate randomly around the dotted line at y=0, and also within the predicted interval 95% of the time.  
A) Model as in Figure 3.9, with an additional SIF-independent differentiation term.  
B) Model as in Figure 3.9, with only SIF dependent differentiation included. 
C+D) The fit of the model to the data presented in MacGregor et al., 2011. Here the data is presented as crosses, and the fit of the model is presented as a line.  
C) Fits of model without SIF independent differentiation to the data.  
D) Fits of model with SIF independent differentiation included.
Figure 3.11  Measuring the goodness of fit of three alternative mathematical models to the data

Here, the blue crosses are the standardised residuals of each data point for each mouse. This is the difference between each data point and the model predictions. The thick red line shows the average, across all mice of the residuals at a particular time point. The dashed lines symbolise the 95% predicted intervals of the model. If a model fits the data well, the red line should fluctuate randomly around the dotted line at y=0, and also within the predicted interval 95% of the time. A) Model C: as in Figure 3.10A, with intermediates classed as slender forms, B) Model D: as A but with intermediate forms classed as themselves, C) Model E: as in B but with SIF-independent differentiating cells terminating at the intermediate stage.
Figure 3.12  The fit of the mathematical model to the data for infections with *T. brucei* of genotype WT/WTγ

The data is represented by filled dots. The coloured lines represent median fits of the model, with dark grey regions being 50% predictive intervals and light grey being 95% predictive intervals, where 50% and 95%, respectively, of future data would be predicted to lie according to the model and the data already observed. The predicted dynamics of SIF concentration is displayed on the lowest panels. A) The mathematical model used involves SIF-dependent and SIF-independent differentiation terms. B) The mathematical model only includes a SIF-dependent differentiation term.
Figure 3.13  The fit of the mathematical model to the data for infections with *T. brucei* of genotype WT/L262Py

The data is represented by filled dots. The coloured lines represent median fits of the model, with dark grey regions being 50% predictive intervals and light grey being 95% predictive intervals, where 50% and 95%, respectively, of future data would be predicted to lie according to the model and the data already observed. The predicted dynamics of SIF concentration is displayed on the lowest panels. (A) The mathematical model used involves SIF-dependent and SIF-independent differentiation terms. (B) The mathematical model only includes a SIF-dependent differentiation term.
Figure 3.14  The fit of the mathematical model to the data for infections with T. brucei of genotype WT/L262Py (AK) clone 1

The data is represented by filled dots. The coloured lines represent median fits of the model, with dark grey regions being 50% predictive intervals and light grey being 95% predictive intervals, where 50% and 95%, respectively, of future data would be predicted to lie according to the model and the data already observed. The predicted dynamics of SIF concentration is displayed on the lowest panels. (A) The mathematical model used involves SIF-dependent and SIF-independent differentiation terms. (B) The mathematical model only includes a SIF-dependent differentiation term.
Figure 3.15  The fit of the mathematical model to the data for infections with *T. brucei* of genotype WT/L262Pγ (AK) clone 2
The data is represented by filled dots. The coloured lines represent median fits of the model, with dark grey regions being 50% predictive intervals and light grey being 95% predictive intervals, where 50% and 95%, respectively, of future data would be predicted to lie according to the model and the data already observed. The predicted dynamics of SIF concentration is displayed on the lowest panels. (A) The mathematical model used involves SIF-dependent and SIF-independent differentiation terms. (B) The mathematical model only includes a SIF-dependent differentiation term.
Figure 3.16  Parameter estimation for each cell line by a mathematical model incorporating both SIF independent and SIF dependent differentiation

The model was fit to the data, with the parameters varied until values were reached that best explain the data. The parameter values are probability distributions, and as such are represented as box and whisker plots, with boxes marked by the 25%, 50% and 75% percentiles, and the whiskers spanning between the 2.5% and 97.5% percentiles.
Parameter estimation for each cell line by a mathematical model incorporating only SIF dependent differentiation

The model was fit to the data, with the parameters varied until values were reached that best explain the data. The parameter values are probability distributions, and as such are represented as box and whisker plots, with boxes marked by the 25%, 50% and 75% percentiles, and the whiskers spanning between the 2.5% and 97.5% percentiles.
Figure 3.18  A second slender peak does not appear at day 7 post infection for akinetoplastic cell lines
Mice were infected with 1000 cells at day 0 with cell lines WT/WTγ (red), WT/L262Pγ +kDNA clone 2 (blue), WT/L262Pγ (AK) clone 1 (green), WT/L262Pγ (AK) clone 2 (black). 5 µl blood samples were taken over the course of the infections, and were processed as IFA samples. After storage in glycine, cells were washed and DAPI stained, allowing cell cycle analysis via flow cytometry. Intact, single cells were gated as shown in Appendix B. Stumpy forms are cell cycle arrested, and so do not enter G2 phase of the cell cycle.
Figure 3.19  Absence of kDNA can be associated with loss of ΔΨm in stumpy forms

Stumpy forms were generated in mice. Cells were harvested at maximum parasitaemia, with approximately 90% stumpy forms, purified by anion exchange chromatography, and placed in HMI-9, 10% FCS. ΔΨm was measured by addition of 100 nM TMRE to the media, +/- 20 µM FCCP, and data was collected by flow cytometry at peak excitation = 549 nm and peak emission = 575 nm. Non-intact cells were gated out of the analysis by removing cell fragments using forward and side scatter, as shown in Appendix B. Cells were also stained with Hoechst 33342 for cell cycle analysis. This experiment was only performed once. A) Mitochondrial membrane potential measurements after 1 hr incubation in media. Blue = -TMRE, Red = +FCCP +TMRE, Orange = -FCCP +TMRE. B) Cell cycle analysis of cells -FCCP +TMRE. Red = population of cells in -ΔΨm gate in A, Orange = population of cells in +ΔΨm gate in A, Green = slender cells of respective genotype (control). C) Graph showing the % of intact cells that were stained TMRE positive. D) Graph showing the median value of TMRE staining of cell lines compared to cell line with genotype WT/WTγ.
4. The role of $F_1F_0$ ATP synthase and kDNA in differentiation and survival of PCF *T. brucei*
4.1 Introduction

*T. b. evansi* and *T. b. equiperdum* strains cannot transform into PCF, at least *in vitro* (Zweygarth and Kaminsky, 1989, Lai et al., 2008, Brun et al., 1998). When fed to tsetse flies, only cells with kDNA establish a midgut infection from a population with 70% AK *T. b. gambiense* (Reichenow, 1940). Chemically induced DK PCF cells are non-viable (Hajduk, 1978). RNAi knockdown of factors required for kDNA replication and division in PCF is also lethal (Wang and Englund, 2001, Wang et al., 2014, Downey et al., 2005, Tyc et al., 2015). The evidence points to kDNA being essential for fly transmissibility (Oppendoes et al., 1976). However, the ability of clonal pleomorphic AK strains to differentiate to the PCF in the tsetse fly midgut has never been assessed.

This lack of PCF viability without kDNA is thought to be due to the lack of the numerous components of the oxidative phosphorylation pathway encoded in kDNA (Vickerman, 1965, Oppendoes et al., 1976). Oxidative phosphorylation was thought to be essential for PCF (Tielens and Van Hellemont, 1998), with the F1F0 ATP synthase harnessing the Δψ generated by the electron transport chain to produce ATP (Williams, 1994). However, the requirement for oxidative phosphorylation in PCF viability has been questioned, at least under *in vitro* conditions (Besteiro et al., 2005). In glucose rich conditions it seems that only ATP production by mitochondrial substrate level phosphorylation is essential (Bochud-Allemann and Schneider, 2002, Coustou et al., 2003), although this does not appear to be the case for all strains (Zikova et al., 2009). However, the kDNA-encoded electron transport chain is still essential in these conditions (Coustou et al., 2003, Van Weelden et al., 2003). In the tsetse fly midgut, proline is thought to be the main energy source (Bursell et al., 1973, Ford and Bowman, 1973, Ter Kuile and Oppendoes, 1992), with glucose only transiently
abundant after bloodmeals (Vickerman, 1985). Hence, in glucose depleted conditions which are more similar to conditions within the tsetse fly midgut, ATP production via oxidative phosphorylation is essential (Lamour et al., 2005, Coustou et al., 2008).

It is expected that the ATP synthase γ mutation will be incapable of compensating for the loss of kDNA in PCF (Jensen et al., 2008). The compensatory γ mutation that functions in BSF requires the AAC to function in import of ATP into the mitochondria (Dean et al., 2013). In the PCF, mitochondrial ATP production is vital to the cell (Bochud-Allemann and Schneider, 2002, Coustou et al., 2008, Coustou et al., 2003), requiring the AAC to function in the direction of ATP export from the mitochondria to fuel processes within the cytosol (Pena-Diaz et al., 2012).

Cells expressing F$_1$F$_0$-ATPase complexes with compensatory γ subunit mutations have been shown to have increased resistance to multiple clinically relevant drugs used to treat animal and human disease (Gould and Schnaufer, 2014). If such mutant trypanosomes were able to differentiate within the fly to the mammalian-infective metacyclic form, they could be transmitted and resistance could spread. Mutations in the C-terminus of ATPase γ subunit in yeast increase affinity for the ATP substrate (Clark-Walker, 2003). However, these mutations cause the reverse ATP synthase reaction to be less efficient, having uncoupled the complex: the yeast K. lactis, when expressing ATP synthase with similar mutations, grows extremely poorly on glycerol, a substrate that requires ATP synthase activity (Chen and Clark-Walker, 1995, Chen and Clark-Walker, 1996, Clark-Walker et al., 2000). The study by Dean et al. (2013) and experiments described in Chapter 2 suggest that subunit γ mutations such as L262P uncouple the F$_1$ and F$_0$ moieties of the T. brucei enzyme, which would be expected to abolish the capacity for ATP synthesis. Thus, the ability of L262P$_γ$ T. brucei mutants to progress through the parasite’s life cycle could be affected.
The generation of L262P-expressing and AK stumpy forms in Chapter 3 presents an opportunity to answer both of these questions: can AK forms survive as PCF, and can kDNA-independent cells be transmitted by tsetse flies?

4.1.1 Hypothesis

The central hypotheses to be tested in this chapter are that (1), due to the essentiality of kDNA in PCF, AK forms of *T. brucei* will not be able to differentiate to the PCF or establish a PCF infection in the tsetse fly midgut, and that (2), in the proline rich environment of the midgut, oxidative phosphorylation should be essential, hence the L262Pγ mutation will affect the cells ability to survive in the fly, manifesting as reduced infection rate in the midgut. Thus, drug resistant L262Pγ expressing cells will be unable to complete the *T. brucei* life cycle or be transmitted cyclically.

4.1.2 Study design

The ability of L262Pγ expressing cells and AK cells to differentiate to the PCF was assessed *in vitro* and *in vivo*. *In vitro*, stumpy to PCF differentiation was assessed by morphology, reentry into the cell cycle and biochemical markers. Tsetse flies were infected with stumpy form AnTat1.1 90:13 cells expressing heterozygous or homozygous L262Pγ, with or without kDNA. Flies were dissected through the course of infection, with the midgut and salivary glands inspected for cells of the appropriate life cycle stage in order to assess whether kDNA-independent cells could differentiate into animal-infective metacyclic forms.
4.2 Materials and Methods

4.2.1 Generation of stumpy forms

Mouse strain MF1 was used throughout this study. All stock mice care was carried out by the staff of the March Building, University of Edinburgh. All mouse handling was carried out by Caroline Dewar, working under the project licence of Professor Keith Matthews, in accordance with conditions of Home Office personal and project licenses.

*T. brucei* strain AnTat1.1 90:13 cell lines WT/WTγ, WT/L262Pγ, L262P/L262Pγ, WT/L262Pγ (AK) and L262P/L262Pγ (AK) were each injected into MF1 mice via an intraperitoneal injection of 200 µl HMI-9 (1000 parasites per injection). No immunosuppressant was used. Parasitaemia was monitored daily in blood obtained via tail snip; a drop of blood was placed on a microscope slide, compressed under a cover slip and viewed with 40x objective. Cell numbers per field of view were estimated by viewing 5 fields of view from these wet smears based on the Rapid Matching method (Herbert and Lumsden, 1976). Morphology was also scored qualitatively from these wet smear slides as in Chapter 3. At peak stumpy form parasitaemia, with a proportion of >90% stumpy forms, trypanosomes were harvested via a cardiac puncture to collect the infected blood. Blood was collected in a 2-ml syringe containing 200 µl 2% sodium citrate (anti-coagulant). Stumpy form trypanosomes were purified from blood using DEAE-cellulose DE52 (Whatman) anionic exchange columns that were preincubated with PSG solution (44 mM NaCl, 57 mM Na₂HPO₄, 3 mM KH₂PO₄, 55 mM glucose, pH 7.8) warmed to 37°C. Blood was loaded onto the column, the column was continuously topped up with warm PSG, and eluate was collected. The density of the cells was measured using a Beckmann Z2 Coulter counter and cells were washed in HMI-9 media (Hirumi and Hirumi, 1989).
4.2.2 *In vitro* differentiation and PCF culture

Cultures of each cell line were set up at a density of $2 \times 10^6$ cells per ml HMI-9. Each culture was split in half, and 6 mM CA was added to one half of each culture (+CA). Both +CA and –CA cultures were left at 37°C for 24 hrs. Microscope slides were prepared by spreading cells on a slide and air drying. Slides were methanol-fixed and DAPI staining was carried out by mounting the slides using 50 µl Prolong Gold Antifade with DAPI (Life Tech.).

After 24 hrs of CA treatment, both +CA and –CA flasks were inspected by microscopy. Newly differentiated PCF cells in +CA were pelleted, washed and resuspended either in SDM79 containing 10 mM glucose (Invitrogen) (Brun and Schonenberger, 1979) or in SDM80 at a density of at least $2 \times 10^6$ cells per ml medium. SDM80 was made from SDM79-CGGGPPTA powder (PAA), an SDM79-based powder that lacks major carbon-containing components (sodium bicarbonate, glucose, glutamine, glutamate, proline, pyruvate, threonine and sodium acetate). All carbon sources except glucose were added back into the solution at the following concentrations: pyruvate 100 mg/l, L-proline 615 mg/l, L-threonine 394 mg/l, L-glutamine 320 mg/l, L-glutamate 24 mg/l, NaHCO$_3$ 2 g/l, sodium acetate 10 mg/l. The media was supplemented with 50 mM N-acetyl D-glucosamine (Sigma) to prevent residual glucose uptake from FCS (F. Bringaud, personal communication). SDM79 and SDM80 were supplemented with 7.5 mg/l hemin and 10% FCS (Invitrogen), and cells were grown at 27°C continuously in these media. Cells were seeded at >$3 \times 10^6$/ml, and cell counts were performed every 2 or 3 days using Beckmann Z2 Coulter counter.
4.2.3 Assessing life cycle stage markers via western blot

2x10^6 cells per sample were washed in PBS, pelleted and resuspended in 10 µl 2x SDS buffer (4% SDS, 20% glycerol, 120 mM Tris-HCL, pH 6.8, 0.2% bromophenol blue, 200 mM DTT).

Samples were either boiled for 10 minutes at 95°C or sonicated for 3 minutes on ice depending on the primary antibody used (see Table 4.1). Denatured samples were loaded onto a Novex NuPAGE Bis-Tris 10% precast gel (Invitrogen), which was run at 150 V with 500 ml ice cold 1x MOPS SDS running buffer (Invitrogen). The gel was blotted onto a methanol-equilibrated PVDF membrane using 1 L ice cold transfer buffer and a Biorad Criterion blotter at 90 V for 45 min, and blocked overnight in 1x TBST (10 mM Tris-HCl, 150 mM NaCl and 0.05% Tween-20) with 10% milk powder at 4°C. Antibody incubations were performed in 1x TBST with 5% milk powder, unless stated otherwise, and washes were performed with 1x TBST. For detection, Enhanced Chemiluminescence reagents 1 and 2 (Amersham) were mixed in equal volumes and incubated with the membrane. The membrane was placed into an X-ray film cassette and used to expose a Kodak BioMax light film, with exposure time depending on intensity of signal produced. Developing was performed by a SRX-101A X ray developer (Konica Minolta).
Table 4.1 Antibodies used for western blotting in this chapter

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Concentration</th>
<th>Sample preparation</th>
<th>Incubation conditions</th>
<th>Secondary</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-EP</td>
<td>1:500</td>
<td>Boiling and sonication</td>
<td>1x TBST, 5% milk</td>
<td>Anti-mouse 1:2000</td>
<td>Cedar Lane Laboratories</td>
</tr>
<tr>
<td>Anti-GPEET</td>
<td>1:1000</td>
<td>Boiling</td>
<td>1x TBST, 5% milk</td>
<td>Anti-rabbit 1:2000</td>
<td>(Ruepp et al., 1997)</td>
</tr>
<tr>
<td>Anti-ATP synthase Tb2</td>
<td>1:2000</td>
<td>Boiling</td>
<td>1x TBST, 5% milk</td>
<td>Anti-rabbit 1:2000</td>
<td>(Subrtova et al., 2015)</td>
</tr>
<tr>
<td>Anti-ATP synthase β</td>
<td>1:2000</td>
<td>Boiling</td>
<td>1x TBST, 5% milk</td>
<td>Anti-rabbit 1:2000</td>
<td>(Subrtova et al., 2015)</td>
</tr>
<tr>
<td>Anti-COX VI</td>
<td>1:500</td>
<td>Boiling</td>
<td>1x TBST, 5% milk</td>
<td>Anti-rabbit 1:2000</td>
<td>(Horvath et al., 2005)</td>
</tr>
<tr>
<td>Anti-COX IV</td>
<td>1:1000</td>
<td>Boiling</td>
<td>1x TBST, 5% milk</td>
<td>Anti-rabbit 1:2000</td>
<td>(Maslov et al., 2002)</td>
</tr>
<tr>
<td>Anti-ASCT</td>
<td>1:300</td>
<td>Boiling</td>
<td>1x TBST, 5% milk</td>
<td>Anti-rabbit 1:2000</td>
<td>(Riviere et al., 2004)</td>
</tr>
<tr>
<td>Anti-EF1α</td>
<td>1:7000</td>
<td>Boiling</td>
<td>1x TBST, 5% milk</td>
<td>Anti-mouse 1:2000</td>
<td>Millipore</td>
</tr>
</tbody>
</table>

4.2.4 Quantitative measurement of EP expression

The protocol as described in Sections 4.2.1 and 4.2.2 were followed. The equivalent of 2x10^6 cells were taken from +CA and –CA cultures at 0 hr, 1 hr, 2 hr, 3 hr, 6 hr, 16 hr and 24 hr after CA addition. Upon collection, samples were transferred in to 5 ml polystyrene round bottom tubes (BD Falcon 352052), centrifuged at 2000 g for 5 minutes and washed in PBS. The cell pellet was fixed in 500 µl PBS with 2% formaldehyde, 0.05% glutaraldehyde overnight. Cells were pelleted, washed three times in PBS, and blocked in PBS with 2% BSA for 1 hr. After a PBS wash, cells were stained with PBS containing 2% BSA and 1/500
anti-EP for 1 hr. Cells were washed in PBS and then PBS containing 2% BSA and 1/1000 anti mouse-Alexa 488 secondary antibody was added and left for 1 hr. Cells were then washed and resuspended in 500 µl PBS containing 5 µg/ml Hoechst DNA staining dye (Life Tech.). After 30 min incubation, cells were analysed by flow cytometry at λ<sub>ex</sub> 495 nm and λ<sub>em</sub> 519 nm using a Beckton Dickinson LSRII machine with BD FACSDiva software. Machine settings were determined by comparing positively and negatively stained samples with the assistance of Martin Waterfall, University of Edinburgh. 2x10<sup>4</sup> events per sample were measured. Results were analysed with FlowJo software.

### 4.2.5 Tsetse fly handling and infections

Mouse infections were carried out to generate the stumpy forms required for fly infections as described in Section 4.2.1. One mouse was infected per cell line, daily tail snips were performed and cardiac punctures were carried out once parasitaemia predominantly consisted of stumpy forms. Blood was mixed with 200 µl 2% sodium citrate acting as an anti-coagulant, and mixed 1:1 with HMI-9 containing 30% FCS and 7.5% glycerol for freezing at -80°C. 200 µl aliquots were transported to LSTM, Liverpool on dry ice.

All fly infections, handling and dissections were carried out at the Acosta-Serrano lab, LSTM. Before an infection was carried out, a blood meal was prepared as follows: one 200 µl aliquot per cell line was thawed at room temperature and mixed with ~2 ml room temperature sterile, defibrinated horse blood.

The *Glossina morsitans morsitans* (Westwood) colony at LSTM (origin Kenya) is maintained at 27°C±2°C and 68–78% relative humidity, with a 12 hr light and 12 hr dark cycle, and is fed on sterile, defibrinated horse blood in the dark using a skin-mimic membrane and a heated mat set to 37°C. Experimental flies were collected no later than 24
hrs after eclosion and briefly chilled at 4°C for initial gender sorting. Male flies were selected. All flies used in this study were teneral (unfed) male adults. These were fed one infected blood meal containing predominantly stumpy form parasites between 0–24 hrs after emergence.

For midgut infections, a minimum of 25 males per repeat were offered a feed on an infected blood meal for 10 minutes. 24 hrs after feeding, flies were then chilled to 4°C and unfed flies, identified by a non-scarlet abdomen, were removed. Flies with a meal visible in the gut were subsequently kept at 27°C until dissection on day 9 post infection. Flies were given uninfected blood meals as above every 2-3 days. Three independent infections were performed for each cell line.

For salivary gland infections, a minimum of 200 flies were fed on a 5 ml infected blood meal for 15 minutes. 24 hrs after feeding, flies were then chilled to 4°C and unfed flies removed. Flies with a meal visible in the gut were subsequently kept at 27°C until dissection at 4 weeks post infection. Flies were given uninfected blood meals as above every 2-3 days.

4.2.6 Tsetse fly dissections

Flies were chilled at 4°C, collected and kept on ice. Dissections were performed on live flies under a dissecting microscope. Dissections were carried out by Caroline Dewar and Aitor Casas of LSTM.

For midgut dissection, flies were secured on their dorsal side by holding down the thorax near the top of the wings with one set of tweezers, while tears were made at the top of the abdomen with a set of sharp tweezers. The abdomen was then pulled away from the thorax, and the midgut was isolated, without tearing, into a 20 µl droplet of ice-cold sterile SDM79. Fat body, testes and other material were cleared away, and the midgut and attached tubules
were disrupted and viewed at 100x magnification. Parasites were scored based on their density, with 1-10 parasites per field of view being scored as +, and >>10 parasites per field of view being scored as ++++. Infected midguts were collected in ice cold sterile SDM79, and smeared across slides that were left to air dry. These slides were methanol-fixed and DAPI staining was carried out by mounting the slides using 50 µl Prolong Gold Antifade with DAPI (Life Tech.).

For salivary gland dissections, flies were secured on their dorsal side as above, and the head was very slowly pulled off the thorax with another set of tweezers. The salivary glands were thus isolated attached to the head into 20 µl ice-cold filtered PBS. Infected salivary glands look swollen, cloudy and are easier to break open, and when viewed under a microscope, parasites can be seen moving within them. After severing at the base of the head, salivary glands were chopped into segments and viewed at 100x magnification to check for infection. Infected glands were collected and pooled in ice-cold SDM79. Frozen stocks were prepared by adding glycerol to a final concentration of 7.5% and frozen at -80°C. Mice infections, monitoring and harvests were then carried out as per Section 4.2.1.

4.2.7 Confirmation of γ genotype in AnTat1.1 90:13 clones

gDNA was extracted using GenElute Mammalian gDNA miniprep kit (Sigma), and the γ subunit gene was amplified via PCR, allowing direct Sanger sequencing of the gel-extracted PCR product to confirm the presence or absence of the L262Pγ mutation.

Standard PCR conditions: 95°C 5 mins, [95°C 1 min, 54°C 1 min, 72°C 2 min] for 30 cycles, 72°C for 10 mins.
Reaction conditions per 25 µl reaction: 14.25 µl dH₂O, 5 µl 5x GoTaq reaction buffer (Promega), 0.2 µl 25 mM dNTPs, 1.5 µl MgCl₂, 0.5 µl 10 µM primer #1 and #2 (Appendix B), 1 µl ~10 µg/ml gDNA template, 0.25 µl GoTaq polymerase (Promega).

Sanger sequencing big dye reactions were carried out by Edinburgh Genomics.

4.3 Results

4.3.1 AK clones express EP procyclin in response to CA

AK AnTat1.1 90:13 cells are able to differentiate to the stumpy BSF (Chapter 3). We first used these AK stumpy forms to see whether AK T. brucei was able to differentiate to the PCF in vitro. Stumpy form cells of genotypes WT/WTγ, WT/L262Pγ, WT/L262Pγ (AK) and L262P/L262Pγ were incubated in HMI-9 with 10% FCS containing 6 mM CA at 37°C for 24 hrs. After this time, no intact AK cells were visible, only debris remained (data not shown). This was in contrast to cell lines with kDNA, where, although some debris was present, there was still a high density of intact cells. Similarly, L262P/L262Pγ clone 3 cells that lack the maxicircle also do not survive 24 hrs of CA treatment.

The morphology of freshly differentiated AnTat1.1 90:13 cells was assessed. Cells looked morphologically PCF (Fig. 4.1): PCF look similar in morphology to long slender BSF. Both the kDNA and nucleus are positioned more towards the centre of the cell than in stumpy forms, with the nucleus positioned further from the kDNA, in a similar arrangement to that found in long slender BSF (Matthews et al., 1995). The unattached part of the flagella is not as long as in the long slender BSF. The morphological and biochemical remodelling of the cell from a stumpy form to a PCF occurs over the period of 10-14 hrs (Ziegelbauer et al.,
1990), and with morphological changes occurring within one cell cycle (Matthews et al., 1995). In dividing PCF cells, the kinetoplast-nucleus arrangement is KNKN rather than KKNN (Robinson et al., 1995, Tyler et al., 2001b) (Fig. 4.1, right hand panel). Less 2K1N and 2K2N cells were observed on microscope slides from cell lines L262P/L262Pγ clones 1 and 2 (data not shown). This observation was not quantified in comparison to other cell lines.

Cells were assessed for EP procyclin expression by western blot (Fig. 4.2A). This is an early marker for PCF differentiation (Vassella et al., 2001a, Acosta-Serrano et al., 2001, Urwyler et al., 2005). As expected, this protein was not detected in slender or stumpy BSF cell lysates. This protein was detected in all PCF cell lysates. The cellular debris left after AK cell lines were treated with CA was also probed, this also showed EP expression, at a reduced level to that seen in kDNA+ cell lines.

To quantify the proportion of the population expressing EP and the time frame in which EP was expressed, a time course was performed. Stumpy cells of each cell line were harvested, purified from blood and resuspended in HMI-9. The cells from each cell line were split in two, with 6 mM CA added to one aliquot (+CA), and the other left untreated (-CA). Samples from each culture were taken at 0, 1, 2, 3, 6, 16 and 24 hrs after CA treatment, stained with mouse anti-EP and anti-mouse Alexa488, and analysed by flow cytometry (Fig. 4.2B and C). As expected, non-induced and -CA cells showed negligible EP expression, confirming that EP expression was due to induction by CA (data not shown).

All intact CA-treated cells began to express EP by 2 hrs after CA addition with the exception of AK cells, where EP expression began at 3 hrs after CA treatment (Fig. 4.2C). From here intact treated cells with kDNA expressed EP with more or less equivalent kinetics. After 6 hrs of CA treatment, EP expression began to plateau for these kDNA+ cells. About 95% of cells from AnTat1.1 90:13 (parental), WT/WTγ, and L262P/L262Pγ clones 1 and 2 cell lines
expressed EP by 24 hrs after CA treatment. Around 90% of cells of cell line WT/L262Pγ clone 1 expressed EP by 24 hrs, and around 75% cells expressing EP in cell line WT/L262Pγ clone 2 by 24 hrs. AK cells had a much reduced level of EP expression at 6 hrs after CA treatment, and the proportion of intact cells expressing EP increased from around 20% to around 60% between 6 and 16 hrs. From here the proportion of AK cells expressing EP plateaued. Noticeably, by 16 and 24 hrs of CA treatment, it took a much longer period of time to collect the $2\times 10^4$ intact cells from AK samples needed for this analysis compared with samples from kDNA+ cell lines as the AK cells were dying by these time points (data not shown). Interestingly, the EP expression of L262P/L262Pγ clone 3, which has lost the maxicircle, follows the same pattern as cells with kDNA, with 90% of cells expressing EP by 24 hrs.

4.3.2 L262P/L262Pγ PCF clones do not enter exponential growth in vitro

To see if freshly differentiated PCF cells expressing ATP synthase L262Pγ could reenter the cell cycle, +CA cells were centrifuged, washed and resuspended in SDM80 after 24 hrs of CA treatment. SDM80 contains depleted glucose levels (estimated 0.15 mM (Lamour et al., 2005)) and N-acetyl D-glucosamine to inhibit uptake of residual glucose (from FCS), and it is in these conditions that PCF are 3000 times more sensitive to oligomycin (Lamour et al., 2005, Coustou et al., 2008), meaning they depend on oxidative phosphorylation to generate sufficient amounts of ATP. In these conditions, freshly differentiated AnTat1.1 90:13 cells expressing WT/WTγ and WT/L262Pγ were able to establish exponential growth in SDM80 (Fig. 4.3A). Despite initiating differentiation to PCF, homozygous L262P/L262Pγ cell lines did not grow in this medium. Cells did survive in these conditions for up to a week, but detached flagella and clumping of cells was visible (data not shown). The motility of
L262P/L262Py cells was also affected. Some cells did not move at all, and the wave of the flagella was distorted in other cells, with the frequency very low and the amplitude quite high (P. Bastin, personal communication).

Cells were also assessed for their ability to express proteins specific to the PCF or those needed for ATP production in the PCF (Fig. 4.4). All PCF cell lines grown in SDM80 showed identical levels of expression of ASCT and ATP synthase subunits β and Tb2. COXVI and COXIV expression was also similar across all cell lines, with the exception of COXVI levels in WT/WTγ cell lines, which were decreased. GPEET was absent in established Lister 427 29:13 cell lines. This is unsurprising as GPEET levels are known to differ between different cell lines (Butikofer et al., 1997), and the 29:13 cell line has been maintained in in vitro culture for an extended period of time. GPEET was present in all freshly differentiated AnTat1.1 90:13 cell lines. A larger smear was visible for L262P/L262Py clone 1 and 2: this could be due to increased phosphorylation of the protein (Mehlert et al., 1999, Butikofer et al., 1997, Butikofer et al., 1999).

4.3.3 SDM79 does not support exponential growth of freshly differentiated AnTat1.1 90:13 cell lines

Proliferation of freshly differentiated cells was also assessed in SDM79 medium. This contains 10 mM glucose, and so allows cells to metabolise both glucose and amino acids. Oxidative phosphorylation is not essential in these conditions (Coustou et al., 2003, Bochud-Allemann and Schneider, 2002, Besteiro et al., 2005), but this is dependent on cell line (Zikova et al., 2009). SDM79 media sustained growth of established PCF strain Lister 427 29:13 cells at a higher growth rate compared to SDM80 (Fig. 4.3A). However, an immediate, severe growth phenotype was observed in all other cell lines, when compared to
growth in SDM80 (representative cumulative growth curves shown in Fig. 4.3A). Cells remained alive, with some clumping visible (data not shown). The growth phenotype was observed regardless of whether cells grown in SDM80 for around 1-2 weeks were added to SDM79, or whether freshly differentiated cells were placed directly into the high glucose medium (Fig. 4.3B, C, dashed lines). The SDM79-induced growth phenotype could be rescued by putting these cells into SDM80 medium (Fig. 4.3B, C). Adaptation to SDM79 medium did seem to occur at variable time points in different clones. WT/WTγ cells began to increase their growth rate after around 400 hrs of growth in SDM79, WT/L262Pγ clone 2 first showed an increased growth rate at around 100 hrs, after which growth plateaus. A second increase in growth rate occurred after 400 hrs of growth in SDM79 media (Fig. 4.3B, C).

### 4.3.4 AK stumpy form trypanosomes do not establish a tsetse fly midgut infection

To experimentally prove that trypanosomes devoid of kDNA cannot establish a PCF midgut infection *in vivo*, teneral tsetse flies were fed with horse blood containing AK stumpy form parasites expressing WT/L262Pγ or L262P/L262Pγ. Teneral flies were also fed with horse blood containing stumpy forms of the AnTat1.1 90:13 parental cell line, the WT/WTγ cell line, and non-acriflavine treated clones of the WT/L262Pγ and L262P/L262Pγ cell lines. At 24 hrs after feeding, flies were sorted. Only flies that had a scarlet-coloured abdomen and therefore could be confirmed to have fed on this first blood meal were retained. At day 9 post infection, the flies were dissected, their midguts were isolated, disrupted, and inspected under a microscope to assess the state of parasitaemia.
The parental cell line was able to differentiate to the PCF and establish a midgut infection in around 85% of flies dissected (Fig. 4.5A). Around 70% of the flies infected with the parental cell line had a high level of infection (Fig. 4.5B). In contrast, there were no infected midguts within flies that had been fed on blood containing either AK *T. brucei* clone (Fig. 4.5A).

### 4.3.5 L262Pγ-expressing stumpy forms can establish a tsetse fly PCF midgut infection

In the slender BSF, 427SM cells expressing L262Pγ from one or both alleles are oligomycin resistant and have structurally uncoupled ATPase complexes, hence are generating Δψm independently of ATPase Fo and therefore kDNA (Chapter 2). Moreover, homozygous L262P/L262Pγ parasites would be expected to be unable to generate ATP via oxidative phosphorylation. Figure 4.5A shows flies infected with WT/L262Pγ cells had a significantly lower midgut infection level than flies infected with cells expressing exclusively WTγ, with around 70% of flies having had infected midguts. Flies infected with L262P/L262Pγ cells had a similar infection rate to the heterozygous γ cell line, at 74%. Figure 4.5B illustrates the scale of infection found within these midguts. For flies infected with cells expressing WT/L262Pγ, 53% of all fly midguts were heavily infected, whereas around 16% had a low level of infection. However, for flies infected with L262P/L262Pγ clone 1, a lower number of midguts, 45%, had a high level of infection, with an increased number of midguts, 29%, containing a low level infection. Cells extracted from the midgut showed some elongation of the cell body, indicative of the longer mesocyclic trypomastigotes near the anterior of the midgut that are generally found around day 6 post infection (Van Den Abbeele et al., 1999) (Fig. 4.6). On the other hand, flies infected with L262P/L262Pγ clone 3, that has lost the maxicircle (Fig. 2.4), did not have any infected midguts. This shows that firstly, without the
maxicircle, cells are unable to establish a midgut infection. Secondly, as this clone has not been treated with acriflavine, this reveals that it is the lack of kDNA that prevents AK cells establishing a midgut infection, rather than the mutagenic actions of acriflavine on nuclear DNA.

Figure 4.5C shows the infection experiment with L262P/L262Pγ clone 2 and parental AnTat1.1 90:13 cell lines. For technical reasons these infections had to be carried out in a different fly facility (still maintained in the same way and by the same personnel). Compared to the study shown in Figure 4B, the % of uninfected fly midguts for the parental strain increased from 22% to 39%, a 77% increase. The percentage of flies with highly infected midguts decreased from 63% to 50%, a 22% decrease. The percentage of midguts with low lying infection remained consistent between the two analyses (Fig. 4.5B and C). Parental cell infections were only performed once in the new insectary, but the switch in facility does seem to have decreased the levels of midgut infection.

For flies infected with L262P/L262Pγ clone 2, the % of fly midguts found uninfected was 56%, with 17% of flies found with a high level of midgut infection and 26% flies found with low level midgut infection (Fig. 4.5C). The rate of low level infection for clone 2 was comparable to the one observed for L262P/L262Pγ clone 1 (Figure 4.5B). However, the percentage of midguts found with high infection levels was much lower for clone 2 (20% compared to 50%) and the percentage of non-infected midguts was correspondingly higher. Based on the results obtained for the parental control cell line, this difference can, at least in part, be attributed to the change in facility. However, biological differences between the clones could also have played a role.
4.3.6 kDNA-independent cell line WT/L262Pγ clone 1 can establish a metacyclic salivary gland infection

Prior to this study, it had not been established whether cells of the strain AnTat1.1 90:13 are able to migrate from the midgut to the salivary glands and differentiate to the metacyclic form. The strain used for the present study had been maintained in the slender form in vitro since culture adaptation and, although it had been routinely passaged through mice to verify BSF pleomorphism, it was not known when it was last passaged through a fly to confirm the maintenance of insect form pleomorphism. 15% of flies that were fed with parental AnTat1.1 90:13 stumpy cells had detectable metacyclic forms within their salivary glands (Fig. 4.7A), thus confirming the ability of this cell line to complete the T. brucei life cycle.

Groups of flies were fed blood meals containing stumpy forms of trypanosomes with genotypes WT/WTγ (one clone), WT/L262Pγ (two clones) and L262P/L262Pγ (two clones). Unfed flies were removed from the experiment 24 hrs after infection, and dissections occurred at 4 weeks post infection. No metacyclic-infected salivary glands were found in flies that had been fed parasites of genotypes WT/WTγ or L262P/L262Pγ (Fig. 4.7A). Out of the two clones with genotype WT/L262Pγ, clone 1 had an approximately 6% salivary gland infection rate, whereas no salivary gland infection was found in flies infected with clone 2 (Fig. 4.7A). This 6% infection rate is significantly lower than the infection rate seen for the parental cell line.

These groups of flies also had their midguts scored for the level of PCF infection found at 4 weeks post infection to ensure that these groups of flies had been infected with PCF successfully. Flies fed on WT/WTγ trypanosomes showed around a 56% midgut infection rate (Fig. 4.7B). Flies fed on either clones 1 or 2 of genotype WT/L262Pγ both showed around a 52% midgut infection rate. Flies fed on either clones 1 or 2 of genotype L262P/L262Pγ both showed around a 40% midgut infection rate. As WT/L262Pγ clone 1 has
a comparable level of midgut infection to WT/WTγ and WT/L262Pγ clone 2 (Fig. 4.7B), we can conclude that the level of midgut infection in the flies infected with these cells did not affect the infection status of the salivary glands in these flies. However this may not be the case for L262P/L262Pγ clones 1 and 2, as the level of the midgut infection in flies infected with these clones is much lower. Statistics cannot be performed on the level of midgut infections with L262P/L262Pγ cell lines, however, as these infections were only performed once.

### 4.3.7 Metacyclic form WT/L262Pγ cells can infect mice

To test if the metacyclic form trypanosomes found within the salivary glands in the parental AnTat1.1 90:13 strain and in WT/L262Pγ clone 1 were animal transmissible, the metacyclic forms of both *T. brucei* strains were retained and individually used to infect mice. The parasitaemia for both mice was monitored daily via tail snip. Slender BSF trypanosomes were visible in both blood smears from day 4, with stumpy forms being visible in both strains from day 8 (Fig. 4.7C). To ensure that cells of original genotype WT/L262Pγ had not reverted to WT/WTγ genotype, these cells were harvested via cardiac puncture and purified from the blood. Genomic DNA was extracted and the ATPase subunit γ gene was amplified and sequenced. A double peak was visible at base 785 for cell line WT/L262P clone 1 (Fig. 4.7D, highlighted). This confirmed that both WT and L262Pγ alleles were detectable at the DNA level.
4.4 Discussion

It has been stated numerous times in the literature that kDNA is essential for PCF *T. brucei*, for example (Schnarwiler et al., 2014, Lai et al., 2008, Chandler et al., 2008). This is expected due to the requirement for oxidative phosphorylation and an electron transport chain utilising kDNA-encoded gene products (Vickerman, 1965, Coustou et al., 2003, Lamour et al., 2005). However, whether kDNA loss causes a loss of fly transmissibility has never been formally tested.

To conclusively answer the question as to whether kDNA is required for development of a PCF midgut infection in the tsetse fly, L262Pγ-expressing AK stumpy form AnTat1.1 90:13 cells were fed to tsetse flies in a blood meal. In contrast to kDNA+ cell lines, PCF infections did not develop for the AK strains, agreeing with the hypothesis that kDNA is essential in PCF. A time course of CA treatment revealed that AK cells do initiate the differentiation process, indicated by the expression of EP procyclin, one of the surface proteins of PCF forms.

To assess the potential for cyclical development of L262Pγ-expressing cell lines in the insect stages of the life cycle, firstly L262Pγ-expressing cell lines were tested for the ability to differentiate to PCF in vitro and in vivo. Morphology, organelle repositioning, expression of molecular markers and ability to reenter the cell cycle were all assessed and found to indicate efficient differentiation. WT/WTγ and WT/L262Pγ AnTat1.1 90:13 cell lines were able to grow in glucose-depleted medium as PCF, unlike L262P/L262Pγ PCF cell lines. However, L262P/L262Pγ clones 1 and 2 did establish a PCF infection within the midgut, albeit at a lower intensity to those found in WT/WTγ or WT/L262Pγ-infected flies. Unexpectedly, one cell line with a WT/L262Pγ genotype was able to migrate to the salivary
glands of the tsetse fly and differentiate to the metacyclic form, disproving our hypothesis that these drug resistant cells could not be transmitted by tsetse flies. These metacyclic cells were able to infect mice and continue in the life cycle.

### 4.4.1 kDNA is required for a midgut PCF infection

AK cells were consistently unable to establish a midgut infection, or differentiate to viable PCF cells in vitro (Fig. 4.5A), hence there is now conclusive evidence that kDNA is required for PCF parasites. As the non-acriflavine treated L262P/L262Pγ clone 3 that has lost the maxicircle was equally unable to infect the fly midgut, this shows that a lack of midgut infectivity was not due to the mutagenic action of acriflavine on nuclear DNA, it was due to the loss of kDNA. The inability of multiple independently-generated AK clones to differentiate to the PCF is unlikely to be due to prolonged in vitro culturing as all cells used in this study have been cultured in vitro for a similar length of time.

*T. brucei* kDNA, like mtDNA of other eukaryotes, encodes subunits of the mitochondrial respiratory chain. This functions in oxidative phosphorylation, where production of the Δψm is coupled to synthesis of ATP via F₁F₀ ATP synthase, and also in the electron transport chain, where redox coenzymes such as NADH are oxidised to regenerate reducing equivalents required for the continuation of metabolism. Within the midgut of the fly, glucose is much depleted, as it is in SDM80. It has been shown that oxidative phosphorylation is important for cellular ATP production; some strains are >1000x more sensitive to F₉ inhibitor oligomycin when grown in the absence of glucose compared to in the presence of glucose (Coustou et al., 2003, Lamour et al., 2005). Similarly, RNAi of succinate dehydrogenase (complex II) abolishes oxidative phosphorylation, causing a growth effect in glucose-depleted media (Coustou et al., 2008), but no growth effect in the presence of glucose (Bochud-Allemann and Schneider, 2002). In the presence of glucose, some strains
of PCF cells grown in vitro seem to depend on substrate level phosphorylation for their ATP production (Coustou et al., 2008, Zikova et al., 2009); but for other strains of PCF cells addition of oligomycin has no effect on intracellular ATP levels in SDM79 (Coustou et al., 2003). Substrate level phosphorylation occurs independently of kDNA. However the electron transport chain is still essential in the presence of glucose; simultaneous treatment with complex IV inhibitor KCN and TAO inhibitor SHAM decreases intracellular ATP levels (Coustou et al., 2003) and kills the cells (Van Weelden et al., 2003). Thus it seems that aerobic respiration is required for PCF cells grown with or without glucose. Presumably due to the number of kDNA-encoded proteins functioning in the PCF, cells cannot compensate for the loss of kDNA in PCF. The ATP synthase γ mutation that compensates for kDNA loss in BSF is not able to compensate for kDNA loss in PCF. The same result would be expected in AK cell lines generated by expression of other γ mutations that compensate for the loss of kDNA upon acriflavine treatment, for example A273Pγ (Dean et al., 2013).

Unlike cells expressing L262Pγ in the presence of kDNA, AK cells are unable to survive 24 hrs of CA treatment. They express PAD1 (Chapter 3), and evidently do have a functional signalling pathway as AK cells do respond to CA in a limited way. They express EP procyclin from 3 hrs after CA addition (Fig. 4.2), comparable to kDNA containing cell lines here and reported elsewhere (Engstler and Boshart, 2004, Szoor et al., 2013), but seem to not reenter the cell cycle. Hence EP expression is independent of kDNA, but reentry into the cell cycle seems to either require the presence of the kinetoplast structure or kDNA-encoded factors, in agreement with Timms et al. (2002).

Reentry into the cell cycle is caused by growth arrested stumpy forms receiving the CA signal (Engstler and Boshart, 2004), with cellular remodelling beginning upon cell cycle reentry at around 6 hrs after CA treatment (Kabani et al., 2009). The proportion of EP positive cells increased at a slower rate in AK cells than in WTγ and L262Pγ expressing cells with kDNA (Fig. 4.2C). It seems that AnTat1.1 90:13 kDNA+ cells reenter the cell cycle
between 3 and 6 hrs after CA treatment, as the largest increase in EP positive cells occurs between these two time points (Fig. 4.2C). AK cells however do not seem to be able to reenter the cell cycle, with EP expression increasing more slowly over time, and EP expression plateauing at around 60% of cells at around 16 hrs after CA treatment due to cell death.

DK cells have been shown not to reenter the cell cycle, evidenced by lack of α-tyrosinated tubulin staining that signifies the start of microtubule extension that occurs during cellular remodelling (Timms et al., 2002). The authors suggested that this demonstrated the presence of a kDNA-dependent checkpoint in the PCF that prevented cell cycle reentry in the absence of kDNA replication. In order to verify that AK cells in this study were unable to reenter the cell cycle, cells could similarly be stained with DAPI and an α-tyrosinated tubulin antibody. However, the lack of cell cycle reentry in the absence of kDNA could have been affected by treatment with the mutagen acriflavine. Additional analysis of a cell line that has lost its entire kDNA repertoire without acriflavine treatment would allow this issue to be resolved.

EP levels are often used as a measure of PCF differentiation efficiency (Jones et al., 2014, Szoor et al., 2013), as it is an early event in differentiation (Vassella et al., 2001a, Acosta-Serrano et al., 2001, Urwyler et al., 2005). A lower expression of EP in AK cells seen here is in contrast to a study where DK cells generated by acriflavine treatment of mice 16 hr before harvest were transformed to PCF by CA (Timms et al., 2002). In that study, DK cells were seen to express EP procyclin with equal dynamics to cells with kDNA. However as transcript and protein turnover occurs after a time lag, kDNA-encoded transcripts and proteins expressed during the stumpy BSF could still have been present and functional at 16 hr after acriflavine treatment. In the stumpy form, A6, NDH subunits and RPS12 are expressed as protein, and CyB and COX subunits are expressed as transcripts (Schnaufer et al., 2002, Michelotti and Hajduk, 1987). This could explain why similar dynamics of EP expression were seen between kDNA+ and DK cells. In the study presented here, no kDNA-
encoded gene products would have been present as acriflavine treatment and induction of differentiation occurred separated by a period of several months.

Interestingly however, a clone retaining a minicircle population but not the maxicircle expressed EP at the same rate as cells with kDNA (Fig. 4.2C, L262P/L262Pγ clone 3), despite the fact that these cells also do not survive after 24 hrs of CA treatment (data not shown). The kinetoplast in these cells is still stainable with DAPI (Fig 2.5B), suggesting that the kDNA structure is present in these cells. The maxicircle is not required for kDNA structural maintenance and segregation (Lai et al., 2008). This suggests that L262P/L262Pγ clone 3 cells are able to reenter the cell cycle after CA treatment to complete PCF differentiation (presumably the cells die after CA treatment as maxicircle encoded gene products are not present). Equally, L262P/L262Pγ cells that are expected to have uncoupled nonfunctional ATP synthase complexes (Chapter 2) are able to reenter the cell cycle and complete PCF differentiation (Fig. 4.2C). These data therefore suggest that it could be the presence of the kinetoplast structure that is required to pass through the cell cycle reentry checkpoint to complete PCF differentiation rather than a kDNA-encoded function like complex V activity. This disagrees with Timms et al. (2002), who came to the conclusion that the function of complex V was required for cell cycle reentry upon PCF differentiation.

4.4.2 Cell lines with the WT/L262Pγ genotype can be transmitted by tsetse flies

One WT/L262Pγ clone (clone 1) was able to complete the life cycle within the fly, producing animal-infective metacyclic form parasites (Fig. 4.7). Considering this one cell line with a verified WT/L262Pγ genotype was able to perform all of the morphological and
biochemical changes required for transmission by the fly, we can conclude that cells with a heterozygous L262Pγ genotype are capable of completing the life cycle of *T. brucei*.

Clone 2 on the other hand did not complete the tsetse fly stages of the life cycle (Fig. 4.7). This could be for a range of reasons unrelated to the genotype of the cell line (Section 4.4.6.1), as the WT/WTγ cell line also was unable to complete the *T. brucei* life cycle. Hence this result does not affect our conclusion that cells expressing WT/L262Pγ can survive the fly stages of the parasite’s life cycle.

Parasites unable to express EP have a lower midgut infection rate than WT parasites, suggesting that expression of EP does provide an advantage to PCF cells in vivo (Vassella et al., 2009, Ruepp et al., 1997). Despite CA treatment inducing lower EP expression in WT/L262Pγ clone 2 in vitro compared to other cell lines (Fig. 4.2C); this does not seem to have affected its ability to colonise the tsetse midgut as infection levels are equivalent to those of WT/L262Pγ clone 1 that has WT levels of EP expression (Fig. 4.2C, 4.5A and B).

Cell lines expressing WT/L262Pγ contain approximately 50% WT ATP synthase complexes and approximately 50% ATP synthase complexes containing L262Pγ that are uncoupled (Fig. 2.20), and hence not productive in generating ATP. There is a slight but reproducible decrease in midgut infection prevalence between WT/WTγ expressing parasites and WT/L262Pγ expressing parasites 9 days post infection (Fig. 4.5A). This suggests that the presence of ATP synthase complexes containing L262Pγ subunits may affect the ability of the cells to survive in vivo, despite the presence of some WT ATP synthase complexes. Although the uncoupling effect of L262Pγ does need to be confirmed in AnTat1.1 90:13 cell lines (Chapter 2), this supports the prediction that, like in yeast, specific mutations at the C-terminus of the γ subunit cause inefficiency of the ATP synthase complex, which in turn causes a growth effect (Chen and Clark-Walker, 1995, Chen and Clark-Walker, 1996, Clark-Walker et al., 2000). The loss of ATP synthase efficiency seem to decrease infection
prevalence in the midgut, agreeing with the observation that oxidative phosphorylation significantly contributes to ATP production in the absence of glucose (Coustou et al., 2008, Lamour et al., 2005). The fact that growth seems less affected by the genotype in vitro (in SDM80, Fig. 4.3A) may be due to growth in culture being very nutrient rich, in contrast to conditions within the tsetse fly, where proline availability may be more limiting (Dyer et al., 2013). Equally, more ATP may be required for survival as PCF in vivo compared with in vitro, meaning that the lower oxidative phosphorylation rate of WT/L262Pγ cells may be the factor limiting growth.

Four weeks post infection there is no difference between the levels of midgut infection (Fig. 4.7), suggesting WT/L262Pγ parasites may have a lower growth rate in vivo than WT/WTγ parasites, and over time, initially low level WT/L262Pγ infections can become more dense and established. The insectary changed location between the midgut dissection and salivary gland dissection experiments (Fig. 4.5A, 4.7B), but this would be expected to affect all experimental flies equally, independent of the cell line they are infected with.

Approximately 25% of flies with parental AnTat1.1 90:13 midgut infections developed salivary gland infections (Fig. 4.7A, B). In flies infected with WT/L262Pγ 1 parasites, only approximately 10% of flies with a midgut infection developed a salivary gland infection. As less WT/L262Pγ parasites are able to reach the salivary glands and differentiate, and these parasites are thought to have less efficient ATP synthase complexes, this could suggest that efficient oxidative phosphorylation becomes more important during the post-midgut tsetse stages of the T. brucei lifecycle. In agreement with this, it has been shown that depletion of glucose, a metabolic substrate that inhibits proline metabolism (Lamour et al., 2005) and therefore oxidative phosphorylation, accelerates metacyclic development in vitro (M. Boshart et al., KMCBM 2015, abstract 4C). ATP is required to fuel extensive migration between fly compartments, survival within diverse harsh environments and morphological and biochemical alterations to the parasite. Hence a decrease in cellular levels of ATP could
be the cause of less WT/L262Pγ parasites being able to fulfil all of the demands of the *T. brucei* life cycle. However it cannot be ruled out that, as only one WT/WTγ clone (parental) and one WT/L262Pγ clone were able to reach the metacyclic stage, the differences observed in their salivary gland infection rate could be due to clone-specific differences.

The significance of being able to complete the *T. brucei* life cycle is that cells expressing L262Pγ - or other mutations that obviate the need for kDNA in BSF - are more resistant to classes of drugs used in the field to treat animal and human disease, including isometamidium and EtBr, since these drugs, at least in part, target the kDNA (Gould and Schnaufer, 2014). This suggests that this trait of multiple drug resistance could be spread across Africa by tsetse flies. It could be argued that maintaining usage of kDNA-targeting prophylactic drugs increases the risk of new strains of kDNA-independent BSF *T. brucei* emerging over time that could also possibly be transmitted by tsetse flies.

There are caveats to the conclusion that tsetse transmission of kDNA-independent cell lines would occur. Firstly, although WT/L262Pγ cells with kDNA can be transmitted by the tsetse fly, exposure to drugs that target kDNA would cause loss of kDNA from these cells, preventing this now AK cell line then being transmitted further by tsetse flies. Hence although this WT/L262Pγ genotype could be spread wider initially by cyclical transmission, once in a host treated with the drugs the cells are more resistant to, parasites would continue to proliferate despite drug treatment, but the opportunity for further transmission from that host would stop. Secondly, it has been observed in our laboratory that although WT/L262Pγ can retain kDNA, they appear to spontaneously become DK at a faster frequency than WT/WTγ cells (S. Cooper, unpublished). From the results presented here, this would cause loss of tsetse transmission. Transmission dynamics would therefore be altered if some cells in a population became less able to maintain a full kDNA repertoire in their daughter cells. Thirdly, a lower infection prevalence in the midgut and salivary glands (Fig. 4.5A, 4.7A)
indicates that these WT/L262P\(\gamma\) cell lines do have a slight competitive disadvantage compared to cells expressing WT/WT\(\gamma\). For example, in a mixed infection it would be expected that WT/WT\(\gamma\) cells would replicate and occupy the midgut lumen faster than WT/L262P\(\gamma\) cells. In nature therefore, these drug-resistant cells could be gradually diluted out from the population over time.

The ability to migrate to the salivary glands would enable cells expressing L262P\(\gamma\) to sexually recombine with other parasites (Gibson, 2015). Hence, if the mutation emerged in an animal-infectious strain of T. b. brucei for example, there is scope for L262P\(\gamma\) expressing cells to continuously be recombining with human-infective parasites to produce parasites with increased drug resistance able to infect humans. This has been documented to be able to occur through inheritance of SRA (Balmer et al., 2011, Gibson et al., 2015).

### 4.4.3 *In vitro* and *in vivo* conditions affect homozygous L262P/L262P\(\gamma\) cells differently

AnTat1.1 90:13 L262P/L262P\(\gamma\) clones 1 and 2 did not grow in glucose-depleted SDM80, unlike WT/WT\(\gamma\) and WT/L262P\(\gamma\) cells (Fig. 4.3). This can be explained by our conclusion that L262P/L262P\(\gamma\) cells only contain uncoupled ATP synthase complexes, incapable of ATP production (although this does remain to be verified in our AnTat1.1 90:13 cell lines, Chapter 2). In glucose depleted conditions, PCF have increased dependence on oxidative phosphorylation (Lamour et al., 2005, Coustou et al., 2008). L262P/L262P\(\gamma\) cells do not die in these conditions, but do not proliferate either, as expected, since cells with no or inefficient ATP synthase complexes will struggle to meet their ATP demands in low glucose conditions.
Surprisingly however, L262P/L262P γ clones 1 and 2 were able to establish a PCF infection in the presumably glucose-depleted midgut (Fig. 4.5). Midgut infections were less dense at day 9 than in flies infected with WT/WT γ or WT/L262P γ cell lines, suggesting that L262P/L262P γ cell lines grow slower than other cell lines in vivo, or die at a faster rate due to either decreased viability or increased susceptibility to being killed in the midgut. This is in accordance with the lack of efficient ATP synthase complexes. There was a lower midgut infection prevalence 4 weeks after infection in flies infected with L262P/L262P γ than flies infected with other parasite genotypes (Fig. 4.7B), suggesting that L262P/L262P γ infections are slightly easier to control by the immune defences of the tsetse fly or that L262P/L262P γ PCF cells can not be sustained as long in glucose-depleted conditions in the midgut.

These contrasting results could imply a number of things. Firstly, that oxidative phosphorylation is important but not essential in glucose-depleted conditions. If the ATP synthase complex in L262P/L262P γ cells is inefficient, cells may be utilising substrate level phosphorylation to generate ATP, fuelling growth in the midgut. Secondly, the ability for substrate level phosphorylation in the absence of glucose may be more possible in vivo than in vitro. Cells do not grow in SDM80, but the cells do not die (Fig. 4.3A). As PCF grow to a much higher density in liquid culture compared with in the midgut, possibly nutrients are depleted faster within liquid culture than in the fly midgut. Despite the fact that tsetse fly energy metabolism competes with trypanosomes for proline (Bursell, 1966), perhaps something within the fly midgut is able to sustain growth of these cells. Threonine or glutamine concentration (Bringaud et al., 2006, Cross et al., 1975) for example may be higher per trypanosome in the midgut, allowing cells to sustain themselves by ATP production through the ASCT cycle (Fig. 1.9, 1.10). This would be in contrast to cells in vitro in SDM80, where minimal ATP production is perhaps maintained through inefficient oxidative phosphorylation, or substrate level phosphorylation from threonine or glutamine. The full scope of the nutrients available in midgut environment is yet to be experimentally
analysed. Other substrates that have not yet been studied may be available in the midgut, but not in \textit{in vitro} media, and could be scavenged by trypanosomes to sustain growth in the midgut, as Leishmania amastigotes in phagolysosomes do (Naderer et al., 2006). Tsetse flies also harbour symbiotic bacteria, one of which, \textit{Sodalis glossinidius}, is known to increase trypanosome susceptibility of the host (Welburn and Maudlin, 1999, Farikou et al., 2010) possibly by metabolic means (Hamidou Soumana et al., 2014). Bloodmeal factors are also known to affect trypanosome infections (Aksoy et al., 2003). Glucose is present in the bloodmeal that was given every 2 to 3 days, which could allow ATP generation by glycolysis. However glucose is thought to remain within the midgut for only 15 minutes (Vickerman, 1985), presumably not long enough for a long lasting effect on cell division. Additionally, the presence of glucose appears to be problematic for the AnTat1.1 90:13 \textit{T. brucei} strain (Fig. 4.3).

No L262P/L262P\textgreek{g} clones were found to be able to infect the salivary glands despite the fact that a midgut infection is sustained in these cell lines (Fig. 4.7A, B). We do not know whether this is due to these clones being unable to progress further due to their ATP synthase genotype or due to an unrelated loss of the ability to differentiate. It is common to observe a loss of fly infectivity in cultured \textit{T. brucei} strains (Vassella et al., 2009), as observed here for WT/WT\textgreek{g} and WT/L262P\textgreek{g} 2.

However with an unfunctional uncoupled ATP synthase complex, these cells may be unable to generate enough ATP to continue past the PCF stage of the life cycle. Efficient ATP generation is required for motility of \textit{T. brucei} (Langousis and Hill, 2014). Indeed, \textit{in vitro}, L262P/L262P\textgreek{g} cells have distorted flagellum movement (P. Bastin, personal communication). While some cells were observed to be immotile, others showed a severe defect in the propagation of the flagellar wave (P. Bastin, personal communication). This was not immediately obvious in parasites isolated from flies, but no videos were taken for later analysis.
Ordered migration between different fly compartments is known to be an integral part of the 
*T. brucei* life cycle (Gibson and Bailey, 2003). A motility defect could influence the 
migration of the cells in the fly, leading to the inability of cells of the L262P/L262Pγ 
genotype to complete the life cycle. An inefficiency in the ATP synthase could lower ATP 
concentrations within the cell, which could reduce the levels available to the ATP dependent 
motors that power the flagella (Hill, 2010). Swimming ability has been shown to be dictated 
by the availability of ATP for the dynein motor in numerous eukaryotic flagellate organisms 
(Kamiya and Okamoto, 1985, Brokaw, 1967, Mitchell et al., 2005, Mukai and Okuno, 2004, 
Christen et al., 1987). Similarities between the reduced motility of these L262P/L262Pγ 
clones and flagellum mutants has been observed (P. Bastin, personal communication). 
Interestingly, a *T. brucei* dynein motor mutant has a motility defect in culture, is unable to 
migrate to the foregut and proventriculus, and cannot infect the salivary glands (Rotureau et 
al., 2014).

A potential motility defect *in vitro* and *in vivo* could be investigated using *in silico* tracking 
to measure the velocity and swimming motion of the cells and, as immotile cells sediment at 
the bottom of culture flasks, indirect analysis of motility can be performed by sedimentation 
assays (Rotureau et al., 2014). Flagellum beating assays could also be performed to analyse 
movement of the cells (Uppaluri et al., 2011, Heddergott et al., 2012).

On a semi-solid surface *in vitro*, early form PCF *T. brucei* exhibit coordinated movement 
dependent on their flagella (Oberholzer et al., 2010, Imhof et al., 2014). This is thought to 
reflect the migration of trypanosomes from the midgut lumen into the ectoperitrophic space. 
L262P/L262Pγ cells with reduced ATP production might therefore have a defect in 
performing social motility. Additionally, cell density appears to affect the ability of cells to 
undergo this coordinated movement (Imhof et al., 2014, Imhof et al., 2015). Hence the low 
density seen in L262P/L262Pγ-infected midguts could be the factor preventing the migration 
from the midgut lumen, preventing colonisation of other fly compartments. Performing
migration assays on agarose plates at differing inoculum densities as in Imhof et al., 2014 would indicate the threshold number of cells required for coordinated migration, and whether L262P/L262Pγ cell lines are able to perform migration to the same level as other cell lines.

Kinesin proteins that govern morphological and organelle rearrangements during the life and cell cycle have ATP-dependent motility essential to their function (Asbury et al., 2003, Hu et al., 2012, Chan et al., 2010, Engelson et al., 2011). In addition, complexes involved in DNA replication and kinases essential for progression through the cell cycle are also dependent on ATP (Li, 2012). This could explain why progression through the cell cycle seems to be halted in L262P/L262Pγ cells grown in SDM80 (section 4.3.1).

Another impact of inefficient ATP synthase complexes could be sustained high levels of Δψm, as it is not being used to drive ATP synthase activity (Houstek et al., 2006, Mattiazzi et al., 2004, Korshunov et al., 1997). This could cause ROS generation and enhanced oxidative stress, damaging cells in a multitude of ways (Turrens, 2003), and thus contribute to the lower growth potential of L262P/L262Pγ-expressing cells in vivo and in vitro. ROS production in T. brucei is thought to occur via NDH2 (Fang and Beattie, 2002) and complex III (Gnipova et al., 2012). This theory could be tested by measuring the Δψm and ROS production of L262P/L262Pγ-expressing cell lines in comparison to other cell lines.

**4.4.4 Why do freshly differentiated cells not grow in SDM79?**

Early PCF, expressing both EP and GPEET procyclins, predominate in the early stage of an infection. Around the same time that GPEET expression falls, parasites penetrate the PM (Vassella et al., 2000, Acosta-Serrano et al., 2001, Van Den Abbeele et al., 1999). Interestingly, growth of freshly differentiated Antat1.1 PCF in SDM79 has been seen to
cause growth inhibition that lasts around 4-6 days, before growth resumes as a population of late PCF (Vassella et al., 2000). This correlates with the effect of growth in SDM79 shown in this work (Fig. 4.3). A reentry into the cell cycle has been shown to correlate with the loss of GPEET (Vassella et al., 2000). This growth arrest can be prevented by adding 10 mM glycerol or depleting media of glucose, and GPEET expression is maintained (Morris et al., 2002, Vassella et al., 2000).

There are molecular markers for early and late stage PCF that could be used to investigate whether cells grown in SDM79 become late stage PCF cells and cells grown in SDM80 remain as early stage PCF parasites (Imhof et al., 2014). The expression of GPEET over time in cells grown in SDM79 and SDM80 needs to be investigated in these AnTat1.1 90:13 cell lines to see whether loss of GPEET correlates with escape from growth arrest in SDM79.

CA acts as a stumpy to PCF differentiation signal via a signalling cascade that involves targeting of the phosphatase PIP39 to the glycosomes (Szoor et al., 2010). The targets and the function of dephosphorylation within the glycosome are not known (Szoor et al., 2014). However, only 50% of proteins in the PCF glycosome are glycolytic enzymes, compared to >90% in the BSF (Misset et al., 1986). Glycosomal remodelling occurs during BSF to PCF differentiation to prime parasites for the change in substrate availability that occurs upon uptake into the fly midgut, with a release from dependence on glucose necessary. Old BSF organelles are turned over and new glycosomes with different enzymatic content are synthesised that are more optimal for survival within the fly midgut (Herman et al., 2008).

Hence parasites in the glucose depleted conditions of SDM80 can maintain growth in these conditions (Fig. 4.3A). However, in SDM79 a period of adaptation may be required for freshly differentiated parasites to allow glycosomal remodelling to utilise glucose more efficiently before growth restarts (Fig. 4.3B, C). This is especially important as it is known that glucose metabolism inhibits amino acid catabolism (Lamour et al., 2005) and glucose is toxic to trypanosomes containing immature glycosomes (Furuya et al., 2002). Supporting
this hypothesis, there appears to be different glycosomal compositions in established cultures of PCF parasites in SDM79 and SDM80 media (Bauer et al., 2013), although this work needs to also be performed on freshly differentiated PCF.

4.4.5 Limitations of this study

4.4.5.1 Does L262Pγ cause F₁Fₒ ATP synthase to be less efficient?

In yeast it has been shown that cells expressing ATP synthase γ with specific point mutations at its C-terminus cannot grow on glycerol, a substrate requiring the complex to function in the ATP synthase direction (Chen and Clark-Walker, 1995, Chen and Clark-Walker, 1996, Clark-Walker et al., 2000). When the yeast equivalent of the L262Pγ mutation was expressed in K. lactis however, cells grew comparably to WT cells on glycerol, suggesting that the mutation did not significantly affect the function of the ATP synthase complex (Schnaufer et al., 2005). The effect of the L262Pγ mutation on ATP synthase reaction kinetics needs to be experimentally verified in our PCF cell lines. Total cytosolic ATP can be measured by numerous methods, including the expression of luciferase in cell lines (Gnipova et al., 2015). If L262Pγ cells grown in glucose depleted SDM80 were inefficient at producing ATP via oxidative phosphorylation, it would be expected that these cells contain less ATP than WT cells.

Kinetic measurements of the F₁Fₒ ATP synthase can be performed from purified samples to provide quantitative analysis. To establish Kₘ (the ADP concentration resulting in the half maximal rate of reaction), measurements would determine the oligomycin-sensitive F₁Fₒ-ATP synthase activity from crude mitochondrial extracts in the presence of varying concentrations of ADP substrate, whereas Vₘₐₓ is the maximal rate of reaction at saturating
substrate concentrations. These studies would reveal if L262Pγ causes a decrease in the velocity or efficiency of the ATP synthase reaction.

Mitochondrial capacity for oxidative phosphorylation can be measured in crude mitochondrial preparations using succinate as substrate (Allemann and Schneider, 2000). If the presence of L262Pγ affected the amount of ATP produced, this would be evident.

The assumed effects of ATP synthase inefficiency (for example decrease in growth rate in vitro, reduced capacity for differentiation in vivo) could also be verified by the production of other ATP synthase subunit mutants or knockouts to see if they produce the same phenotypes. Mutations should be chosen that have been seen to cause reduction in ATP synthase activity, a reduction in ATP synthase complex and a homolog of that subunit should be present in T. brucei. Missense mutations or knockdown of ATP synthase subunit ε have been shown to cause this phenotype (Mayr et al., 2010) (Havlickova et al., 2010), as has T. brucei specific subunit Tb1 (Subrtova et al., personal communication).

4.4.5.2 Is completion of the life cycle by WT/L262Pγ cells dependent on downregulation of the L262P allele?

A possible explanation for how clone WT/L262Pγ 1 is able to complete the life cycle despite having approximately half its F1F0 ATP synthase complexes uncoupled is that there is upregulation of WTγ or downregulation of L262Pγ at the protein level. This would produce higher levels of WTγ ATP synthase complex allowing potentially a greater production of ATP per cell. We know that in the long slender BSF, the ratio of WTγ and L262Pγ transcripts is approximately 1:1 in these clones (Chapter 2). Pyrosequencing could be used to assess the levels of WT and L262Pγ allele transcripts in the PCF and metacyclic stages from parasite DNA. Ideally parasite RNA would be obtained from infected flies by flash freezing infected fly material as in Ooi et al., 2015.
Unfortunately, at the protein level, the amount of WT and mutated γ subunit cannot be measured to see whether the L262Pγ allele is functional. The presence of coupled F₁Fₒ synthase prevents detection of uncoupled complex by hrCNE and western blot (Chapter 2). Drug sensitivity assays could be used to see if L262Pγ is still being expressed, as WT/WTγ cells are sensitive to acriflavine, EtBr and oligomycin ((Dean et al., 2013, Gould and Schnaufer, 2014), Chapter 2). However, obtaining the amount of parasite material required for this analysis from flies would be very difficult due to the bottlenecks experienced in fly infections (Dyer et al., 2013). Even infected fly compartments contain few parasites (Welburn and Maudlin, 1997, Van Den Abbeele et al., 1999). In vitro differentiation to the metacyclic form could be used to produce the number of cells required (Kolev et al., 2012). Metacyclic forms are non-replicative, further complicating the use of an Alamar Blue assay, which relies on exponential growth of parasites in the presence of the test compound. The metacyclic forms are animal-infective, hence the analysis could be performed on the long slender BSF of the parasite isolated from mice. If these cells grow in medium supplemented with EtBr or acriflavine, the L262Pγ allele is functional. However, there is further depression of mitochondrial morphology and activity between the metacyclic and long slender BSF stages (Brun et al., 1984, Bohringer and Hecker, 1975), possibly affecting the consistency of subunit expression between the two stages. Although we do not yet have a comparison between the levels of ATP synthase/ATPase found in the metacyclic and slender BSF forms, there is likely to be a further decrease in ATPase levels in the slender BSF, so a non 1:1 expression ratio between WT and L262P γ subunits could be overridden by this downregulation.
4.4.6 Outlook

4.4.6.1 Why do cell lines WT/WTγ and WT/L262Pγ clone 2 not differentiate to the metacyclic form?

Despite cell lines of the genotypes WT/WTγ and WT/L262Pγ being able to differentiate to the metacyclic form, metacyclic forms of the clones WT/WTγ and WT/L262Pγ 2 were not found in any of the salivary glands despite 400 flies having been infected with each of these cell lines. Salivary gland dissections are challenging: the head is removed and pulled away from the body, with the salivary glands slowly emerging from the body attached to the head. If they snap, they recoil back into the thorax, and are difficult to find. In an infected fly, only one of the two salivary glands can be infected, so it is possible that if only one salivary gland was dissected out, some infected salivary glands were lost during dissection. However, the probability of losing one or both salivary glands during dissection is identical for flies infected with all cell lines, hence it is probable that no salivary gland infections resulted from these cell lines as reported (Fig. 4.7).

As these AnTat1.1 90:13 cell lines had been maintained in BSF culture, it is possible that there was selection for parasites that had lost genes required for progression from the PCF in the midgut of the tsetse fly. There are known mutations that can result in loss of mature salivary gland infection despite colonisation of the midgut. Knockout of either M KK-1, PSSA-2 or all procyclins (EP and GPEET) can affect the ability to establish a mature salivary gland infection (Morand et al., 2012, Fragoso et al., 2009, Vassella et al., 2009). Although these genes are known to be important in salivary gland infection, the actual roles these proteins play is uncertain.

There are a multitude of possibilities as to why these cell lines cannot form metacyclic forms. We did not dissect out and inspect the PV, hence we do not know if these cell lines
were able to reach and colonise the PV. This is needed to inspect whether these clones can first differentiate to the epimastigote form within the PV. This differentiation requires extreme organelle repositioning that appears to be mediated by multiple RNA binding proteins that could be mutated (Sharma et al., 2008, Subota et al., 2011). The epimastigote form could be unable to attach to the salivary glands, a process that signals reentry into the cell cycle, expression of meiotic genes and further differentiation (Tetley and Vickerman, 1985, Sharma et al., 2009, Rotureau et al., 2012, Peacock et al., 2011). Additionally, cells may be unresponsive to the currently unknown environmental cues that allow ordered migration and differentiation.

Sequencing the genomes and transcriptomes of these clones in order to look for mutated genes could highlight protein candidates that could play roles in the many currently underprobed processes occurring within the tsetse fly.

4.4.6.2 Are L262P/L262Pγ cell lines able to differentiate into the other fly stages in the life cycle?

L262P/L262Pγ cell lines were similarly found not to be able to colonise the salivary glands and form metacyclic forms. As only two clones have been tested, flies should be infected with additional clones in order to see whether this is a clone-specific effect, as seems to be the case with cell lines WT/WTγ and WT/L262Pγ 2. Metacyclic differentiation could be an extremely rare event in these cell lines, hence a larger number of flies should be infected and dissected. To circumvent these problems, differentiation to the metacyclic form can now be analysed in vitro by overexpressing RBP6 (Kolev et al., 2012). Questions still remain as to whether this upregulation is physiological, as this differentiation negates the requirement for cross-talk between parasite and the internal environment of the tsetse fly (Ooi and Bastin, 2013). However, the experiment could answer the question as to whether L262P/L262Pγ cell lines are physically capable of performing the morphological and cellular remodelling
required for differentiation to the animal-infective metacyclic stage, and at what efficiency differentiation can occur at in comparison to other cell lines.

4.4.6.3 How can L262P/L262Pγ cells generate ATP?

If L262P/L262Pγ cells have inefficient ATP synthase complexes, and yet can establish midgut infections, cells could be generating their mitochondrial ATP via substrate level phosphorylation. To support parasite cell division in the midgut, there could be upregulation of the partial Krebs cycle involving SCoAS, using proline or glutamine as sources, and with increased excretion of succinate.

Metabolomic studies on parasites isolated from the tsetse fly midgut using $^{13}$C-proline, $^{13}$C-threonine or $^{13}$C-glutamine and metabolic flux analysis could allow us to understand how these L262P/L262Pγ clones are generating ATP to divide and survive within the harsh environment of the tsetse fly midgut. A comparison of metabolomic results from cells isolated from midguts and in vitro culture could indicate why in vitro culture cannot support growth of these L262P/L262Pγ clones. Metabolomic investigation of the midguts from infected and non infected flies could reveal other possible substrates for trypanosomes. However studies on PCF isolated from midguts would be challenging. Isolating the number of PCF required from midguts of flies would require many flies and much dissection time. Equally, tsetse fly midguts contain bacterial symbionts, which could contaminate results, and/or outgrow midgut-harvested PCF in in vitro conditions. Another possibility is that these symbionts could provide substrates for trypanosomes; this has not yet been investigated.

ATP production using different substrates can be measured in organello (Allemann and Schneider, 2000). The assay could be performed on mitochondria isolated from cells extracted from midguts and from in vitro cultured cells to indicate which pathways are being utilised to generate ATP in the different cell lines and in the different growth conditions. Succinate triggers oxidative phosphorylation (Fig. 1.9), whereas pyruvate triggers the ASCT
cycle and α-ketoglutarate triggers the SCoAS reaction. Addition of complex II inhibitor malonate or Fo inhibitor oligomycin would demonstrate the amount of ATP produced from oxidative phosphorylation.
4.5 Figures

Figure 4.1 After treating stumpy cells for 24 hrs with CA, cells look morphologically PCF
Stumpy cells isolated from mice were treated with CA for 24 hrs, mounted on microscope slides and DAPI stained. Compared to the stumpy form, cells are thinner, with a more pointed posterior end. Kinetoplast and nucleus (stained) are found more towards the middle of the cell. Cells have reentered the cell cycle (right hand panel), showing 2K1N and 2K2N configurations.
Figure 4.2  

EP procyclin is expressed by CA-induced AK cells

A) Western blot to assess EP expression. Stumpy cells were treated with CA for 24 hrs and 2x10⁶ cells were analysed with anti-EP (top panel) and anti-EF1α as loading control (bottom panel). Slender and stumpy WT/WTγ cells were also analysed as negative controls for EP expression. B) EP expression measured by flow cytometry. The equivalent of 2x10⁶ stumpy cells were fixed before and 24 hrs after CA treatment and stained with mouse anti-EP and anti-mouse Alexa 488 antibodies. Non-intact cells were gated out of the analysis using forward and side scatter, as shown in Appendix B. Red= parental AnTat1.1 90:13 cells +CA 24 hrs, Blue= parental AnTat1.1 90:13 cells –CA 24 hrs, Orange= parental AnTat1.1 90:13 cells 0 hrs. These control populations were used to define ‘–EP’ and ‘+EP’ gates for the time course experiment. The ‘–EP’ gate was set to include all counts up to the maximum fluorescence detected for –CA cells. All counts to the right of this gate were defined as ‘+EP’. C) Analysis of EP expression over the course of 24 hrs post CA treatment using the –EP/+EP gates defined in B. n=2, the equivalent of 2x10⁶ cells were analysed per time point.
Figure 4.3 Newly differentiated L262P/L262Pγ cells are unable to proliferate in vitro; glucose is toxic to freshly differentiated cells
A) Cells were treated with CA in HMI-9 for 24 hrs and transferred to either SDM80 (residual glucose, solid lines) or SDM79 medium (10 mM glucose, dashed lines; also indicated by ‘+’ next to the genotype in the chart legend) at a density of 2x10⁶/ml. Density was assessed by counting the cells over time, beginning 24 hrs after CA treatment (time point 0 h in the chart). Cells were counted once a day; after each counting, cells were diluted to a density of at least 3x10⁶/ml. B, C) WT/WTγ (B) and WT/L262Pγ clone 2 parasites (C) were initially grown in either SDM80 (-glucose, solid lines) or in SDM79 (+glucose, dashed lines) and transferred to the other type of medium at the times indicated by the arrows.
Figure 4.4  PCF specific markers can be detected in L262Pγ-expressing PCF cells

Cells were harvested after 48 hrs in SDM80. 2x10⁶ cells were analysed by western blot with the antibodies indicated. Anti GPEET was a kind gift from the Roditi lab, anti ASCT was a kind gift from the Bringaud lab.
Figure 4.5  Experimental infection of tsetse fly midguts with kDNA independent and AK trypanosomes

Flies were infected with trypanosomes of the genotypes shown. The infection status of the midguts were checked by dissecting flies 9 days after infection. A) % of flies with an infected midgut. w = none detected. Infections performed before insectary relocation, n=3. P-values were derived using a paired, 2-tailed Student’s t-test. * = P<0.05. B) % of flies found with a high level of midgut infection at >>10/field of view (+++), a low level of midgut infection at ~10/field of view (+), and no detectable midgut infection (-). n=3 for all mutated parasite lines. C) As B, but infections performed three months after insectary relocation. For cell line L262P/L262Pγ clone 2, n=3.
Figure 4.6   The morphology of PCF cells within the midgut
Flies were infected with trypanosomes of the genotypes shown. The midguts were dissected out of the flies 9 days after infection. Homogenised infected midgut tissue was added to PBS and spread on microscope slides (left panel). Tissue was also filtered and spread on microscope slides (right panels). Slides were DAPI stained to visualise nuclear and kDNA.
Figure 4.7  Generation of kDNA-independent metacyclic form trypanosomes

A) Experimental infection of tsetse fly salivary glands with kDNA dependent and independent trypanosomes. Flies were infected with trypanosomes of the genotypes shown. The infection statuses of the salivary glands were checked by dissecting flies 4 weeks after infection. For infections with genotypes WT/WTγ, WT/L262Pγ 1 and WT/L262Pγ 2, n=2. For the remaining infections, n=1. P-values were derived using a paired, 2-tailed Student’s t-test. ** = P<0.01. B) The midgut infection rate for flies from the same cohort that had their salivary glands dissected. C) Infection of mice with metacyclic form trypanosomes isolated from fly salivary glands. Tail snips were performed daily and DAPI stained blood smears were collected on days stated. D) Chromatogram confirming the genotype of metacyclic WT/L262Pγ clone 1 trypanosomes. Parasites were harvested from a mouse at peak parasitaemia by cardiac puncture, trypanosomes were purified and gDNA was extracted. ATP synthase γ was amplified via PCR and sequenced.
5. General discussion
The mitochondrion and kDNA maintenance and expression have been implicated in playing essential functions in the life cycle and cell cycle of *T. brucei*, but their precise roles, how they change over time, and how these changes are regulated is poorly understood. Here, using kDNA-independent and AK cell lines, we have been able to specify roles that kDNA plays and does not play during life cycle progression.

The life cycle of *T. brucei* dictates that parasites must be able to tolerate and adapt to a diverse but ordered range of environmental conditions. In each niche environment, the parasite must respond to signals in order to differentiate into forms better adapted to survival within those conditions. The changes involved in differentiation include entry into the cell cycle, morphology and metabolism. Control of metabolism is crucial as particular nutrients are not available in certain environments, and the parasite must be able to maintain production of ATP in order to drive the differentiation process and survive. Regulation of the mitochondrion is central to this control. Within the mammalian bloodstream, these extracellular parasites utilise the glucose-rich conditions. It is well documented that the slender BSF use a mitochondrion that does not function in oxidative phosphorylation (Verner et al., 2015). The essential Δψm is produced unusually by the *F*₁*F*₀ ATP synthase acting in reverse (Nolan and Voorheis, 1992, Brown et al., 2006, Schnaufer et al., 2005, Vercesi et al., 1992), as the proton pumping complexes of the respiratory chain are absent. Glycolysis is upregulated for ATP production and the first seven steps are localised within the glycosomes (Michels et al., 2006). Upon receiving a self-generated density-dependent differentiation signal, SIF, cells respond by arresting in the cell cycle (Vassella et al., 1997), prolonging host survival. A host of other changes allow preadaptation to survival within the tsetse fly midgut, as stumpy forms are the form transmissible to the insect vector (Nolan et al., 2000, Engstler and Boshart, 2004, Dean et al., 2009). Mitochondrial energy metabolism is known to be upregulated, allowing α-ketoglutarate to also be used as a metabolic substrate.
(Bienen et al., 1993), at least in vitro. Structural elaboration of the mitochondrion also begins, with cristae formation in preparation for the oxidative phosphorylation within the PCF (Vickerman, 1965). Once ingested by the tsetse fly during a bloodmeal, the surface protein of the stumpy form parasite, PAD1, is able to receive the metabolic differentiation signal from within the fly midgut (Dean et al., 2009). This signals another set of ordered cellular changes. Cells reenter the cell cycle (Matthews and Gull, 1994b), allowing colonisation of the midgut. Proline found within the fly is used as the respiratory substrate. The mitochondrion thus becomes more upregulated in function, with classical oxidative phosphorylation thought to predominate as the pathway producing ATP and $\Delta \Psi m$ (Lamour et al., 2005). From here the parasite performs a series of defined migrations between fly compartments coordinated with extreme morphological changes and asymmetric cell divisions (Ooi and Bastin, 2013). After passing through the PM from the midgut lumen, the parasite moves towards the foregut, through the PV and up into the salivary glands (Dyer et al., 2013). Due to the difficulty of culturing these stages in vitro, mitochondrial activity in these stages is currently unexplored. The animal-transmissible metacyclic form found within the salivary glands in bioenergetic terms have a more downregulated and deelaborated mitochondrion than in previous stages (Brun et al., 1984), in preparation for survival as slender BSF that they will differentiate into once within the mammalian bloodstream.

The mitochondrion of T. brucei contains an essential and elaborately structured genome. Expression of this genome involves an intricate and highly involved process of mRNA editing as many genes encoded within this kDNA (kinetoplast) are encrypted. As the kDNA encodes subunits of the mitochondrial ribosome, ATP synthase and respiratory chain complexes, not all protein products are required for each life cycle stage. Control of kDNA expression is therefore crucial for the regulation of mitochondrial activity and for adaptation to different metabolic substrates. Accordingly, expression and editing of the kDNA transcripts is tightly coordinated within the life cycle. The dependency on glycolysis in the
BSF means that complexes III and IV of the respiratory chain need not be expressed, however as $\Delta\psi_m$ production is dependent on the ATP synthase complex in BSF, the subunit of ATP synthase $F_o$ encoded in kDNA, A6, is required for the entire cycle, as is ribosomal subunit RPS12 (Dean et al., 2013). In fact, one theory for the evolution of extensive RNA editing is that a process was required to prevent erosion of the kDNA repertoire not necessary for each life cycle stage (Speijer, 2006).

Despite the essentiality of kDNA through the life cycle, there are naturally occurring subspecies of T. b. brucei that survive without kDNA in the BSF. These cells have been shown to express mutated ATP synthase $\gamma$ subunit (Schnaufer et al., 2005, Dean et al., 2013) (Lai et al., 2008). In this regard, T. b. evansi and equiperdum have similarity to petite-negative yeast, with expression of mutated $\gamma$ subunit allowing survival in the absence of mitochondrial DNA (Chen and Clark-Walker, 1995). In yeast the presence of such mutations not only permits survival of cells with gross deletions in mtDNA (mtDNA$^-$), or its complete loss (mtDNA$^0$), but actively selects for such ‘petite’ cells. The presence of mutated $\gamma$ is thought to uncouple the actions of $F_o$ from $F_1$, resulting in a proton leak (Mueller, 2000). Since such a proton leak would dissipate the essential $\Delta\psi_m$ through $F_o$, a lethal event, cells that have lost the mtDNA-encoded $F_O$ subunits have a strong growth advantage. In trypanosomes, expression of such mutated $\gamma$ subunits does not appear to have the same effect, as a seemingly intact kDNA network is still visible in these cells (personal observations). In Chapter 2, we aimed to understand how T. brucei cells expressing L262P$\gamma$ (a mutation known to obviate the requirement for kDNA in the BF stage) are able to compensate for the loss of kDNA, and tolerate the possible proton leak. Multiple cell lines were generated expressing heterozygous or homozygous mutated ATPase $\gamma$. It was confirmed that cell lines expressing L262P$\gamma$ had functionally uncoupled $F_1F_o$ ATPase complexes. In the presence of homozygous L262P$\gamma$, in several independent transfectants there seemed to have been selection for cells that had lost expression of A6, supporting the
theory that mutant γ causes a proton leak via F_o subunit A6. It appears that, in these clones, loss of A6 caused structural disruption of the F_1F_o ATPase complex, presumably as a consequence of destabilisation of F_O in the absence of A6. There were indications that clone-specific adaptation in the presence of L262Pγ occurred to maintain Δψm production, but the precise nature of these adaptations remains to be elucidated. Further work is needed to quantify the effect of L262Pγ on kinetics of the ATPase reaction and the impact of using alternative mechanism of generating Δψm on the cell as a whole.

AK and DK subspecies T. b. evansi and equiperdum are deemed to be monomorphic. Although they are found in the slender BSF mammalian bloodstream, they are only rarely found differentiated to the stumpy form (Section 1.10.2), and are thus mechanically transmitted. In Chapter 3, we aimed to investigate whether loss of kDNA was directly linked to a loss of BSF pleomorphism. For the first time, mutant ATPase γ was expressed in a pleomorphic strain of T. brucei (AnTat1.1 90:13), able to differentiate to the stumpy life cycle stage, and acriflavine treatment produced AK cell lines. Mice infections revealed that AK AnTat1.1 90:13 cell lines could differentiate to the stumpy form (Fig. 5.1), unexpectedly showing that T. b. evansi and equiperdum are monomorphic not due to the loss of kDNA. An infection time course confirmed that slender to stumpy differentiation occurred in AK cells over the same time scale as kDNA+ cells expressing WTγ or L262Pγ. The data was then fitted to a SIF-dependent mathematical model for stumpy formation and in vivo infection by Dr Nick Savill (Macgregor et al., 2011). A key prediction by the model was a shorter lifespan of AK stumpy cells compared to kDNA+ cells. Although nuclear DNA damage caused by acriflavine treatment cannot be completely ruled out as a cause of this lifespan decline, it appears that kDNA-encoded gene products may be required to sustain the short life span of the non-proliferative stumpy form. Complex I subunits would be a potential candidate as it has been reported that Δψm in the stumpy form is sensitive to complex I inhibitor rotenone (Bienen et al., 1991).
It has long been assumed that kDNA is essential for the PCF due to the requirement for oxidative phosphorylation within the fly midgut, but this has never been directly proven. The fact that AnTat1.1 90:13 AK cells can differentiate to the stumpy form allowed us to conclusively probe this question in Chapter 4. Cells lacking maxicircle or all kDNA did not initiate midgut infections, proving that kDNA is essential for the PCF in vivo (Fig. 5.1).

During in vitro differentiation, AK cells were able to express EP, a surface protein of the PCF, but did not survive CA treatment.

L262Pγ expressing cells with – as far as could be determined – intact kDNA were able to infect the tsetse fly midgut, surprisingly even L262P/L262Pγ expressing cells that are expected to have inefficient ATP synthase activity, or lack it altogether (Clark-Walker, 2003, Dean et al., 2013). The lack of efficient ATP synthase activity in homozygous L262Pγ cell lines remains to be confirmed in direct biochemical assays, but indirect evidence presented in this thesis suggests that oxidative phosphorylation is not essential for PCF growing within the midgut. WT/L262Pγ cells were able to initiate a metacyclic salivary gland infection, demonstrating that, despite the expectation that roughly half of the F₁F₀ complexes in these cells constitute a proton leak, these drug resistant parasites are able to complete the T. brucei life cycle and theoretically can be spread by tsetse flies. Freshly differentiated AnTat1.1 90:13 cells were incapable of growing in vitro in the presence of glucose, suggesting that stumpy to PCF differentiation primes cells to life in the absence of glucose, and further adaptation is required to allow glucose metabolism. This metabolic rerouting and the basis for glucose toxicity remain to be investigated.
5.1 So why do kinetoplastids need a kinetoplast?

The use of kDNA-independent cell lines in this study has generated considerable new insight into understanding the role of kDNA through the *T. brucei* life cycle. Significant inroads have been made into the molecular mechanism of kDNA-independence in the BSF, strongly suggesting that compensatory mutations in subunit γ not only allow survival in the absence of kDNA, but are incompatible with having complete and fully functional kDNA. We now know that kDNA plays no role in slender to stumpy differentiation, and only influences cell cycle reentry during stumpy to PCF differentiation. There are implications that kDNA is required for maintenance of Δψm and to preserve the limited lifespan of the stumpy form. We have shown that kDNA is essential for the PCF in the tsetse fly midgut. Our findings support the theory that kDNA has a role in maintaining the capacity of the respiratory chain to balance the redox potential in the mitochondrion and/or maintaining Δψm in PCF rather than due to the essentiality of oxidative phosphorylation in PCF.

As discussed in each chapter, further work is required to completely rule out alternatives to these conclusions, for example non-mitochondrial effects of acriflavine that might have affected AK stumpy longevity, and ability of L262Pγ F₁Fₒ to function in ATP synthesis. Work is also required to answer a multitude of new questions that have been raised. What are the differences between the molecular mechanisms of different compensatory ATPase γ mutations found within naturally occurring DK subspecies? How do stumpy forms generate mitochondrial ATP and Δψm? What environmental differences are responsible for the proliferation of L262P/L262Pγ PCF cells within the fly midgut but not *in vitro*?
5.2 Outlook

Inevitably, this work leads on to important wider questions that remain about the impact of kDNA on the cell biology of T. brucei. Regarding life cycle differentiation, how are the differentiation signals like SIF and CA received in different environments of the life cycle able to cause controlled mitochondrial upregulation? How are kDNA expression and editing coordinated with this? How is complex V function regulated through this differentiation? Is cristae formation during differentiation governed by oligomerization of complex V, as in some other organisms (Zick et al., 2009)? Equally, how is the mitochondrion downregulated first in the metacyclic form, and then again in the BSF? Many differentiation and migratory signals within the fly stages are unknown, making this analysis extremely challenging. Mitochondrial function and its impact on the cellular metabolism in epimastigote and metacyclic stages also remain to be probed.

Regarding the role of the mitochondrion in metabolism, it has been recently shown that the mitochondrion plays an essential role in generating acetate for de novo fatty acid synthesis (Mazet et al., 2013, Huang et al., 2013). Equally, succinyl CoA synthetase has also been recently shown to be essential in the BSF (Zhang et al., 2010b). That these two ATP-producing pathways that were thought to be repressed in BSF mitochondria are in fact essential suggests that we are overlooking a more intricate metabolic network in the BSF mitochondria. How does the compensatory mechanism for viability in the absence of kDNA impact on these essential mitochondrial pathways?

The present study demonstrated that kDNA is required for differentiation to the PCF (Chapter 4). In respiration-deficient human cells lacking mitochondrial DNA, aspartate has been shown to compensate for the loss of mtDNA by circumventing the requirement for electron acceptors in these cell lines (Birsoy et al., 2015, Sullivan et al., 2015). Whether this is also true in trypanosomes remains to be examined. Concerning the other stages of the life cycle, the requirement for kDNA is now understood, however, the roles that particular
kDNA-encoded proteins play in these stages still require elucidation. For example, is complex I required to generate Δψm in the stumpy form? The metabolism occurring in less experimentally-tractable stages also requires further probing.

Equally, the mechanisms of kDNA replication and division and mitochondrial fission that occur every cell cycle during proliferative life stages are also unknown (Hammarton, 2007, Farr and Gull, 2012, Povelones, 2014). Nuclearly-encoded proteins have been implicated in these processes (for example (Morgan et al., 2004, Chanez et al., 2006, Clayton et al., 2011, Milman et al., 2007)) but no significant insight has been produced on control of these critical parts of the cell cycle. The kDNA-independent cell line promises to be a useful tool in the analysis of potential candidates, some of which, if essential, could prove to be novel drug targets. Equally this work supports the idea that there is a kDNA-dependent cell cycle reentry checkpoint during differentiation to the PCF (Chapter 4)(Timms et al., 2002). The molecular basis of this checkpoint requires elucidation.

The functions, if any, of maxicircle genes MURF2, MURF5, CR3 and CR4 are still unknown. CR3 and CR4 are preferentially edited in the BSF (Stuart et al., 1997, Corell et al., 1994), with MURF2 being edited in the BSF and PCF (Feagin and Stuart, 1988). Hence it seems that if these proteins are expressed in the BSF, they become dispensable in the presence of an ATPase γ mutation, like RPS12. The prospect of alternative editing also expands the kDNA-encoded proteome (Ochsenreiter et al., 2008b), potentially generating multiple novel proteins with roles in mitochondrial biogenesis and regulation, for example AEP-1, shown to be a component of the TAC (Ochsenreiter et al., 2008a). Kinetoplast-independent cell lines again could prove an important control for analysing the roles of these proteins, along with new reverse genetic techniques to study kDNA-encoded protein function (Szmpruch et al., 2015).
In *T. brucei*, mechanical transmission seems to be linked with kDNA loss. There are different possibilities as to the order in which adaptation events may have occurred in the evolution of naturally occurring DK *T. b. brucei* subspecies. Firstly, parasites could have adapted towards mechanical transmission (via adaptations currently unknown), allowing the parasite to escape the tsetse belt, rendering most kDNA redundant. Some selective pressure could then have selected for the mutation of ATPase γ, allowing full loss of functional kDNA, and the adoption of the alternative mechanism to generate Δψm. However, what is not clear in this scheme is what selective advantage kDNA independence would offer, and why mechanically transmitted *T. brucei* have not retained the ability to express A6 and RPS12, unlike mechanically transmitted American strains of *T. vivax* (Greif et al., 2015). Equally, why is there no selection for cells containing mutated ATPase γ in *T. vivax*? This could be due to differences in metabolism, meaning that the alternative mechanism of generating Δψm would not be supported in *T. vivax*. It could also suggest that there are differences between the structures of complex V in *T. vivax* and *T. brucei*, so that a mutation in subunit γ does not uncouple the enzyme, for example. Alternatively, the structure of the *T. vivax* F₁Fₒ complex could be highly sensitive to mutations, perhaps because it is integral for maintaining mitochondrial architecture in the BSF, for example in the generation of cristae that are present in *T. vivax* BSF mitochondria (Vickerman, 1962a, Vickerman, 1962b), or necessary for efficient mitochondrial function (Zick et al., 2009).

In an alternative evolutionary scenario, an ATPase γ mutation could arise first, allowing the parasite to begin to lose kDNA. This work provides a mechanism for how an ATPase γ mutation could select for parasites with loss of kDNA (Chapter 2). The loss of kDNA could then somehow increase the chances of mechanical transmission. One theory was that kDNA loss might in turn cause loss of competence for stumpy differentiation and, hence, loss of self-limiting parasitaemia (Schnaufer, 2010). The resulting high density of slender cells
would enhance mechanical transmission (Desquesnes et al., 2009). This can now be refuted as kDNA loss does not affect slender to stumpy differentiation (Chapter 2). Another theory is that the lack of kDNA could dampen immune system activation (Zhang et al., 2010a, Collins et al., 2004, Oka et al., 2012), which could allow a more chronic infection to occur. Simulations with a mathematical model have countered this as AK parasites seem to be more immunogenic than kDNA+ parasites (Chapter 2). Thus, understanding how kDNA loss and mechanical transmission are linked remains an open question.

Mitochondria are recognised as being highly involved in programmed cell death in eukaryotes, with the mitochondrion believed to have a role in controlling the type of cell death performed (Kroemer, 1997, Kroemer et al., 2007). The role of cell death in single-celled parasites is thought to be used to control parasite numbers, allowing survival of the parasite in the face of limited resources and the host (Welburn and Maudlin, 1997, Welburn et al., 1989, Figarella et al., 2005, Luder et al., 2010). Indeed, programmed cell death may play a role in the limited life span of stumpy cells (Duszenko et al., 2006). Δψm loss is traditionally a hallmark of programmed cell death (Zamzami et al., 1995, Ly et al., 2003, Zong and Thompson, 2006). Interestingly, the data in Chapter 3 here suggests that loss of Δψm could have a role in stumpy cell death. The mechanism for limitation of life span in stumpy cells remains to be fully elucidated.

Many proteins acting in apoptosis are absent in Kinetoplastids (Kaczanowski et al., 2011), however cell death phenotypes are similar to those displayed in other eukaryotic cells (Smirlis and Soteriadou, 2011), with mitochondrial dysfunction such as ROS production being heavily implicated (Das et al., 2008, Das et al., 2001, Irigoin et al., 2009, Sen et al., 2004, Mehta and Shaha, 2004, Ridgley et al., 1999). The pathways involved in these processes and impact of the kDNA and mitochondrion remain to be elucidated.
Crucially, understanding mitochondrial function and regulation is becoming an important aspect of controlling human disease. Many conditions have now been recognised as having a mitochondrial component, including cancer, diabetes and Alzheimer’s disease (Weinberg and Chandel, 2015, Kroemer, 1997, Sivitz and Yorek, 2010, Piaceri et al., 2012). African trypanosomes make an ideal tool in which to study mitochondrial regulation. With each cell containing a singular mitochondrion, heteroplasmy is not an issue. In *T. brucei* importantly the cycle of mitochondrial regulation can be studied in one controllable genetic background.
5.3 Figures

Chapter 3 of this thesis shows that AK parasites are able to perform slender to stumpy differentiation. Mathematical modelling suggested that AK stumpy forms have a lower lifespan than kDNA+ stumpy forms. Chapter 4 of this thesis shows that AK stumpy forms cannot complete differentiation to the PCF. WT/L262P parasites are able to complete the life cycle, suggesting that they can be transmitted by tsetse flies. L262P/L262P parasites enter growth arrest in vitro, but do establish PCF midgut infections in vivo. However, it seems that L262P/L262P parasites are unable to differentiate further in the life cycle.
6. Appendices
Appendix A: A quantification of the expression levels of ATPase γ alleles in *T. b. evansi* strains

**Introduction**

A comparison of the sequences of ATPase γ subunits between strains of *T. b. brucei*, the naturally occurring DK subspecies *T. b. evansi*, and an acriflavine-induced DK *T. b. brucei* strain had previously revealed several polymorphisms (Schnaufer et al., 2005, Lai et al., 2008). Subsequently, these mutations had been introduced into bloodstream form Lister 427 *T. b. brucei* to investigate whether they, as similar mutations in petite negative yeast, are able to compensate for the loss of mitochondrial DNA, and, as such, permit generation of the essential mitochondrial membrane potential independent of the ATPase-F₀ moiety (Dean et al., 2013). Mutations L262Pγ and A273Pγ were found to be fully sufficient to allow the loss of kDNA. Cells expressing this mutation therefore showed increased resistance to treatment with the drug acriflavine, a DNA intercalator that preferentially binds kDNA, preventing its replication (Manchester et al., 2013). Introduction of a single A281-γ allele, as found in many naturally occurring DK subspecies (Table 1.2), on its own appeared to be insufficient and acriflavine-treated cells appeared to require additional adaptations before they showed normal growth (Dean et al., 2013). Analysis of γ subunit cDNA from *T. b. evansi* STIB810 had found that 90% of transcripts originated from the mutated A281-γ allele (Lai et al., 2008), suggesting that the mutated γ allele is somehow transcribed in preference to the WT allele. Could such differential allelic expression be part of the additional compensatory mechanism that appeared to be required to allow naturally occurring DK subspecies with the A281-γ mutation to remain viable despite loss of kDNA? Conceivably, an increase in the proportion of mutated γ subunits would increase the number of ATPase-F₁ complexes capable of functioning independently of F₀, the complexes that are efficiently contributing to production of the Δψm via the AAC.
Initially, ATPase subunit \( \gamma \) 5’ and 3’ UTRs were compared between kDNA\(^+\) and DK \( T. brucei \) subspecies, in order to investigate whether any polymorphisms are found in \( T. b. evansi \) and \( T. b. equiperdum \) \( \gamma \) UTRs that could influence the level of mutant \( \gamma \) allele expression. Pyrosequencing was then employed to quantify the proportion of ATPase \( \gamma \) subunit transcripts originating from each allele in three strains of \( T. b. evansi \) to verify the original published observation.

**Materials and Methods**

**Table 6.1 Primers used in this chapter**

<table>
<thead>
<tr>
<th>Primer #</th>
<th>Name of primer</th>
<th>Sequence of primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Amplification of ( \gamma ) 5’ UTR F</td>
<td>GCGGAATTTCGAAGCAGATGACACCTAA</td>
</tr>
<tr>
<td>2</td>
<td>Amplification of 3’ end of ( \gamma ) R</td>
<td>ATAGGATCCCTACTTGTTACTGCCCTTCCAG</td>
</tr>
<tr>
<td>3</td>
<td>Amplification of ( \gamma ) 3’ UTR F</td>
<td>GCGGGATCCTATTTGTTCATAATGCGGTAGGTATGC</td>
</tr>
<tr>
<td>4</td>
<td>Amplification of ( \gamma ) 3’ UTR R</td>
<td>GCGGAAGACCTTGCTGCGAGCCACTCT</td>
</tr>
<tr>
<td>5</td>
<td>Pyrosequencing F ( \gamma ) SNP region</td>
<td>ACGGATAAGGACACCGGACTTC</td>
</tr>
<tr>
<td>6</td>
<td>Pyrosequencing R ( \gamma ) SNP region</td>
<td>Biotin-GTTTGCTGAAGCGCCAACTCC</td>
</tr>
<tr>
<td>7</td>
<td>Pyrosequencing Seq ( \gamma ) SNP region</td>
<td>CGCGATTTCAGCCTC</td>
</tr>
<tr>
<td>8</td>
<td>Pyrosequencing F ( \gamma ) deletion region</td>
<td>Biotin-GTATTACGCGCGCTAAATT</td>
</tr>
<tr>
<td>9</td>
<td>Pyrosequencing R ( \gamma ) deletion region</td>
<td>TTGCCTTCAACGAACTCA</td>
</tr>
<tr>
<td>10</td>
<td>Pyrosequencing Seq ( \gamma ) deletion region</td>
<td>CCTTCAACGAACCTCA</td>
</tr>
<tr>
<td>11</td>
<td>pCR-BLUNT insert F</td>
<td>GTAAAACGACGCCCAG</td>
</tr>
<tr>
<td>12</td>
<td>pCR-BLUNT insert R</td>
<td>CAGGAAACAGCTATGAC</td>
</tr>
</tbody>
</table>
Amplification of ATPase γ 5’ and 3’ UTRs

ATPase γ subunit (TriTrypDB gene ID Tb927.10.180) 5’ UTR + ORF and 3’ UTR were amplified with Phusion High Fidelity DNA polymerase (Fisher Scientific), using genomic DNA (gDNA) from strains of T. b. evansi (STIB810, STIB805, AnTat3/3) and T. b. equiperdum (STIB818) with conditions shown below. Each PCR reaction was assessed using gel electrophoresis using a 1% agarose gel, and PCR products of the correct size were gel purified via NucleoSpin extract kit (Machery-Nagel).

Temperature cycles for 5’ UTR+ORF PCR: 98°C 30 seconds, [98°C 10 seconds, 58°C 30 seconds, 72°C 90 seconds] for 35 cycles, 72°C 10 minutes.

Temperature cycles for 3’ UTR PCR: 98°C 30 seconds, [98°C 10 seconds, 64°C 30 seconds, 72°C 30 seconds] for 35 cycles, 72°C 10 minutes.

Reaction conditions per 50 µl PCR reaction: 10 µl 5x reaction buffer, 1 µl 10 mM dNTPs, 2.5 µl 10 µM each primer (#1 and #2 for 5’ UTR+ORF, #3 and #4 for 3’ UTR, Table 6.1), 0.5 µl Phusion High Fidelity polymerase, 32.5 µl dH2O, 1 µl 50 ng gDNA template.

Ligation of ATPase subunit γ and γ UTRs into Zero BLUNT

Each gel-extracted amplicon was ligated into the pCR-Blunt plasmid using the Zero BLUNT PCR cloning kit (Life Technologies).

10 µl reaction conditions: 1 µl 25 ng pCR-Blunt, 2 µl 5x T4 DNA ligase buffer, 1 µl (5 U/µl) T4 DNA ligase and up to 5 µl gel-extracted PCR product amplified by Phusion DNA polymerase (Fisher Scientific), mixed and incubated at room temperature overnight.
2 µl ligation mix was transformed into 50 µl *E. coli* XL1-Blue. Transformed cells were spread onto agar plates containing 50 µg/ml kanamycin (Sigma) and incubated overnight at 37°C. Colonies from each transformation were picked into 3 ml LB broth and plasmid DNA prepared using PeqGold miniprep kit.

**Sequencing**

Sanger sequencing big dye reactions were carried out and analysed by Edinburgh Genomics, as per their protocol. Direct sequencing was carried out on the gel-purified products of PCR reactions detailed above to identify SNPs present in these amplicons, using the same primers as those used for the PCR reaction. Sequencing was also carried out on PCR products ligated into pCR-Blunt using primers #11 and #12 (Table 6.1).

**Infection of Sprague Dawley rats with *T. b. evansi***

Sprague Dawley rats were infected with 500 µl freshly thawed *T. b. evansi* strains AnTat3/3 or STIB805 via IP injection. Parasitaemia was monitored and judged as per the Rapid Matching method (Herbert and Lumsden, 1976), and at peak parasitaemia of approximately 2.5 x10⁸/ml a cardiac puncture was administered in order to collect the infected blood.

**Purification of trypanosomes from blood**

DEAE-cellulose DE52 (Whatman) anionic exchange columns were pre-incubated with filtered PSG solution (44 mM NaCl, 57 mM Na₂HPO₄, 3 mM KH₂PO₄, 55 mM glucose, pH 7.8) warmed to 37°C. Blood was loaded onto the column, the column was continuously topped up with warm PSG and eluate was collected. The density of the cells was measured using a Beckmann Z2 Coulter counter, and 1x10⁹ cells were pelleted at 1300 x g for 10 minutes and put on ice.
RNA extraction

RNA extraction was performed via the Life Technologies TRI reagent protocol. Certain precautions were taken during this protocol: the protocol was carried out in a fume hood, double layered gloves were worn, benches and pipettes were cleaned thoroughly prior to the protocol with 70% ethanol and RNaseZAP (Ambion), and filter tips were used to reduce RNase contamination. RNA pellets from $1 \times 10^9$ cells were resuspended in 10 ml ice cold TRI reagent solution (Ambion), and frozen at -80°C. The lysate was thawed for use at room temperature, 1 ml bromo-chloro-3-propanol (BCP) was added and the solution was vortexed. After a 15 minute incubation at room temperature, the lysate was centrifuged at 12,000 x g for 15 minutes at 4°C. The aqueous phase was collected and 5 ml isopropanol was added. After vortexing and a 10 minute incubation at room temperature, the mixture was centrifuged at 12,000 x g at 4°C for 15 minutes. The pellet was washed in 10 ml 75% ethanol and centrifuged at 12,000 x g for 10 minutes at 4°C, and the final pellet was air dried before being dissolved in 100 µl DEPC water. The RNA solution was stored at -80°C.

*T. b. evansi* STIB810 RNA was gratefully received from Dr Hassan Hashimi. This was the RNA that had been used in Lai et al., 2008, the paper where the original observation had been published.

DNase treatment of extracted RNA

Removal of contaminating DNA was performed as via the Ambion DNA-free DNase kit. 40 µl RNA was mixed with 4 µl DNase I 10x buffer and 0.5 µl DNase I, and incubated at 37°C for 30 minutes. An additional 0.5 µl DNase I was then added, and the mixture was incubated for a further 30 minutes at 37°C. After vortexing, 4 µl inactivation reagent was added and the solution was mixed for 2 minutes, and then centrifuged at 10000 x g for 90 seconds. 40 µl of supernatant was transferred to a fresh Eppendorf tube.
Nano Agilent analysis of RNA

The integrity of the DNase-treated RNA was assessed using the Agilent Bioanalyzer nanochip kit. Briefly, the chip priming station was set to ‘C’, and an RNA Nano chip was positioned in the station. 1 µl Nano dye concentrate was mixed with 65 µl of spin filtered (1500 x g for 10 minutes) Nano gel matrix, and vortexed thoroughly. 9 µl was pipetted into the ‘G’ well for the RNA Nano chip, and was dispensed by pressing down of the plunger for 30 seconds. 9 µl of the gel-dye mix was then pipetted into the two wells above well ‘G’, 5 µl Nano marker was pipetted into all remaining wells and 1 µl heat-denatured RNA ladder (70°C for 2 minutes) was pipetted into the ladder well. 1 µl of each sample was loaded into each of the sample wells before the chip was vortexted for 60 seconds at 2400 rpm. The chip was inserted into the Agilent 2100 Bioanalyzer to start the run.

Reverse transcription of T. b. evansi RNA

Reverse transcription was performed using the protocol for Life Technologies SuperScript III. 25 ng RNA in 11 µl dH2O was mixed with 1 µl 10 mM dNTPs and 1 µl 200 ng/µl random hexamer primers (NEB), and the solution was incubated at 65°C for 5 minutes, and then put on ice for at least 1 minute. 4 µl 5x first strand buffer, 1 µl 0.1 M DTT, 1 µl RNaseOUT (40 units/µl) (Invitrogen) and either 1 µl Superscript III Reverse Transcriptase (+RT) or 1 µl dH2O (-RT) were then added. The following reaction conditions were used: 25°C 5 minutes, 50°C 60 minutes, 70°C 15 minutes.

1 µl RNase H (NEB) was then added, and the mixture was incubated at 37°C for 20 minutes to remove RNA contamination. To stop the reaction, the temperature was increased to 90°C for 10 minutes.
Design of pyrosequencing primers and PCR of pyrosequencing templates

PCR and sequencing primers were designed by the Biotage PSQ Assay Design software, and were ordered from Sigma (Table 6.1, Figure 6.2A). PCR reactions were performed on 60 ng 
*T. b. brucei* and *T. b. evansi* gDNA and cDNA to amplify the ~60 bp surrounding the two polymorphism (the ‘SNP region’ and the ‘deletion region’), using primers #5 and #6 (SNP region) and #8 and #9 (deletion region) (Table 6.1). Controls were generated by performing these PCR reactions on plasmid DNA containing cloned versions of each γ allele. This provided samples that should score 100% WT (‘allele 1’) or 100% mutated γ allele (‘allele 2’) in the pyrosequencing analysis. Additionally, plasmid DNA for each allele was mixed at a 1:1 ratio and the PCR reaction was performed using this mixture as template to provide a 50% WT, 50% mutated γ allele control.

PCR temperature cycle conditions: 95°C 5 minutes, [95°C 1 minute, 54°C 1 minute, 72°C 2 minutes] for 30 cycles, 72°C 10 minutes.

PCR reaction conditions (25 µl volume): 14.25 µl dH₂O, 5 µl 5x GoTaq reaction buffer (Promega), 0.2 µl 25 mM dNTPs, 1.5 µl MgCl₂, 0.5 µl 10 µM each primer, 1 µl ~10 µg/ml gDNA template, 0.25 µl GoTaq polymerase (Promega).

5 µl of each reaction was analysed by 2% agarose gel electrophoresis.

Pyrosequencing

Pyrosequencing was performed using Qiagen reagents and a Biotage Pyromark instrument (Figure 2.8). 0.5 µl pyrosequencing primer (#7 for SNP region, #10 for deletion region, Table 6.1) was added to 11.5 µl annealing buffer in each well of a Biotage pyrosequencing plate. In a round bottomed microtitre plate, 30 µl water, 38 µl binding buffer, 2 µl
streptavidin beads and 5 µl PCR product were added to each well and placed on a shaker to mix. Nucleotide, enzyme and substrate solutions were added into dispensing tips in volumes advised by the PSQ Assay Design programme. Using the hedgehog vacuum (Biotage), the contents of the wells containing the PCR products were washed in 70% ethanol for 5 seconds, denatured in 0.2 M NaOH for 5 seconds and washed in 10 mM Tris acetate pH 7.6 for 5 seconds. The vacuum was switched off, and the hedgehog holding the beads with PCR product bound was lowered into the pyrosequencing plate and shaken. This plate was incubated at 80°C for 2 minutes, cooled and then placed in the instrument for the pyrosequencing run to commence.

Three individual pyrosequencing runs were carried out with each sample in triplicate. Each pyrosequencing run was carried out with a separate PCR reaction providing the template. Peak height assessment was performed by Biotage PSQ software. In the SNP position, the base detected can either be T or C, hence the % T value for the SNP region was calculated by the programme using the following formula:

\[
\frac{\text{height of T peak}}{\text{height of T peak} + \text{height of C peak}} \times 100
\]

With % T value being the % of subunit γ alleles within the amplified material that have been identified as WT, i.e. having the nucleotide T at the SNP position, nucleotide 142, in γ.

The % No Deletion value for sequence TAGAAA (A281-) vs. TAGCAGAAA (WT; sequence deleted in A281- in italics) was calculated manually by the following formula:

\[
\frac{\text{height of C peak}}{\text{height of first G peak}} \times 100
\]

with the first G base (underlined) taken as an indication of a peak of intensity of 1.
With % No Deletion value being the % of subunit γ alleles within the amplified material that have been identified as WT, i.e. having the codon GCT at nucleotide 841-843 in subunit γ.

**Results**

No consistent polymorphisms are present within ATPase γ subunit UTRs from DK subspecies ATP synthase γ subunit coding sequences and 5’ and 3’ UTRs were amplified using gDNA from strains of *T. b. evansi* (STIB810, STIB805, AnTat3/3) and *T. b. equiperdum* (STIB818). Each gel-extracted amplicon was ligated into pCR-Blunt and transformed into *E. coli* XL1-Blue. Colonies were picked and plasmid DNA prepared and sequenced. Sequencing was performed on both the gel-purified and the cloned PCR product. No consistent polymorphisms were found within the 5’UTR between *T. b. brucei* Lister 427 and the various DK strains (data not shown). One polymorphism was found between the alleles of the 3’ UTR in *T. b. evansi* STIB805, but this polymorphism was not present in any other strains (data not shown). Within the coding region of subunit γ itself, a T/C polymorphism at nucleotide 142 was found that was always consistent with the presence or absence of the A281- mutation (bases 840-842 ‘TGC’ of the wild type ATPase subunit γ CDS) in the strains analysed (data not shown). Only in STIB818 was the T in the SNP position found in the same allele as the deletion. In strains STIB810, STIB805, AnTat3/3, T was found in the WTγ allele.

**Generation of cDNA from strains of *T. b. evansi***

In the absence of an obvious polymorphism in the γ UTRs, it was decided to investigate both the region containing the SNP (referred to here as the ‘SNP region’, bases 115-169 of ATPase γ subunit CDS) and the region containing the deletion (the ‘deletion region’, bases 809-863 of ATPase γ subunit ORF) using pyrosequencing to quantify the ratio of WT to...
mutated γ allele at the gDNA and cDNA level. By doing this, we could verify the original observation that the mutated allele was more highly expressed at the cDNA level (Lai et al., 2008). Pyrosequencing is a sequencing technique that, via an enzyme-coupled DNA polymerase reaction, detects light produced in proportion to the frequency of the allele in a sample, and as such allows to quantitate the relative expression levels of alleles of the same gene (Figure 2.8).

Total RNA isolated from T. b. evansi AnTat3/3, STIB805 and STIB810 was reverse transcribed; mock reactions minus reverse transcriptase (-RT) were included for each sample. PCR amplification of ATPase subunit γ using +RT and –RT reactions was performed to assess the degree of gDNA contamination in the –RT control. These reactions were analysed via gel electrophoresis, and showed no detectable amplicon in the –RT controls (data not shown).

**Generation of the pyrosequencing template**

Sequencing had shown the link between the WT γ allele having a T nucleotide at the SNP position (base 142 of ATPase γ ORF) and the A281- mutated γ allele having a C nucleotide in this position seemed to always be consistent (except in STIB 818, where it was the other way round) (data not shown), hence the SNP region could also be assessed, acting as an additional indicator marker for the relative expression levels of the A281-γ allele. In order to assess any potential upregulation on the mRNA level we needed to confirm that in the genomic DNA the ratio between the two alleles was 1:1. Hence PCR reactions were performed on T. b. evansi cDNA and gDNA to amplify ~60 bp regions surrounding the SNP and the A281 deletion. All PCR and sequencing primers were designed by the Pyrosequencing Assay Design program (Figure 6.2A), and specificity was confirmed by BLAST analysis against the T. brucei TREU 927 genome (www.TriTrypDB.org).
Pyrosequencing quantification of ATPase γ subunit allelic expression

Gel electrophoresis of these PCR reactions showed only the expected ~60 bp amplicons and no bands in the negative controls (data not shown). These PCR products were subjected to pyrosequencing followed by analysis with the Biotage PSQ software. Positive controls were generated by using cloned alleles of *T. b. evansi* AnTat3/3, either individually to mimic homozygosity or mixed in a 1:1 ratio to mimic heterozygosity. The WT and A281- alleles are referred to here as ‘allele 1’ and ‘allele 2’, respectively. Figure 6.2B shows the output of these control pyrosequencing reactions. The polymorphic regions of the sequences are highlighted, with the reaction detecting only a T base in the SNP region for allele 1 (Figure 6.2Bi), and only a C base being detected in this region for allele 2 (Figure 6.2Bii). Pyrograms for the heterozygous controls are shown in Figure 6.2C. These were generated by using as templates mixtures of cloned alleles in a 1:1 ratio. Here, the T and C bases are of equivalent height in the SNP region (Figure 6.2C, left).

The analysis of the deletion region is more complicated as the deletion of the GCT codon (AGC in reverse orientation, as the sequencing primer is designed in reverse, see Figure 6.2A) from one or both alleles alters not only the heights of the peaks corresponding to these bases, but also the peak heights for subsequent bases. Approximately, the patterns seen in the peak heights are:

- **A281-/A281-γ**
  - T A G 3A G 2A
  - (see Fig. 6.2Biv)

- **WT/WTγ**
  - T A G C A G 3A
  - (see Fig. 6.2Biii)

- **WT/A281-γ**
  - T A G $^{1/2}$C 2A G 3A
  - (see Fig. 6.2Cii)

Consequently the C base (in bold) was used as a marker for the presence or absence of the deletion; compared with the second G base (underlined), the C base peak is either absent
(A281-/A281-γ), present at an intensity of 1 (WT/WTγ) or present at an intensity of 1/2 (WT/A281-γ).

These peak values generated by the PSQ software were then normalised to the score for T. b. brucei Lister 427 WT gDNA, which contains only WT γ; hence this score was set to 100% No Deletion, and the others were judged in comparison to this.

Tables 6.2 and 6.3 show that the gDNA and cDNA results are remarkably similar for each strain and suggest that (i), as expected, the two alleles have the same copy number (presumably one), and (ii), that there is no preference on the mRNA level for the A281- allele over the WT allele in any of the strains.

Comparison between the averages in Tables 6.2 and 6.3 shows a slightly higher proportion of alleles are detected as WT when the SNP region was used to quantify alleles than when using the deletion region. Approximately half of the amplicons in all samples were identified as WT using the deletion region to quantify the allelic proportion, whereas around 60% of the same amplicons were identified as WT using the SNP region.

Table 6.4 shows when a cloned WT subunit γ sequence (allele 1) was analysed this technique scored this control as approximately 96% WT when quantifying using either SNP or deletion region. The cloned A281- subunit γ sequence (allele 2) was scored as ~4% WT using the SNP region for quantification, and ~1% WT using the deletion region. The 1:1 mix of both allele 1 and 2 minipreps that should contain equal proportions of WT and mutated γ was scored as containing approximately 54% WT γ allele and 46% mutated γ allele. Thus, pyrosequencing measured the allelic ratios accurately with a margin of error of about 5-10%.
Taken together, the pyrosequencing data suggest that all three isolates investigated express mRNAs for the WT and A281-\(\gamma\) allele at approximately equal ratios.

**Discussion**

This pyrosequencing quantification showed that the mRNA expression levels for the A281- and WT subunit \(\gamma\) alleles are approximately equivalent for all three *T. b. evansi* strains investigated (Tables 6.2 and 6.3). An upregulation in transcription of the mutated \(\gamma\) allele is therefore not a mechanism used as part of the compensation for loss of kDNA in these *T. b. evansi* strains.

Previous literature had suggested that there was an upregulation in the levels of mutated \(\gamma\) subunit RNA when compared to the levels of WT \(\gamma\) subunit (Lai et al., 2008). This work does not support these findings. The previous study had been performed via ligating PCR-amplified ATPase \(\gamma\) subunit cDNA into a cloning vector, sequencing these clones and quantifying the frequency of sequenced alleles. The authors reported a frequency of 10% WT allele and 90% mutated allele. An explanation for their result could be more of the clones containing mutated allele were chosen at random to sequence, perhaps not unlikely since only 10 clones were sequenced in total. Other possible explanations, for example the mutated allele being preferentially amplified from cDNA or being preferentially ligated into the cloning vector, seem more unlikely.

Table 6.4 shows there is a certain error to be expected when using this technique to quantify mutations in alleles. For example in around 3.5% of cases, a cloned WT subunit \(\gamma\) allele (allele 1) was assigned as mutated when investigating genotype via either the SNP region or the deletion region. For a cloned mutated allele (allele 2), in around 4.5% of cases this was assigned as WT when investigated via the SNP region, and for the deletion region this
occurred in around 1.5% of cases. Indeed, when a 1:1 mix of cloned WT and mutated γ alleles was investigated, the result suggested a 5-10% margin of error.

Similarly, when the SNP region was used to quantify the % of amplicon identified as being WT γ allele the score was between 60-64% for all *T. b. evansi* samples, whereas the % of amplicon identified as WT γ allele using the deletion region is consistently lower at between 52-55% (Tables 6.2 and 6.3). There are possible reasons for this:

1. The parasite population that the genetic material was isolated from may not have been genetically homogenous. The WT γ allele may not always contain a T at the SNP position. If the SNP has no biological function there would be no evolutionary advantage in retaining the consistency. The sequencing used to initially search for SNPs in γ was not exhaustive, there could have been clones that contained both the T WT nucleotide at the SNP position and the deletion mutation that were not detected. The T is the WT base at the SNP position, and so if a few alleles containing the deletion also contain the T → C substitution this could explain why T is the base that is found slightly higher frequency.

2. There could be some bias in the pyrosequencing PCR reaction for the T-containing allele being used as the template. Even in the 1:1 mix of the two alleles, the WT allele was deemed to have a higher abundance when analysed by this technique (Table 6.4). How this bias occurs is not uncertain; there is no obvious reason why one T base in the middle of a PCR template would be preferably replicated over the C-containing allele.
**Conclusion**

The fact that three strains of *T. b. evansi* do not upregulate the level of A281-γ allele, at least not on the level of mRNA, suggests that this is not part of the compensatory mechanism for this γ mutation.

We have not however investigated the expression level of the two γ subunits at the protein level. The A281-γ mutation has been subsequently shown to uncouple the F₁F₀ ATPase (C. Dewar and L. Woodcock, unpublished). Hence in heterozygous WT/A281-γ cell lines, an upregulation in expression of the mutated subunit may function to increase the proportion of productive uncoupled complexes present. This could yet be shown to be part of the F₀-independent mechanism of efficient Δψm generation in these cell lines.
Figures

A

B

i

ii

iii

iv

C

345
Figure 6.1  Pyrosequencing analysis of ATPase γ from DK subspecies of *T. brucei*

A) Schematic showing the T/C SNP (top) and Ala281 deletion regions (bottom) of ATPase subunit γ (polymorphisms indicated by blue shading), the primers used for PCR, and the sequencing primers used for the pyrosequencing reactions. Circle marks biotinylated primer. The sequencing primers (outlined in red) for the SNP and deletion regions are in the forward and reverse orientation, respectively. B) Pyrosequencing output, showing positive ‘homozygous’ controls for the SNP region (i+ii) and the deletion region (iii+iv). WT/WTγ = left, A281-/-A281- γ = right. These were generated by performing the reaction on cloned wt (i and iii) or Ala281-γ alleles (ii and iv). The area of variation due to the mutation is highlighted in yellow. E= enzyme injected into reaction mix. S= substrate injected into reaction mix. For the SNP region, the first A and the second T are negative controls, with these nucleotide triphosphates being dispensed at these positions to show the peak height for a negative reading i.e. readings when a nucleotide is dispensed and not incorporated into the elongating strand due to it being non complementary to the template strand expected. For the deletion region the first base, G, fulfils this role. The approximate peak height of one is indicated by the red dotted line. The Gs used for normalisation are underlined. C) Pyrosequencing output for a ‘heterozygous’ 50% wt, 50% A281- control. SNP region = left. Deletion region = right. The plasmids containing either WTγ or A281-γ were mixed in a 1:1 ratio in order to generate a heterozygous mixture of both alleles. The approximate peak height of one is indicated by the red dotted line. The G used for normalisation is underlined.
Table 6.2  Pyrosequencing results quantifying the % of \( \gamma \) alleles in the amplicon mixture that are WT from the SNP position

i.e. T was identified as being in the SNP position. Peaks were quantified and analysed as detailed in Materials and Methods by the pyrosequencing analysis software, and given as averages from 3 separate pyrosequencing runs. Each run contained each sample in triplicate. Standard deviations of the averages were generated by square rooting the average of the variances.

<table>
<thead>
<tr>
<th>% T (WT)</th>
<th>gDNA</th>
<th>cDNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>( T. b. evansi ) AnTat3/3</td>
<td>62.12 +/- 2.74</td>
<td>62.32 +/- 1.31</td>
</tr>
<tr>
<td>( T. b. evansi ) STIB805</td>
<td>60.17 +/- 2.36</td>
<td>63.38 +/- 1.86</td>
</tr>
<tr>
<td>( T. b. evansi ) STIB810</td>
<td>60.83 +/- 2.92</td>
<td>63.55 +/- 2.28</td>
</tr>
</tbody>
</table>

Table 6.3  Pyrosequencing results quantifying the % of \( \gamma \) alleles in the amplicon mixture that are WT from the deletion region

i.e. no TGC deletion was identified. Peaks were quantified by the pyrosequencing analysis software and analysed as detailed in Materials and Methods, and given as averages from 3 separate pyrosequencing runs. Each run contained each sample in triplicate. Standard deviations of the averages were generated by square rooting the average of the variances.

<table>
<thead>
<tr>
<th>% NO DELETION (WT)</th>
<th>gDNA</th>
<th>cDNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>( T. b. evansi ) AnTat3/3</td>
<td>53.57 +/- 3.44</td>
<td>53.90 +/- 1.94</td>
</tr>
<tr>
<td>( T. b. evansi ) STIB805</td>
<td>52.28 +/- 1.16</td>
<td>54.66 +/- 1.74</td>
</tr>
<tr>
<td>( T. b. evansi ) STIB810</td>
<td>52.84 +/- 1.73</td>
<td>53.71 +/- 1.77</td>
</tr>
</tbody>
</table>
Table 6.4 Pyrosequencing results for the control PCR reactions

A quantification of the % of γ alleles in the amplicon mixtures that are WT, i.e. T was identified as being in the SNP position and no TGC deletion. Peaks were quantified and analysed as detailed in Materials and Methods by the Pyrosequencing analysis software, and these averages are a result of 3 separate pyrosequencing runs. Each run contained each sample in triplicate. Standard deviations of the averages were generated by square rooting the average of the variances. Minipreps used as templates used for PCR reaction to generate template for pyrosequencing reaction: Allele 1 = WT γ allele cloned into Zero BLUNT, allele 2 = mutated γ allele cloned into Zero BLUNT, 1:1 1+2 mix = a 1:1 mixture of allele 1 and allele 2 minipreps.

<table>
<thead>
<tr>
<th>CONTROLS</th>
<th>%T</th>
<th>% NO DELETION</th>
</tr>
</thead>
<tbody>
<tr>
<td>Allele 1</td>
<td>96.45 +/- 0.60</td>
<td>96.3 +/- 2.41</td>
</tr>
<tr>
<td>Allele 2</td>
<td>4.35 +/- 0.37</td>
<td>1.40 +/- 0.37</td>
</tr>
<tr>
<td>1:1 1+2 mix</td>
<td>54.28 +/- 1.95</td>
<td>45.55 +/- 1.24</td>
</tr>
</tbody>
</table>
### Appendix B: For Chapter 2

#### Table 6.5 Genotypes of cell lines generated in this study

<table>
<thead>
<tr>
<th>Cell line name</th>
<th>Cell line background</th>
<th>Genotype</th>
<th>Acridine treated?</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT/WTγ clone A</td>
<td>Lister 427SM</td>
<td>$ATP\gamma/\Delta ty: atp\gamma\text{WTPURO}$ $Tb\text{ATP}p\Delta\text{TAP}$ $T\text{ETR T7RNAP}$</td>
<td>N</td>
</tr>
<tr>
<td>WT/L262Pγ clone A</td>
<td>Lister 427SM</td>
<td>$ATP\gamma/\Delta ty: atp\gamma L262P\text{PURO}$ $Tb\text{ATP}p\Delta\text{TAP}$ $T\text{ETR T7RNAP}$</td>
<td>N</td>
</tr>
<tr>
<td>WT/L262Pγ (AK) clone A</td>
<td>Lister 427SM</td>
<td>$ATP\gamma/\Delta ty: atp\gamma L262P\text{PURO}$ $Tb\text{ATP}p\Delta\text{TAP}$ $T\text{ETR T7RNAP}$</td>
<td>Y</td>
</tr>
<tr>
<td>L262P/L262Pγ clone A</td>
<td>Lister 427SM</td>
<td>$\Delta\text{atp}: atp\gamma L262P\text{BSD}/\Delta ty: atp\gamma L262P\text{PURO}$ $Tb\text{ATP}p\Delta\text{TAP}$ $T\text{ETR T7RNAP}$</td>
<td>N</td>
</tr>
<tr>
<td>L262P/L262Pγ (AK) clone A</td>
<td>Lister 427SM</td>
<td>$\Delta\text{atp}: atp\gamma L262P\text{BSD}/\Delta ty: atp\gamma L262P\text{PURO}$ $Tb\text{ATP}p\Delta\text{TAP}$ $T\text{ETR T7RNAP}$</td>
<td>Y</td>
</tr>
<tr>
<td>WT/WTγ clone B</td>
<td>Lister 427SM</td>
<td>$ATP\gamma/\Delta ty: atp\gamma\text{WTPURO}$ $Tb\text{ATP}p\Delta18\text{TAP}$ $T\text{ETR T7RNAP}$</td>
<td>N</td>
</tr>
<tr>
<td>WT/L262Pγ clone B</td>
<td>Lister 427SM</td>
<td>$ATP\gamma/\Delta ty: atp\gamma L262P\text{PURO}$ $Tb\text{ATP}p\Delta18\text{TAP}$ $T\text{ETR T7RNAP}$</td>
<td>N</td>
</tr>
<tr>
<td>WT/L262Pγ (AK) clone B</td>
<td>Lister 427SM</td>
<td>$ATP\gamma/\Delta ty: atp\gamma L262P\text{PURO}$ $Tb\text{ATP}p\Delta18\text{TAP}$ $T\text{ETR T7RNAP}$</td>
<td>Y</td>
</tr>
<tr>
<td>L262P/L262Pγ clone B</td>
<td>Lister 427SM</td>
<td>$\Delta\text{atp}: atp\gamma L262P\text{BSD}/\Delta ty: atp\gamma L262P\text{PURO}$ $Tb\text{ATP}p\Delta18\text{TAP}$ $T\text{ETR T7RNAP}$</td>
<td>N</td>
</tr>
<tr>
<td>L262P/L262Pγ (AK) clone B</td>
<td>Lister 427SM</td>
<td>$\Delta\text{atp}: atp\gamma L262P\text{BSD}/\Delta ty: atp\gamma L262P\text{PURO}$ $Tb\text{ATP}p\Delta18\text{TAP}$ $T\text{ETR T7RNAP}$</td>
<td>Y</td>
</tr>
<tr>
<td>WT/WTγ clone C</td>
<td>Lister 427SM</td>
<td>$ATP\gamma/\Delta ty: atp\gamma\text{WTPURO}$</td>
<td>N</td>
</tr>
<tr>
<td>WT/L262Pγ clone C</td>
<td>Lister 427SM</td>
<td>$ATP\gamma/\Delta ty: atp\gamma L262P\text{PURO}$</td>
<td>N</td>
</tr>
<tr>
<td>WT/L262Pγ (AK) clone C</td>
<td>Lister 427SM</td>
<td>$ATP\gamma/\Delta ty: atp\gamma L262P\text{PURO}$</td>
<td>Y</td>
</tr>
<tr>
<td>L262P/L262Pγ clone C</td>
<td>Lister 427SM</td>
<td>$\Delta\text{atp}: atp\gamma L262P\text{BSD}/\Delta ty: atp\gamma L262P\text{PURO}$</td>
<td>N</td>
</tr>
<tr>
<td>L262P/L262Pγ (AK) clone C</td>
<td>Lister 427SM</td>
<td>$\Delta\text{atp}: atp\gamma L262P\text{BSD}/\Delta ty: atp\gamma L262P\text{PURO}$</td>
<td>Y</td>
</tr>
<tr>
<td>WT/WTγ</td>
<td>AnTat1.1 90:13</td>
<td>$ATP\gamma/\Delta ty: atp\gamma\text{WTPURO}$</td>
<td>N</td>
</tr>
<tr>
<td>WT/L262Pγ clone 1</td>
<td>AnTat1.1 90:13</td>
<td>$ATP\gamma/\Delta ty: atp\gamma L262P\text{PURO}$</td>
<td>N</td>
</tr>
<tr>
<td>WT/L262Pγ clone 2</td>
<td>AnTat1.1 90:13</td>
<td>$ATP\gamma/\Delta ty: atp\gamma L262P\text{PURO}$</td>
<td>N</td>
</tr>
<tr>
<td>WT/L262Pγ (AK) clone 1</td>
<td>AnTat1.1 90:13</td>
<td>$ATP\gamma/\Delta ty: atp\gamma L262P\text{PURO}$</td>
<td>Y</td>
</tr>
<tr>
<td>WT/L262Pγ (AK) clone 2</td>
<td>AnTat1.1 90:13</td>
<td>$ATP\gamma/\Delta ty: atp\gamma L262P\text{PURO}$</td>
<td>Y</td>
</tr>
<tr>
<td>L262P/L262Pγ clone 1</td>
<td>AnTat1.1 90:13</td>
<td>$\Delta\text{atp}: atp\gamma L262P\text{BSD}/\Delta ty: atp\gamma L262P\text{PURO}$</td>
<td>N</td>
</tr>
<tr>
<td>L262P/L262Pγ clone 2</td>
<td>AnTat1.1 90:13</td>
<td>$\Delta\text{atp}: atp\gamma L262P\text{BSD}/\Delta ty: atp\gamma L262P\text{PURO}$</td>
<td>N</td>
</tr>
<tr>
<td>L262P/L262Pγ clone 3</td>
<td>AnTat1.1 90:13</td>
<td>$\Delta\text{atp}: atp\gamma L262P\text{BSD}/\Delta ty: atp\gamma L262P\text{PURO}$</td>
<td>N</td>
</tr>
<tr>
<td>L262P/L262Pγ (AK)</td>
<td>AnTat1.1 90:13</td>
<td>$\Delta\text{atp}: atp\gamma L262P\text{BSD}/\Delta ty: atp\gamma L262P\text{PURO}$</td>
<td>Y</td>
</tr>
</tbody>
</table>
### Table 6.6  Primers used in Chapter 2

Key: F = forward, R = reverse

<table>
<thead>
<tr>
<th>Primer #</th>
<th>Amplification target</th>
<th>Sequence of primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>ATPase γ F</td>
<td>CGCGCGCCGATGCGGTTAATTCAAAAG</td>
</tr>
<tr>
<td>2</td>
<td>ATPase γ R</td>
<td>ATAGGGATCCCTACTTGGTTACTGCCACTCCAAAG</td>
</tr>
<tr>
<td>3</td>
<td>Maxicircle gene A6 F</td>
<td>AAAAAATAAGTATTTTGATATTATAAAAG</td>
</tr>
<tr>
<td>4</td>
<td>Maxicircle gene A6 R</td>
<td>TATATATACCTTTGTATGAT</td>
</tr>
<tr>
<td>5</td>
<td>Maxicircle gene ND4 F</td>
<td>TGTTGACTTACAGGAAAG</td>
</tr>
<tr>
<td>6</td>
<td>Maxicircle gene ND4 R</td>
<td>ATCTATACCCGTTGGAAT</td>
</tr>
<tr>
<td>7</td>
<td>Maxicircle gene ND7 F</td>
<td>ATGACTACATGATAAGTA</td>
</tr>
<tr>
<td>8</td>
<td>Maxicircle gene ND7 R</td>
<td>AAGAATGGGAGATACAGGAAAG</td>
</tr>
<tr>
<td>9</td>
<td>Maxicircle gene ND5 F</td>
<td>TGTTTATATACAGGTTACCTTTATG</td>
</tr>
<tr>
<td>10</td>
<td>Maxicircle gene ND5 R</td>
<td>CCCTAAACTTCATCCGAGTACAG</td>
</tr>
<tr>
<td>11</td>
<td>Minicircle type A-like F</td>
<td>GGGTTGATTAGTCCGAG</td>
</tr>
<tr>
<td>12</td>
<td>Minicircle type A-like R</td>
<td>CGAAATAGCACGAGT</td>
</tr>
<tr>
<td>13</td>
<td>LipDH F</td>
<td>ATACCAATGTGATGGAATCGACGAAAAG</td>
</tr>
<tr>
<td>14</td>
<td>LipDH R</td>
<td>ATACCAATAGTGTGATGGAATCGACGAAAAG</td>
</tr>
<tr>
<td>15</td>
<td>Pre-edited ND7 F (qPCR)</td>
<td>GCCGGGCGGAGGATTATT</td>
</tr>
<tr>
<td>16</td>
<td>Pre-edited ND7 R (qPCR)</td>
<td>GATCTACGCTCCCCCTTTTCCAAAG</td>
</tr>
<tr>
<td>17</td>
<td>Edited ND7 F (qPCR)</td>
<td>GCATCCGCCGACGACAG</td>
</tr>
<tr>
<td>18</td>
<td>Edited ND7 R (qPCR)</td>
<td>CGTACCAGCATGAAATAAATCTTAAT</td>
</tr>
<tr>
<td>19</td>
<td>Pre-edited A6 F (qPCR)</td>
<td>TTTGCCCTTCCAAACTTTTGAAG</td>
</tr>
<tr>
<td>20</td>
<td>Pre-edited A6 R (qPCR)</td>
<td>AATCTATAACTCCAAACTACAAACCTTCC</td>
</tr>
<tr>
<td>21</td>
<td>Edited A6 F (qPCR)</td>
<td>GATTATAATGCTGTTCAATAGTAT</td>
</tr>
<tr>
<td>22</td>
<td>Edited A6 R (qPCR)</td>
<td>CAAAACCAACAAACAAATACAAATCAAAAC</td>
</tr>
<tr>
<td>23</td>
<td>Pre-edited RPS12 F (qPCR)</td>
<td>CGACGGAGAGCTCTTTTGAATA</td>
</tr>
<tr>
<td>24</td>
<td>Pre-edited RPS12 R (qPCR)</td>
<td>CCCCCCACCCTATTTT</td>
</tr>
<tr>
<td>25</td>
<td>Edited RPS12 F (qPCR)</td>
<td>CTTGACGTGTTTACCCGAACT</td>
</tr>
<tr>
<td>26</td>
<td>Edited RPS12 R (qPCR)</td>
<td>CAATCTGACCGACCTCCATGTAAGT</td>
</tr>
<tr>
<td>27</td>
<td>ND4 F (qPCR)</td>
<td>TTTCAGACAAATACCTTGAATAAAAAAA</td>
</tr>
<tr>
<td>28</td>
<td>ND4 R (qPCR)</td>
<td>CGGAATGGACACGACAGAC</td>
</tr>
<tr>
<td>29</td>
<td>18S rRNA F (qPCR)</td>
<td>TGGTAAGGATCCCCGCTGTA</td>
</tr>
<tr>
<td>30</td>
<td>18S rRNA R (qPCR)</td>
<td>TATCAGGACGCTGGAAG</td>
</tr>
<tr>
<td>31</td>
<td>Pyrosequencing L262Pγ F</td>
<td>AGTTCACGTGTTTACCCGAACT</td>
</tr>
<tr>
<td>32</td>
<td>Pyrosequencing L262Pγ R</td>
<td>CAATCTGACCGACCTCCATGTAAGT</td>
</tr>
<tr>
<td>33</td>
<td>Pyrosequencing L262Pγ Seq</td>
<td>CAACAGAGAAGATATACGCTCG</td>
</tr>
</tbody>
</table>
Figure 6.2  Gating strategy for flow cytometry
i) Intact cell gate, determined by forward and side scatter. Intact cell gate was used for 427SM cells and AnTat1.1 90:13 cells. ii) Singlet cell gate, determined by Hoechst 33342 staining. Singlet cell gate was used for 427SM and AnTat1.1 90:13 cells. iii) $+\Delta \psi$ and $-\Delta \psi$ gates determined by TMRE staining on 427SM cells, iv) $+\Delta \psi$ and $-\Delta \psi$ gates determined by TMRE staining on AnTat1.1 90:13 cells. Intact gate was used for all flow cytometry analysis in this thesis. Singlet cell gate was used for all flow cytometry on slender cells in this analysis.
Appendix C: for Chapter 3

Assessing the error in scoring morphology by PAD1 IFA

In order to analyse the error in morphology scoring, scores for morphology (via the DAPI staining of blood smear), and counts for PAD1 positive cells (via anti PAD1 IFA) were compared. One mouse was infected using each cell line and triplicate samples were taken from each infection at three time points, early in the infection at day 4, intermediate to the infection, day 6 and towards the end of the first peak of parasitaemia at day 7. These samples allowed us to make three sets of blood smears and three repeats of the PAD1 IFA per time point, per mouse. Morphology scores and PAD1 positive counts were performed by two individuals.

Comparing the two sets of morphology scores that had been assessed from the same slides, there is greater inaccuracy with scoring morphology at later time points with a higher proportion of stumpy forms (Fig. 6.3A). In these populations, cells with an intermediate morphology can often be difficult to distinguish from stumpy cells (see Figure 3.6). There was a tendency for individual 1 to score a higher % of cells as stumpy forms in comparison to individual 2. Both sets of counts for PAD1 positive cells from the PAD1 IFA slides are very similar (Fig. 6.3B), with the relationship between the two sets of counts showing an ideal gradient of 1.

Figure 6.3C compares the relationship between each individual’s stumpy score and PAD1 positive count separately for each cell line. This shows that in general, the relationship between scores and counts is the same, with % stumpy forms by morphology being underscored compared to % cells that stain for PAD1. Therefore, if the number of cells being staining positively for PAD1 is a correct representation of the number of stumpy cells in the population, the number of stumpy forms recognised by morphology is lower than it should
be. On average, individual 2 tends to underscore for stumpy forms more extremely than individual 1. For example, for WT/WTγ with around 80% of cells positively staining for PAD1, individual 1 scored cells as around 70% stumpy forms (i), whereas individual 2 scored cells as around 60% stumpy forms (v). This is consistent across cell lines: with WT/L262Pγ, around 80% of cells stained PAD1 positive, individual 1 morphology score was around 80% (ii), individual 2 morphology score was around 60% (vi). For WT/L262Pγ (AK) 1, a population of 90% PAD1 stained cells was scored as around 80% by individual 1 (iii) and around 70% by individual 2 (vii). For WT/L262Pγ (AK) 2, a population of 90% PAD1 stained cells was scored as around 70% by individual 1 (iv) and around 60% by individual 2 (viii). This suggests that PAD1 begins to be expressed before all of the morphological characteristics of stumpy forms are present, in agreement with (Macgregor et al., 2012), and that both individuals are being more stringent in scoring parameters than necessary. The presence or absence of kDNA does not affect the ability to score for morphology, as the underscoring for stumpy forms via morphology is consistent across all cell lines. Additionally, cells devoid of kDNA are as comparably stumpy in morphology as a stumpy form with kDNA.

The over scoring of stumpy forms compared to PAD1 staining would be a problem. Judging some intermediates to be stumpy forms would lead to a greater degree of inconsistency due to the range of morphology characteristics that are considered to be intermediate between true slender and stumpy forms. As our issue seemed to be that we were being too rigorous with our judging criteria, as long as there was consistency between the results from the two individuals doing the scoring we would be able to trust judging stumpy proportion within a population by morphology scoring.
Figure 6.3  Assessment of the accuracy of the morphology scoring of two individuals
Mice were infected with 1000 cells at day 0 with cells expressing either WT/WTγ (red), WT/L262Pγ +kDNA clone 2 (blue), WT/L262Pγ (AK) clone 1 (green) and WT/L262Pγ (AK) clone 2 (black). Tail snips were performed daily, and samples were taken on days 4, 6 and 7. A) A comparison between the morphology scoring of individual 1 and 2. 3 blood smears were taken at each time point. The population of cells were scored as having slender, intermediate or stumpy form morphology from DAPI-stained dry blood smears. B) A comparison between the counts for PAD1 positive cells of individual 1 and 2. 3 samples for PAD1 IFA were taken at each time point. The slides for A and B were assessed by two individuals, and the scores of these two individuals for each time point per cell line were plotted against each other. The dashed line is a line of gradient 1. The solid black line is the line of best fit.
C) A measure of the accuracy of morphology scoring for each cell line individually. One mouse was infected with a cell line expressing WT/WTγ (red), WT/L262Pγ + kDNA (clone 2) (blue), WT/L262Pγ (AK) clone 1 (green) or WT/L262Pγ (AK) clone 2 (black). Tail snips were performed in day 4, day 6 and day 7. Per time point, 3 dry blood smears were prepared and DAPI-stained for morphology scoring; 3x 5 μl blood was taken to prepare antiPAD1 IFAs. Slides were blinded. Morphology scores and PAD1 positively stained cells were recorded for each time point by 2 individuals independently. The results for each time point for each mouse were plotted against each other for individual 1 (i, ii, iii, iv) and then for individual 2 (v, vi, vii, viii). The dashed line had a gradient of 1. The solid line is the line of best fit.
Appendix D: The mathematical model for trypanosome within-host dynamics

The quantitative data generated in Chapter 3 was incorporated into a mathematical model by Dr Nick Savill, University of Edinburgh. Details of how the model was constructed; how the model was fitted; and how the parameters were estimated, as described by Dr Savill, are detailed below.

Construction of the model

The model is constructed as follows. Let the concentration of non-committed slender cells at time \( t \) be \( L(t) \). The initial infection is at time \( t = 0 \). Non-committed slender cells replicate at rate \( \alpha \) (i.e., a cell-cycle time of \( \ln(2)/\alpha \)). They are cleared by a time-dependent immune response at rate \( r_L(t) \). They become committed to differentiate at rate \( \beta_b + \beta_f f(t) \), where \( f(t) \) is SIF concentration, \( \beta_b \) is the background, SIF independent differentiation rate and \( \beta_f \) is the SIF dependent differentiation rate. Therefore the differential equation that describes the dynamics of non-committed slenders is:

\[
\frac{d}{dt} L(t) = \left[ \alpha - \beta_b - \beta_f f(t) - r_L(t) \right] L(t)
\]

Let the age of differentiated cells since becoming committed to differentiation be \( \alpha \) and let \( d(\alpha, t) \) be the age density distribution of differentiated cells at time \( t \).

Differentiated cells fall into two classes: i) replicating, committed slender cells and ii) non-replicating stumpy cells. Committed slender cells replicate at rate \( \alpha \), are assumed to be
cleared by the immune system at the same rate as non-committed slender cells ($r_c(t)$), and develop into stumpy cells at age $\tau_c$. Stumpy cells do not replicate, they are assumed to be cleared by the immune response at a different rate $r_s(t)$ and they die at age $\tau_s$. Thus the partial differential equation that describes the dynamics of the age density distribution of committed cells is

$$\frac{\partial}{\partial t} d(a, t) + \frac{\partial}{\partial a} d(a, t) = -d(a, t) \times \begin{cases} r_c(t) - \alpha & \text{if } 0 \leq a < \tau_c \\ r_s(t) & \text{if } \tau_c \leq a < \tau_s \end{cases}$$

The boundary conditions on these equations are determined by differentiation of non-committed slender cells into age $a = 0$, i.e., $d(0, t) = [\beta_h + \beta_f f(t)]L(t)$, and stumpy death at age $\tau_s$, i.e., $d(\tau_s, t) = 0$.

Let $C(t)$ be the total concentration of committed slender cells, let $S(t)$ be the total concentration of stumpy cells, and let $T(t)$ be the total concentration of all cells. These are given by:

$$C(t) = \int d(a, t) da$$
$$S(t) = \int d(a, t) da$$
$$T(t) = L(t) + C(t) + S(t)$$

SIF is produced by both non-committed and committed slender cells. SIF is removed at rate $\gamma$. Therefore the differential equation describing the dynamics of SIF concentration is:

$$\frac{d}{dt} f(t) = L(t) + C(t) - \gamma f(t)$$

Note that, because SIF is not measured, its concentration is on a dimensionless scale.
The immune response against trypanosomes is multifactorial and highly complex, and only qualitatively understood at best. A detailed mathematical model of the immune response is, therefore, of little use when no data is available to fit to. Instead, we use a simple step function to represent an immune response switching from an inactive to an active state at a time $T$ post infection. The strengths of the immune responses against slender and stumpy cells are assumed to be different. They are given by the equations

\[
    r_L(t) = \begin{cases} 
        0 & \text{if } t < T \\
        \phi_L & \text{if } t \geq T 
    \end{cases}
\]

and

\[
    r_S(t) = \begin{cases} 
        0 & \text{if } t < T \\
        \phi_S & \text{if } t \geq T 
    \end{cases}
\]

where $\phi_L$ is the removal rate of slender cells and $\phi_S$ is the removal rate of stumpy cells.

Naive mice are infected with non-committed slender cells at a concentration $L_0$. Therefore the initial conditions are $L(0) = L_0$, $d(a, 0) = 0$ for all $a$ and $f(0) = 0$. These imply $C(0) = S(0) = T(0) = 0$. All variables and parameters are listed in Table 6.7.
Table 6.7  Variables and parameters used in the model

<table>
<thead>
<tr>
<th>Independent variables</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>$t$</td>
<td>Time since infection</td>
<td>h</td>
</tr>
<tr>
<td>$a$</td>
<td>Age of differentiated cells</td>
<td>h</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Dependent variables</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>$d(a, t)$</td>
<td>Age distribution of differentiated cells</td>
<td>cells $\mu^3 \cdot h^{-1}$</td>
</tr>
<tr>
<td>$r_L(t)$</td>
<td>Immune-mediated clearance rate of all slender cells</td>
<td>h $^{-1}$</td>
</tr>
<tr>
<td>$r_S(t)$</td>
<td>Immune-mediated clearance rate of stumpy cells</td>
<td>h $^{-1}$</td>
</tr>
<tr>
<td>$f(t)$</td>
<td>SIF concentration</td>
<td>dimensionless</td>
</tr>
<tr>
<td>$L(t)$</td>
<td>Concentration of non-committed slender cells</td>
<td>cells $\mu^3$</td>
</tr>
<tr>
<td>$C(t)$</td>
<td>Concentration of committed slender cells</td>
<td>cells $\mu^3$</td>
</tr>
<tr>
<td>$S(t)$</td>
<td>Concentration of stumpy cells</td>
<td>cells $\mu^3$</td>
</tr>
<tr>
<td>$T(t)$</td>
<td>Total concentration of all cells</td>
<td>cells $\mu^3$</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Parameters</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>$\alpha$</td>
<td>Replication rate of slender cells</td>
<td>h $^{-1}$</td>
</tr>
<tr>
<td>$\beta_b$</td>
<td>SIF independent differentiation rate</td>
<td>h $^{-1}$</td>
</tr>
<tr>
<td>$\beta_f$</td>
<td>SIF dependent differentiation rate</td>
<td>h $^{-1}$</td>
</tr>
<tr>
<td>$\gamma$</td>
<td>SIF removal rate</td>
<td>h $^{-1}$</td>
</tr>
<tr>
<td>$\tau_C$</td>
<td>Lifespan of committed slender cells</td>
<td>h</td>
</tr>
<tr>
<td>$\tau_S$</td>
<td>Lifespan of stumpy cells</td>
<td>h</td>
</tr>
<tr>
<td>$T$</td>
<td>Time until activation of immune response</td>
<td>h</td>
</tr>
<tr>
<td>$\varphi_L$</td>
<td>Immune clearance rate of slender cells</td>
<td>h $^{-1}$</td>
</tr>
<tr>
<td>$\varphi_S$</td>
<td>Immune clearance rate of stumpy cells</td>
<td>h $^{-1}$</td>
</tr>
<tr>
<td>$L_0$</td>
<td>Initial concentration of slender cells</td>
<td>cells $\mu^3$</td>
</tr>
</tbody>
</table>
Model fitting and parameter estimation

Likelihood

For particular numerical values of the model parameters, the model is solved numerically for each mouse. In order to quantify the fit of the model with these parameters to the data we proceed by calculating the log-likelihood of the model solution at each data point.

Parasite density is estimated by observing a field of cells and estimating the number of parasites in the field. Due to the difficulty of observing many moving parasites in a microscopic field, density estimates are categorised into 0, 1, 2, 3, 4, 5, 6, 8, 10, 12, 16, 20, 24, 32, 48, 64, and 92 parasites per field. Let $\rho_{t_i}$ be the expected density of parasites at time $t_i$ (which is obtained from the model). The volume of blood $v$, in a microscopic field is $v = 25.6 \times 10^{-8} \mu l$. Then the expected number of parasites per field is $\lambda = v \rho_{t_i}$. The number of parasites $N$ in a field is Poisson distributed with parameter $\lambda$. If $N$ equals 0 to 5 then the likelihood of $\rho_{t_i}$ is equal to $\frac{\lambda^N e^{-\lambda}}{N!}$. If $N$ is greater than 5 then we assume that the number of parasites lies within a range. The start of the range is the midpoint between the previous category and the assigned category. For example, if the number of parasites in a field is estimated to be about 48 parasites, then the assigned category is 48, the start of the range is $N_t = (32 + 48)/2 = 40$ parasites. Similarly, the end of the range is the midpoint between the next category and the assigned category, for example $N_u = (48 + 64)/2 = 56$. The likelihood of $\rho_{t_i}$ is then equal to $\sum_{N_t=N_i}^{N_u} \frac{\lambda^N e^{-\lambda}}{N!}$ which equals $Q(N_u, \lambda) - Q(N_t, \lambda)$ where $Q$ is the normalised incomplete Gamma function.

The likelihood function also includes the proportion of parasites that are slenders at a time $t_i$. The number $X$ of parasites that have slender morphology is Binomially distributed with
parameters $M$ and $p$ where $M$ is the number of parasites observed and $p$ is the predicted proportion that are slenders (obtained from the model). Thus the likelihood of $p_{t_i}$ is proportional to $p_{t_i}^{x_{t_i}} (1 - p_{t_i})^{M_{t_i} - x_{t_i}}$.

The parameter posterior distribution is found by multiplying the likelihood, which is the product of likelihoods at each time point, by the prior distributions, which are taken from (Macgregor et al., 2011). The prior on $\beta_p$ was $N(0.01, 0.01^2)$, a normal distribution truncated at 0. Samples from the posterior are drawn using an adaptive population based Markov chain Monte Carlo algorithm with power posteriors (Miller et al., 2010, Savill et al., 2009).
7. References


La Greca, F. & Magez, S. (2011). Vaccination against trypanosomiasis: can it be done or is the trypanosome truly the ultimate immune destroyer and escape artist? Hum Vaccin, 7: 1225-33.


