Interplay of Trypanosoma cruzi and host metabolism and impact on parasite intracellular growth

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Interplay of Trypanosoma cruzi and host metabolism and impact on parasite intracellular growth

A dissertation presented

by

Sheena Shah-Simpson

to

The Committee on Higher Degrees in Biological Sciences in Public Health

in partial fulfillment of the requirements

for the degree of

Doctor of Philosophy

in the subject of

Biological Sciences in Public Health

Harvard University

Cambridge, Massachusetts

August 2017
Interplay of *Trypanosoma cruzi* and host metabolism and impact on parasite intracellular growth

Abstract

The protozoan parasite *Trypanosoma cruzi*, which causes Chagas disease, is capable of infecting and replicating within metabolically distinct tissues such as the heart, skeletal muscle, and adipose tissue, driving disease pathogenesis. However, nutrient requirements of the replicative intracellular amastigote form and knowledge of how host metabolism affects parasite growth remain ill defined. Studying the metabolism of intracellular amastigotes has been difficult given the overlap between parasite and mammalian metabolic pathways and the challenges of obtaining enough amastigote material for biochemical characterization, necessitating the development of new methodologies. In this dissertation we study amastigote and host metabolism during *T. cruzi* infection using novel techniques.

Predictions that amastigotes oxidize fatty acids for energy generation were investigated using microscopy and extracellular flux analysis, which revealed that intracellular amastigotes take up a form of exogenous palmitate, likely palmitoyl-CoA, which stimulates amastigote mitochondrial respiration. Knockdown of host *CPT1*, to reduce entry of cytosolic fatty acyl-CoA into mitochondria for beta-oxidation, increased parasite intracellular growth, pointing to a role for host cytosolic long chain fatty acyl-CoA pools in sustaining intracellular parasite replication.

To characterize how *T. cruzi* metabolically adapts to intracellular infection, we paired transcriptome data with functional data from an optimized mitochondrial stress test for different life cycle stages. Transcriptome and extracellular flux analysis revealed increased metabolic capabilities of intracellular amastigotes compared to extracellular trypomastigotes or insect stage epimastigotes. However, amastigote growth in different host metabolic states did not induce transcriptional metabolic reprogramming or substantial alterations to the bioenergetic
profile, suggesting that increased amastigote metabolic plasticity aids adaptation to multiple different intracellular milieus.

To determine whether *T. cruzi* infection also modulates host metabolism to improve intracellular infection, we examined the impact of infection on host mitochondrial respiration and glucose uptake. Inhibition of amastigote mitochondrial respiration in infected monolayers with ELQ300 revealed that host cells increase mitochondrial respiration during infection, though that increase is dispensable for parasite replication. However, labeling assays demonstrated that infection drives increased glucose uptake in the host, and the amastigote accesses host cytosolic glucose which it uses to support energy generation, biosynthetic processes, and replication. In summary, the data indicate that amastigotes increase their metabolic capacity and modulate host metabolism to increase access to key nutrients, which fuels intracellular replication.
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Acknowledgements

I would first like to thank my advisor Dr. Barbara Burleigh for her support and guidance throughout my thesis work. I am incredibly grateful for all the time she took to talk about science and all things related, no matter what else she had going on, and her support of my scientific exploration. Her mentorship and belief in me has played a huge role in my development as a scientist, and I thank her for that.

The Burleigh lab has provided a fantastic working environment over the last five years. I am indebted to Dr. Kacey Caradonna, Dr. Yan Zhao, and David Ndegwa for teaching me the basics of mammalian cell culture, showing me how to work with *T. cruzi*, and creating a lab environment I couldn’t say no to. Thanks to Dr. Prasad Padmanabhan, Dr. Rafael Polidoro, and Dr. Jose Calzada for all the coffee time, pranks, and great discussion about science, peanut butter, and life. My mentees Rebecca Mandt and Camila Pereira were a ton of fun to work with, and their contributions helped shape my thesis. Finally, a huge thanks to Dr. Rebeca Manning, Dr. Monica Marcolino, Dr. Peter Dumoulin, and Dr. Gaëlle Lentini for their invaluable advice and ability to liven up any conversation with their dancing skills and animal stories.

I would also like to thank the HSPH parasitology group. PIs and members of the Wirth, Duraisingh, Marti, Catteruccia, and Dvorin labs were great at creating a positive environment while providing critical feedback and advice. In particular, Dr. Sabrina Absalon, Dr. Selina Bopp, Dr. Allison Demas, and Dr. Tomoyo Kato have provided valuable advice and assistance over the years. In addition, a big thank you to Nelson Knudsen and Chih-Hao's lab for all the reagents, protocols, and advice. A big thank you also to the members of the Marti and Fortune labs for creating such a fun working environment on the 8th floor (Deepali Ravel, Kassie Dantzler, Dr. Niggi Brancucci, Eli Gerrick, Nate Hicks, and Dr. Jeremy Rock, among others), and to the Duraisingh lab for going out of their way to welcome us to the 2nd floor.

I would also like to thank my dissertation advisory committee, Dr. Dyann Wirth, Dr. Chih-Hao Lee, and Dr. Nika Danial for their significant contributions to my scientific development.
through great discussion and for the reagents, protocols, and contacts which aided my project. In addition, thanks to my exam committee for participating in my defense: Dr. Michele Klingbeil, Dr. Eric Rubin, Dr. Marianne Wessling-Resnick, and Dr. Dyann Wirth.

The administrative teams for the BPH program and IID were a huge help throughout my thesis work. A big thank you to Dr. Marianne Wessling-Resnick, Dr. Brendan Manning, Holly Southern, Deirdre Duckett, and Tom Brazda for their guidance through the program. Another big thank you to Andi Sabaroff, Joann Garrido, Maura Meagher, and Ryan Greene for answering all my random questions about booking rooms, getting card access, and everything HCOM.

Finally, a big thanks to all my friends and family for their constant love, support, and belief in me. My roommates, friends, and fellow PhD students, Deepali Ravel, Nicole Espy, and Kasia Baranowski, have alternatively commiserated with me and celebrated with me, and have always provided great advice to keep moving forward. Thanks to my parents, who are an inspiration and whose encouragement has been a driving force in my success so far, and to my sister, who is always there to keep me from going crazy. Lastly, a big thank you to Michael Tomko for never begrudging the way I plan my life around experiments, while making sure I never forget how to enjoy myself.
Dedication

This work is dedicated to my family for their love, encouragement, and inspiration.
Chapter 1

Introduction
1.1 Life cycle and transmission of *Trypanosoma cruzi*

*Trypanosoma cruzi*, the causative agent of Chagas disease is estimated to infect between 5.7-9.4 million people worldwide [1,2], which has an annual burden of 806,170 disability-adjusted life-years and $627.5 million in healthcare costs [3]. Transmitted by multiple members of the Triatominae subfamily with differing geographic ranges within the Americas [4,5], the parasite has a complex lifecycle and has been identified in over 42 mammalian species including domesticated animals, forest-dwelling animals, and animals like rodents that live in peridomestic spaces [6,7], giving rise to domestic and sylvatic cycles of infection [8].

Vector transmission is the leading route of human infection (reviewed in [9]). In the triatomine insect, *T. cruzi* epimastigotes multiply in the midgut, pass into the hindgut, and differentiate into metacyclic trypomastigotes (Figure 1.1) [9]. During a blood meal or immediately following it, the triatomine defecates [5], releasing the infective metacyclic trypomastigotes on the skin of a mammalian host. Scratching at the site of the bite or subsequent contact with mucosal surfaces allows the parasite to enter the human host [9]. Metacyclic trypomastigotes then invade nucleated mammalian cells and begin to differentiate into amastigotes within a parasitophorous vacuole that is formed around the parasite from host membranes during invasion [10]. Following escape from the parasitophorous vacuole, amastigotes replicate by binary fission while in direct contact with the host cell cytoplasm [9,11]. After multiple rounds of replication, amastigotes transform into trypomastigotes, burst out of the host cell, and move throughout the body. Trypomastigotes can be picked up by a triatomine through a blood meal or invade a new mammalian cell, differentiate into amastigotes, and continue the infection cycle within the mammalian host [9].

Though the majority of Chagas disease is found in South and Central America, where both the parasite and its triatomine vectors are located [9], immigration to North America, Europe, and Australia has resulted in cases across the globe [12]. Additionally, congenital transmission [13] and infected blood transfusion [14] or organ transplantation [15,16] has given
Figure 1.1. Lifecycle of *Trypanosoma cruzi*. Reprinted with permission from [9].
rise to new *T. cruzi* infections in non-endemic countries. In endemic areas, oral transmission by ingestion of infected triatomines or contaminated food has also been documented, and results in more severe clinical manifestations than vector transmission [17,18].

### 1.2 Chagas disease: Disease development, diagnosis, and current management

In the early 1900s, Carlos Chagas, a Brazilian hygienist and microbiologist, identified *T. cruzi* as an infectious agent, determined its route of transmission to humans via a triatomine vector, and described clinical manifestations of disease as well as some animal reservoirs (reviewed in [19,20]). Acute *T. cruzi* infection is usually symptomless, though it can cause general clinical features such as fever and muscle pain. Inflammation at the site of entry can help identify infection, especially the unusual Romaña’s sign, a periorbital swelling associated with infection through the conjunctiva [9,21]. Occasionally, acute infection results in death due to severe myocarditis or meningoencephalitis [9,21,22]. During acute infection, extracellular trypomastigotes are readily detected in fresh blood by microscopy [23], but as the patient immune system controls infection, the peripheral parasitemia drops and *T. cruzi* trypomastigotes are no longer detectable by microscopy [24,25].

Most patients never develop symptoms but remain seropositive for *T. cruzi* in what is considered the indeterminate phase [25]. However, up to 10% of infected individuals display mild polyneuropathy [9,26], while 10-20% eventually develop gastrointestinal disorders ranging from mild motility disorders to severe megaoesophagus, which must be managed by surgery [27–29]. Cardiac disease is the most common and severe type of disease induced by *T. cruzi* infection, with 14-45% of infected individuals showing symptoms ranging from slight arrhythmia and an enlarged heart, to thromboembolism, aneurysm, or congestive heart failure [19,21,27,29–31]. Geographic location and parasite genetic background is broadly linked with human clinical disease [4]. Digestive disorders seem to be more common in Southern Cone countries of Argentina, Chile, and Uruguay, but the data linking parasite genetic background to
disease pathology remains scarce, partially because parasite load in peripheral blood during chronic disease remains low, making molecular characterization difficult [4]. Diagnosis of chronic Chagas disease generally relies on serology and clinical symptoms [23,32].

The pathogenesis of chronic Chagas disease is incompletely understood. During acute infection in rodents, *T. cruzi* intracellular amastigotes are found throughout the body [33–36], while during chronic infection parasites exhibit tropism for adipose tissue, skeletal muscle, the muscular layer of the gastrointestinal tract, and the heart [34,36,37], the latter two of which are specifically impaired during clinical disease progression. Recent work in mice indicates that *T. cruzi* primarily resides in the gut, with varying rates of dissemination to other tissues based on the strain [36]. During chronic disease, multiple foci of inflammation, necrosis, and interstitial fibrosis are found across the myocardium but are rarely observed with whole parasites, which gave rise to autoimmune theories of disease pathogenesis [19]. However, work by Lewis *et al.* (2016) and PCR detection of *T. cruzi* kinetoplast DNA indicate that parasite levels are positively correlated with cardiac disease progression in both mice and humans [34,38,39]. Host immune system activity is also closely linked with disease progression, as patients with cardiac disease exhibit increased pro-inflammatory markers and decreased anti-inflammatory markers compared to patients in the indeterminate phase [40]. Together, the data suggest a new model for disease progression, whereby repeated *T. cruzi* infection of target tissues activate focal immune responses and induce physiological changes that drive disease progression [22,24,36,41,42].

In 1965, nifurtimox was introduced for treatment of Chagas disease by Bayer [43,44]. Nifurtimox’s efficacy against *T. cruzi* derives from production of oxygen radicals [45,46] and is effective in treating acute infection [47]. However, a high percentage of patients do not complete the 2-3 month regimen due to adverse events including vomiting, anorexia, and neuropathy [44,48]. In 1971, Roche introduced benznidazole [43], which has a less well defined mechanism of action, but may involve covalent bonding of nitroreductive intermediates or other interactions.
with parasite components such as DNA, lipids, and proteins [49,50]. Similar to nifurtimox, benznidazole is effective in clearing parasitemia if given during acute infection [51,52], and is preferred over nifurtimox due to its shorter drug regimen, 2 months, and higher tolerability [9,29,53]. During chronic disease, benznidazole treatment reduces the likelihood of a positive T. cruzi PCR test and reduces antibody titers, but does not result in complete serological reversion and cure in most patients [51,53,54]. Viotti et al. (1994) originally described that treatment during chronic disease helped halt disease progression, but results from the more recent and much larger BENEFIT trial indicate that benznidazole treatment during chronic disease in adults does not alter disease progression [53], likely because the parasite is still present. The intracellular amastigote form has been posited to hinder parasite clearance in chronic disease during drug treatment by hiding within tissues as a quiescent form [51,55]. Currently, benznidazole treatment is recommended during acute infection and should be considered for chronic infection based on the patient [9,23], but treatment of chronic disease relies largely on symptom management. Patients with cardiomyopathy are treated as they would be for heart failure due to other causes [9], and digestive megasymphdromes are managed with surgery [56]. As most T. cruzi infections remain undiagnosed until chronic disease sets in, new therapeutics that clear parasitemia during indeterminate and chronic infection or halt disease progression are needed.

1.3 T. cruzi metabolism

Glycolysis

The T. cruzi genome encodes all the components of glycolysis [57], though T. cruzi glycolysis differs from mammalian glycolysis in its location and regulation. After entering the parasite through a transporter capable of taking up both glucose and fructose [58,59], glucose is trafficked to an organelle termed the glycosome, that was first discovered in the related parasite T. brucei [60]. A member of the peroxisome family, glycosomes are unique organelles that
contain the first 6 steps of glycolysis in *T. cruzi*, generating 3-phosphoglycerate that is transported to the cytosol for the remaining reactions and net ATP generation (Figure 1.2) (reviewed in [57,61]). Unlike most organisms, *Trypanosome* glycolysis is not allosterically regulated by feedback inhibition. Glucose-6-phosphate does not inhibit *T. cruzi* hexokinase activity [62–64], and neither citrate nor ATP regulate phosphofructokinase activity [63]. Instead, compartmentation of glycolysis within the glycosome has been shown to substitute for classic feedback inhibition by preventing cytosolic ATP from increasing hexokinase and phosphofructokinase activity to the point where sugar phosphates build up to toxic levels within the cell [57,65–67]. In addition, modeling in *T. brucei* indicates that when cells lack allosteric regulation of hexokinase, compartmentation of glycolysis enables parasites to recover from periods of glucose deprivation [65]. Hysteretic behavior of hexokinase is also predicted to aid parasite adaptation to changing environments [68]. In *T. brucei*, glycosome turnover is associated with differentiation [69], and is hypothesized to contribute to additional metabolic adaptation in changing nutrient environments as parasites alter what is packaged within new glycosomes [67,70].

Early studies determined that *T. cruzi* metabolizes glucose to succinate and alanine in the glycosome as part of glucose fermentation that occurs under both aerobic and anaerobic conditions [71–74], which is thought to balance glycosomal redox and energy states (Figure 1.2) [57]. Glucose also feeds the tricarboxylic acid cycle (TCA cycle) [57] and fuels mitochondrial respiration in all life cycle stages [75]. Insect-stage epimastigotes vary use of glucose as they grow, consuming it most efficiently in mid-log phase when they are replicating most actively [74,76], even though glucose is predicted to be limiting within the insect midgut [57]. Trypomastigotes also transport and utilize glucose, and are predicted to rely heavily on it in the mammalian host bloodstream [57,75,77]. Amastigote utilization of glucose, however, is contested. Axenically derived amastigotes have been shown to take up and metabolize glucose [78,79], though intracellular-derived amastigotes do not express the transporter and show no
Figure 1.2. *T. cruzi* glycolysis and TCA cycle. Reprinted with permission from [57]. Enzymes: 1) hexokinase/glucokinase, 2) phosphoglucone isomerase, 3) phosphofructokinase, 4) aldolase, 5) triose phosphate isomerase, 6) glyceraldehyde-3-phosphate dehydrogenase, 7)
Figure 1.2 (Continued) phosphoglycerate kinase (glycosomal), 8) phosphoglycerate kinase (cytosolic), 9) phosphoglycerate mutase, 10) enolase, 11) pyruvate kinase, 12) pyruvate dehydrogenase complex, 13) citrate synthase, 14) aconitase, 15) NADP-linked isocitrate dehydrogenase, 16) α-ketoglutarate dehydrogenase complex, 17) succinate thiokinase, 18) succinate dehydrogenase, 19) fumarate hydratase (mitochondrial), 20) malate dehydrogenase (mitochondrial), 21) phosphoenolpyruvate carboxykinase, 22) malate dehydrogenase (glycosomal), 23) fumarate hydratase (glycosomal), 24) NAD-linked fumarate reductase, 25) pyruvate-phosphate dikinase, 26) alanine dehydrogenase, 27) fumarate hydratase (cytosolic), 28) NADP-linked malic enzyme (mitochondrial), 29) NADP-linked malic enzyme (cytosolic), 30) alanine aminotransferase, 31) fructose-1,6-bisphosphatase. Boxed in red are aerobic fermentation products.
glucose transport activity [77]. Additionally, glucose is thought to be scarce within the host cell cytosol [57]. However, another study using a different strain of *T. cruzi* showed intracellular amastigotes increase mitochondrial respiration in response to glucose [75], which may indicate strain-dependent differences in parasite fuel utilization.

*T. cruzi* is also capable of gluconeogenesis [80]. In the related parasite *Leishmania*, fructose-1,6-bisphosphatase is required for intracellular amastigote growth and is posited to provide glucose for cellular needs like glyco-conjugate biosynthesis in glucose-limiting conditions [81]. In *Toxoplasma gondii*, which replicates within a wide range of host cells similar to *Trypanosoma cruzi*, gluconeogenesis and glycolysis are active simultaneously, which is thought to regulate flux through glycolysis and connected pathways such as sugar nucleotide and glyco-conjugate biosynthesis and potentially aid adaptation to nutrient conditions [82]. While *T. cruzi* gluconeogenic activity throughout the life cycle is unknown, it may aid parasite adaptation to changing intracellular environments.

**TCA cycle and electron transport chain**

*T. cruzi* has a single mitochondrion that extends throughout the body of the parasite with the mitochondrial DNA encoded in maxi and minicircles that are catenated and condensed, forming a discoid shape termed the kinetoplast [83–85]. Early work identified evidence of TCA cycle activity within *T. cruzi* [71,86], and additional studies demonstrated that the TCA cycle is functional within all life cycle stages [75], unlike in the related parasite *T. brucei* where the bloodstream form lacks multiple TCA cycle enzymes and cytochromes despite their expression during other life cycle stages [87]. The genes encoding all TCA enzymes in *T. cruzi* have now been identified and expression localized to the mitochondria (Figure 1.2) [57,88,89].

The presence of a functioning electron transport chain (ETC) was also demonstrated for all life cycle stages in *T. cruzi* [88,90,91]. However, complex I has limited function in energy and reactive oxygen species (ROS) generation [92]. Instead, an NADH-dependent fumarate
reductase regenerates NAD$^+$ from NADH and uses fumarate to produce succinate, which fuels the ETC via complex II, succinate dehydrogenase [92–97]. Complex II and III are the main sites of ROS generation in T. cruzi [98,99], and trypomastigotes have elevated complex II and III activity and levels of ROS formation relative to epimastigotes, which is predicted to activate parasite ROS defense pathways and protect against the mammalian immune system [99]. Recent genetic manipulation of the parasite ETC resulted in significant impairment of parasite intracellular replication [100], as did selective targeting of cytochrome b with a small molecule [101].

T. cruzi oxidizes multiple substrates for energy generation, including glucose, fatty acids, and amino acids [75,102,103]. Epimastigotes are thought to primarily use proline, which is readily available in the insect midgut, despite exhibiting a preference for the more limited glucose, while trypomastigotes are predicted to rely most heavily on glucose from the host bloodstream [57,75,104,105]. Amastigote substrate preferences however are not well established. Reports of amastigote glucose utilization are mixed, while isolated amastigotes have been shown to transport and oxidize multiple amino acids [75,77]. Current predictions hold that amastigotes rely on fatty acids and amino acids for energy generation based on increased expression of fatty acid beta-oxidation enzymes and the ability of isolated amastigotes to utilize amino acids [57,77,106], though no studies have yet examined intracellular parasite metabolite access. Complicating matters, T. cruzi exhibits strain-dependent differences in iron and cytochrome content, resulting in measurable differences in respiratory rates [107]. Parasite genetic background may therefore also impact other components of metabolism. The successful use of selective small molecules to impair parasite respiration and growth [101] suggests that understanding amastigote substrate preferences and utilization may yield additional targets for limiting T. cruzi infection.
**Fatty acid metabolism**

*T. cruzi* utilizes fatty acids for multiple cellular functions including membrane synthesis and energy generation. The parasite primarily relies on an elongase system for synthesis of long chain fatty acids (LCFA) and very long chain fatty acids (VLCFA) [108–111]. While conventional elongases add carbons to make LCFA longer, elongase 1 (Elo1) in trypanosomes synthesizes fatty acids *de novo* by adding carbons from malonyl-CoA to butryl-CoA or similar four-carbon chains to generate C10-CoA in the endoplasmic reticulum [109,110,112,113]. The modular elongase system allows for continued elongation of fatty acids—Elo2 elongates fatty acids to C14-CoA, Elo3 extends those to C18-CoA, and Elo4 generates C24-CoA and C26-CoA from LCFA [109–111] (Figure 1.3c)—and may help regulate which fatty acid species are produced [112]. In *T. brucei*, most fatty acid synthesis occurs in the elongase pathway, while a type II fatty acid synthase (FAS) system produces octanoate and palmitate, which are predicted to be generated and used within the mitochondria [114]. *T. cruzi* is similarly predicted to have a functioning type II FAS system while relying primarily on elongases for fatty acid synthesis [112,113].

*T. cruzi* trypomastigotes and epimastigotes also scavenge exogenous lipids, potentially using a fatty acid transport protein expressed at the cell membrane [112]. Exogenous fatty acids are incorporated into parasite neutral lipids and phospholipids [102,103,115], which are used for membrane synthesis and glycosylphosphatidyl inositol (GPI) synthesis, which anchors glycoproteins to the parasite cell membrane [116–119]. GPI is required for the development of intracellular amastigotes, and its deficiency results in abrogated mammalian infection, making GPI biosynthesis a potential drug target [120]. In addition, both synthesized and scavenged fatty acids are modified by desaturases [108,115,121,122], and chemical inhibition of the desaturases significantly impairs epimastigote replication [123,124], although the effect of the compounds on intracellular amastigote growth has not yet been tested.
Figure 1.3 Trypanosomatid fatty acid pathways. Reprinted with permission from [113]. Similar to *T. brucei* (shown in diagram above), *T. cruzi* is thought to (a) generate phosphatidylinositol for GPI synthesis in the endoplasmic reticulum using alkyl-glycerolphosphate made in the glycosome from long chain fatty acyl-CoA. (b) Acetyl-CoA is used by mitochondrial fatty acid synthase to generate C8 to C16. (c) In the endoplasmic reticulum, ELO1 and ELO2 use malonyl-CoA to elongate butyryl-CoA to C14, which can be incorporated into GPI anchors. ELO3 elongates C14 to C16 or C18 for fatty acid desaturation and/or incorporation into phospholipids and GPI. ELO4 elongates C16 and C18 up to C24 or C26.
Both epimastigotes and trypomastigotes evidence a preference for scavenging palmitate over other LCFA [102], which they utilize for beta-oxidation or incorporation into neutral lipids and phospholipids. Interestingly, trypomastigotes divert a higher percentage of scavenged palmitate towards beta-oxidation than epimastigotes, which primarily incorporate palmitate into other lipid species [102,103] and may reflect the fact that epimastigotes are a replicative form while trypomastigotes are not. In one study, isolated intracellular amastigotes demonstrated higher rates of palmitate oxidation than trypomastigotes or epimastigotes, but rates were negligible in all three life cycle stages relative to glucose oxidation [75]. The ability of amastigotes to incorporate exogenous fatty acids into membrane components has not been explored. Interestingly, parasites with knockout of the fatty acid transporter or fatty acid oxidation genes enoyl-CoA hydratase 1 and 2 fail to establish persistent infection in mice and exhibit attenuated growth as intracellular amastigotes (R. Tarleton, personal communication) [125], suggesting that access to fatty acids and fatty acid oxidation play important roles in sustaining intracellular parasite replication.

1.4 Host cellular metabolism and intracellular infection

Establishment of infection by intracellular pathogens requires adaptation of host and pathogen at cellular and metabolic levels. Innate immune responses, pathogen-mediated signaling, and host cellular metabolic sensing all converge to manipulate host metabolic activity and support host cell survival and/or provide the intruder with increased nutrient access (Figure 1.4) [126]. *T. cruzi* has been shown to promote host cell survival during infection by activating host metabolic phosphatidylinositol-3 kinase (PI3K)/AKT or immune NF-κB signaling pathways to inhibit apoptosis [127,128]. The antiapoptotic effect is mediated in part by the secreted parasite proteins trans-sialidase and cruzipain [128–130]. Depending on the host cell type, though, infection can induce metabolic changes that impair pathogen survival. In macrophages, *T. cruzi* infection promotes increased oxidative metabolism and nitric oxide generation, which aids
Figure 1.4 Network of major metabolic pathways and regulators in infected cells. Reprinted with permission from [126]. Blue arrows indicate exchange of metabolic intermediates. Green arrows indicate regulation by key metabolic modulators (green oval). Red arrows indicate potential regulation of host metabolic networks by an intracellular pathogen, and red triangles and circles indicate surface factors or secreted molecules that could be used to direct the host.
parasite clearance [131]. Innate immune responses can also modulate metabolism to impair pathogen survival. During coxsackievirus B3 infection, type I interferons increase glucose uptake and ATP generation in fibroblasts in a PI3K/AKT-dependent manner, which is thought to fuel energy requirements of anti-viral responses [132]. T. cruzi infection has similarly been shown to induce a robust type I interferon response in multiple cell types [133–136], though the impact of T. cruzi on host glucose metabolism specifically and cellular metabolism generally has not been fully explored in non-immune cells.

A genome-wide functional screen in HeLa identified multiple host metabolic pathways as important for supporting T. cruzi intracellular growth [137]. In particular, impairment of host fatty acid oxidation, respiration, or nucleotide metabolism through RNA interference (RNAi) significantly reduced amastigote growth and pointed towards host AKT as a central regulator of parasite proliferation [137]. In addition profiling of early transcriptomic responses to T. cruzi infection in HeLa revealed upregulation of fatty acid metabolism and decreased phospholipid metabolism, resulting in an increase in cellular lipid accumulation [138]. However, the mechanism by which these host pathways support intracellular parasite growth, whether direct provision of substrates, production of energy or redox intermediates, or other means, remains unexplored. Even the nutritional requirements of amastigotes remain unknown though they increase exponentially in an infected cell during replication, reaching over 100 amastigotes per cell within a few days. Understanding the complex interplay of host metabolism with T. cruzi infection may yield useful insights into host and parasite metabolic pathways that can limit infection.

1.5 Systemic alterations in host metabolism during T. cruzi infection

T. cruzi infection is associated with altered metabolism in infected tissues and dysregulation of metabolic regulators such as the hormones adiponectin and insulin. Acute and chronic infection in mice results in dysfunctional mitochondrial respiration in the heart and skeletal muscle [139–
Additional study of acute disease has indicated that the heart increases glucose and fatty acid uptake during *T. cruzi* infection, but decreases pentose phosphate pathway and TCA cycle activity [144]. These changes may predict disease progression, as decreased TCA cycle activity can indicate metabolic stress, while increased fatty acid uptake could contribute to lipotoxicity in the heart [144]. Analysis of the plasma proteome during chronic Chagas disease reveals dysregulation of multiple lipid metabolism and molecular transport proteins in rodents that are known to correlate with cardiovascular function and disease [145]. In humans with chronic indeterminate or symptomatic disease, examination of peripheral blood mononuclear cells has shown decreased expression of glycolytic and ETC enzymes [146]. The contribution of these tissue level changes in host metabolism to regulating parasite growth or driving disease progression is poorly understood during *T. cruzi* infection, though the systemic nature of the metabolic transformation suggests a possible combination of immune and hormonal-mediated changes in metabolism.

*T. cruzi* infection of adipose tissue has been identified in chronic disease in both mice and humans [37,147]. During acute symptomatic infection, animals lose weight, have increased intracellular cholesterol levels but decreased triglyceride content in adipose tissue, and temporarily decrease adiponectin production, which normally acts to reduce inflammation and increase insulin-sensitivity [37,134,148]. Although adiponectin production increases again after a few weeks, the total amount of white adipose tissue is vastly reduced by that time, which may result in less total adiponectin, contributing to the increased inflammation seen in adipose tissue [134]. Interestingly, a high fat diet in mice reduces mortality during acute Chagas disease [149,150]. Increased adipogenesis and decreased lipolysis lead to decreased levels of serum triglycerides and free fatty acids during infection in obese mice compared to lean mice, and decreased intracellular cholesterol levels in cardiac tissue [149,150]. Intracellular cholesterol has been shown to aid *T. cruzi* invasion, which may explain the reduction in cardiac parasite load in obese mice and decreased mortality, though the parasite burden in adipose tissue
increases [148,149]. Treatment with metformin to reduce the hyperglycemia that accompanies obesity further reduces mortality during acute *T. cruzi* infection [150]. However, metformin has also been shown to reduce ROS production and attenuate pro-inflammatory responses [151,152], which may contribute to host survival during acute Chagas disease [150].

Data regarding serum glucose levels in Chagas patients are conflicting, ranging from hypoglycemic to hyperglycemic based on the study, which may be due to small sample sizes, employment of different methodologies, and lack of repeated sampling [153–155]. However, insulin levels are decreased during acute and chronic Chagas disease [37,153,155]. In acute mouse infection, insulin and adiponectin levels are significantly decreased, though oral glucose tolerance remains unchanged [37]. In addition, mice evidence severe hypoglycemia [37], which might be due to glucose utilization by the parasite or reduced gluconeogenesis in the liver caused by liver infection and the corresponding inflammatory response [155,156]. Strain-dependent infection of the pancreas suggests some parasite strains may directly disrupt insulin signaling [153]. However, no alteration in pancreatic β-cell number has been identified, suggesting hypoinsulinemia does not result from *T. cruzi*-mediated destruction of pancreatic islets. Instead, impaired fusion of the secretory granules that contain insulin with the plasma membrane has been noted in a rodent model of chronic disease, although the underlying mechanism remains unknown [155]. Interestingly in humans, higher rates of diabetes or hyperglycemia are associated with patients with cardiac manifestations of Chagas disease, but not patients with indeterminate disease or gastrointestinal manifestations [154].

Experiments in mice reveal that controlling host metabolism can reduce disease progression, necessitating a stronger understanding of how *T. cruzi* infection shapes metabolism. Antioxidant treatment in rats restored normal plasma expression of a number of lipid metabolism proteins that mark cardiovascular dysfunction and are dysregulated during *T. cruzi* infection [145]. Similarly, inhibition of splenocyte ROS generation with apocynin reduced cardiac disease progression, but did not affect parasite burden [157], and treatment with
resveratrol, a sirtuin 1 and AMPK agonist, or metformin, an AMPK agonist, reduced oxidative and inflammatory stress in the heart and improved ventricular function [142,158]. In contrast, treatment of obese, T. cruzi-infected mice with atorvastatin, a common statin used to reduce cholesterol and triglyceride levels, resulted in increased lipid accumulation in the heart and higher tissue parasite loads, likely due to the increase in LDL receptor expression [159], which is known to facilitate T. cruzi invasion [160]. Additionally, atorvastatin treatment resulted in elevated levels of inflammation and increased mortality during acute infection [159]. Improving our understanding of how T. cruzi infection interacts with different host metabolic states is warranted given the evidence that controlling immunometabolism can minimize disease pathogenesis and that drug regimens for treating metabolic syndromes like hyperlipidemia, which is on the rise in Latin America [161], may be contraindicated for Chagas disease.

1.6 Summary of aims

This dissertation seeks to understand how T. cruzi amastigotes interact with host nutrient pools and metabolic processes to support intracellular replication. In Chapter 2 we ask whether intracellular amastigotes access and utilize host fatty acids for energy generation, as has been predicted [106], to support growth. Using microscopy and extracellular flux analysis, we demonstrate that T. cruzi amastigotes obtain long chain fatty acyl-CoA from their environment for use in beta-oxidation. Manipulation of exogenous fatty acid sources and RNAi-mediated impairment of host fatty acid transport between the cytosol and mitochondria reveal small changes in parasite replication that point to host pools of long chain fatty acyl-CoA as substrates supporting T. cruzi replication.

In Chapter 3 we establish a methodology to profile the bioenergetics of the different life cycle stages of T. cruzi and use it to determine how T. cruzi metabolic plasticity and capacity alters based on life cycle stage or intracellular environment. Using media composition to push host metabolism towards a more oxidative or glycolytic state, we demonstrate that amastigotes
grown in different environments do not exhibit signs of substantial metabolic remodeling by transcriptome analysis or bioenergetic profile.

In Chapter 4 we ask whether *T. cruzi* infection modulates host metabolism to support intracellular parasite growth. Uptake assays and extracellular flux analysis reveals that *T. cruzi* infection increases host glucose consumption and mitochondrial respiration, though only glucose supports parasite replication. Additional labeling experiments reveal that amastigotes access glucose within the host cell cytosol and utilize it for energy generation and biosynthetic processes. The works here confirms that *T. cruzi* intracellular amastigotes access and utilize multiple carbon sources, highlights their flexibility with regard to nutrient source, and demonstrates that *T. cruzi* infection modulates host metabolism in ways that support parasite growth.

1.7 References


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Chapter 2

Characterization of the interplay between host fatty acid metabolism and intracellular
Trypanosoma cruzi replication

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experiments and analyzed the data. KLC provided induced pluripotent stem cell-derived
cardiomyocytes.
2.1 Abstract

The intracellular protozoan parasite *Trypanosoma cruzi* exhibits a tropism for colonizing and replicating in tissues rich in lipids such as the heart, vascular smooth muscle, and adipose tissue. Despite the link between disease progression and intracellular infection in these tissues, little is known regarding host metabolic pathways that sustain *T. cruzi* intracellular growth. An unbiased RNA interference screen conducted in HeLa cells identified a number of host fatty acid metabolism genes that impact intracellular *T. cruzi* replication. Additionally, host fatty acid oxidation is upregulated *in vivo* during *T. cruzi* infection, and *T. cruzi* intracellular amastigotes increase expression of a number of fatty acid metabolism genes. However, the mechanism by which host fatty acid oxidation supports *T. cruzi* replication is not understood. Here we sought to determine whether *T. cruzi* amastigotes access long chain fatty acids from the host cytosol to support intracellular replication. Microscopy demonstrates that amastigotes scavenge fatty acids from the host, while bioenergetics analysis confirms the prediction that amastigotes oxidize long chain fatty acyl-CoA. Utilizing RNA interference, we demonstrate that knockdown of host *CPT1*, which controls uptake of long chain fatty acyl-CoA into the mitochondria for beta-oxidation, increases amastigote intracellular replication, but depletion of exogenous lipids alone does not impact parasite replication. Our work confirms that exogenous fatty acids are utilized by intracellular *T. cruzi* and suggests that host cytosolic long chain fatty acyl-CoA pools play a role in supporting intracellular replication.

2.2 Introduction

The intracellular protozoan parasite *Trypanosoma cruzi* causes the development of chronic Chagas disease in a subset of infected individuals [1]. This progressive disease is characterized by parasite persistence in tissues like the heart and vascular smooth muscle [2–4], which promotes damaging host immune and physiologic responses that drive pathology [1,5]. While mechanisms by which *T. cruzi* intracellular amastigotes persist and replicate within diverse host
cell types are not well described, the parasite’s tropism for metabolically active, lipid rich tissues like the heart and adipose tissue [2,6] suggests lipids may serve as an important nutrient source.

In mice, *T. cruzi* infection has been shown to alter plasma levels of lipid metabolism proteins, which return to normal upon treatment, suggesting a role for dysregulation of host lipid metabolism in disease pathogenesis [7]. Moreover, infection alters cardiomyocyte mRNA expression, predicting an increase in fatty acid oxidation (FAO) [8]. A recent RNA interference (RNAi) screen in HeLa cells performed by our group identified multiple genes involved in lipid metabolism in the peroxisome and mitochondria as important for supporting *T. cruzi* intracellular replication [9]. Specifically, knockdown of peroxisomal FAO of very long chain fatty acyl-CoA (VLCFA-CoA) to produce long chain fatty acyl-CoA (LCFA-CoA), as well as pyruvate dehydrogenase kinase 4 (*PDK4*), which phosphorylates pyruvate dehydrogenase and results in increased FAO in cells, resulted in reduced intracellular *T. cruzi* growth. However, knockdown of carnitine palmitoyl-transferase 1 (*CPT1*), which controls flux of LCFA-CoA into the mitochondria, increased intracellular parasite numbers [9], suggesting host cell cytosolic LCFA-CoA pools support intracellular amastigote growth.

*T. cruzi* has been shown to utilize fatty acids for beta-oxidation in the insect-epimastigote stage and extracellular trypomastigote stage [10,11] and employs elongases as the primary mechanism for fatty acid synthesis and fatty acid elongation [12–14]. Transcriptomic analysis revealed that both pathways, FAO and fatty acid elongation, are upregulated in the intracellular amastigote stage relative to the extracellular trypomastigote stage [15] (see Appendix 1, Figure A1.2B,E), potentially supporting the parasite’s biosynthetic and energy needs during replication. Proteomic analysis further confirms that levels of FAO enzymes are elevated in amastigotes [16]. Interestingly, parasites with knockout of the fatty acid transporter (R. Tarleton, personal communication) or enoyl-CoA hydratase 1 and 2 fail to establish persistent infection in mice.
Together the data suggest that *T. cruzi* access to exogenous fatty acids and FAO are important in supporting mammalian infection.

To understand how fatty acids and host lipid metabolism aids *T. cruzi* intracellular replication, we examined the ability of amastigotes to access and utilize fatty acids, and the effect of manipulating potential fatty acid sources on intracellular growth. Here we demonstrate that amastigotes take up exogenous fatty acids, which they can utilize for energy generation. However, removal of exogenous lipids or knockdown of host *CPT1* results in mild replication phenotypes. Combined, our results suggest that intracellular amastigotes utilize fatty acids for energy generation, but have flexible fuel requirements.

### 2.3 Results

**T. cruzi** intracellular amastigotes take up and utilize exogenous fatty acids

Knockout of the *T. cruzi* fatty acid transporter (R. Tarleton, personal communication) or enoyl-CoA hydratase 1 and 2 significantly impairs amastigote replication [17,18]. We therefore sought to determine if intracellular amastigotes take up exogenous palmitate for utilization in FAO. Incubation of infected human foreskin fibroblasts (HFF) with BODIPY-labeled palmitate (BODIPY-C16) resulted in cytosolic fluorescence of HFF and intracellular amastigotes, with bright puncta next to the amastigote kinetoplast, a structure composed of circular mitochondrial DNA (Figure 2.1A; white arrowheads). Following a 3-hour chase, the BODIPY label was largely excluded from amastigotes (Figure 2.1B). Similar labeling of host and amastigote cytoplasm was seen in induced pluripotent stem cell derived-cardiomyocytes (iPSC-CM; Figure 2.1C). Analysis of the ability of isolated intracellular amastigotes to use fatty acids for energy generation revealed an increase in the oxygen consumption rate (OCR) when palmitoyl-CoA, but not palmitate, was provided (Figure 2.1D,E). Together the data suggest that intracellular amastigotes access LCFA-CoA from the cytosol of multiple host cell types, which can be used for amastigote fatty acid beta-oxidation.
Figure 2.1. Amastigotes utilize exogenous palmitate. 1 μM BODIPY-labeled palmitate (BODIPY-C16) was added to the media of infected HFF for one hour then cells were fixed (A) or washed and fixed after a 3 h chase (B). DAPI staining shows the host nucleus, amastigote nuclei (round and lightly stained), and amastigote kinetoplast (discoid and brightly stained). White arrowheads point to examples of BODIPY puncta next to kinetoplasts. (C) Infected induced pluripotent stem cell-derived cardiomyocytes (iPSC-CM) were incubated with 10 μM BODIPY-C16 for 1 h before fixation. (D-E) Assessment of isolated amastigote fatty acid oxidation by Seahorse. BSA and 50 μM palmitate (D) or palmitoyl-CoA (E) were added prior to the assay start. Basal respiration was calculated and a student’s t-test was applied. * p < 0.05.
Exogenous palmitate can partially rescue multiple carbon source depletion

As intracellular amastigotes can access and utilize LCFA-CoA, we tested whether exogenous lipids support intracellular amastigote growth. Cells cultured in high glucose concentrations have been shown to increase reliance on glycolysis despite functioning oxidative phosphorylation (OXPHOS) [19–21]. To better model the metabolic state of cells in vivo, exogenous glucose was replaced with galactose to increase host cell reliance on mitochondrial OXPHOS relative to glycolysis (see Figure 3.3), potentially by increasing host fatty acid metabolism. The switch from glucose to galactose resulted in a small decrease in amastigote growth in HFF, while additional withdrawal of lipids failed to have an effect (Figure 2.2A). However, depletion of glutamine and lipids resulted in a significant decrease in amastigote replication, which was partially rescued by supplementation with palmitate. The same trends were identified in amastigote replication within iPSC-CM (Figure 2.2B). Exogenous lipids therefore support amastigote replication, either directly or by promoting the health of the host cell, but are not essential. However, mammalian cells also generate fatty acids, which may support parasite growth.

Targeting host CPT1 with siRNA increases amastigote intracellular replication

The RNAi screen in HeLa indicated that knockdown of genes involved in peroxisome synthesis and peroxisome metabolism of VLCFA-CoA decreased intracellular amastigote numbers [9]. However, knockdown of CPT1C, which transports LCFA-CoA from the cytosol into the mitochondria, increased parasite numbers, raising the possibility that the cytosolic concentration of LCFA-CoA is important for amastigote replication. To validate the effect of host CPT1C knockdown on intracellular amastigote growth, we targeted each CPT1 isoform in HeLa (CPT1A, CPT1B, CPT1C) with siRNA and infected cells two days later with trypomastigotes. At 48 hours post infection (hpi), cells reverse transfected with siRNA targeting CPT1B or CPT1C had increased numbers of amastigotes per infected cell (Figure 2.3A). As HeLa is a transformed cell line, which results in metabolic changes that may alter responses to pathogens [22,23], we
Figure 2.2. Exogenous palmitate partially rescues amastigote growth during multiple carbon source depletion. (A) HFF were infected with *T. cruzi* and cultured with the indicated substrates in DMEM + 1 mM pyruvate. At 66 hpi the relative number of parasites and host cells were assessed by β-Glo and CellTiter-Fluor respectively. Graph shows mean ± standard deviation (SD) for 16 technical replicates per condition. One-way ANOVA with Tukey’s multiple comparisons test was applied. (B) iPSC-derived cardiomyocytes were infected with *T. cruzi* and cultured with the indicated substrates in DMEM + 1 mM pyruvate. At 48 hpi monolayers were fixed and parasite numbers were assessed by microscopy. Black line indicates median. Kruskal-Wallis test with Dunn’s multiple comparisons test was applied. * p < 0.05, ** p < 0.01, **** p< 0.0001.
targeted each CPT1 isoform in HFF, a primary cell line relevant to early T. cruzi infection [24].

Here, targeting CPT1A and CPT1B resulted in increased intracellular amastigote numbers per infected cell (Figure 2.3B). Since knockdown of CPT1 might decrease host cell division and thereby increase the number of amastigotes per cell, trypomastigotes were dyed with the fluorescent dye CFSE and allowed to replicate as amastigotes before their isolation from host cells. The number of divisions each amastigote underwent was then determined based on the dilution of CFSE intensity by flow cytometry (Figure 2.3C; lower panel), which prevents changes in host cell division from confounding analysis of parasite proliferation, and showed an increase in amastigote replication following host CPT1A or CPT1B knockdown (Figure 2.3C).

Analysis of RNA levels revealed that siCPT1A reduced transcript levels of the target in both uninfected HeLa (Figure 2.3D; blue) and HFF (Figure 2.3E; blue). However, the increase in amastigote replication in HFF after knockdown of CPT1A was associated with an increase in CPT1B expression (Figure 2.3B,G; blue). Interestingly, siCPT1B was associated with increased amastigote numbers and CPT1B expression in both HeLa (Figure 2.3A,F; red) and HFF (Figure 2.3B,G; red). Simultaneous targeting of CPT1A and CPT1B in HFF likewise resulted in increased CPT1B expression and higher amastigote numbers (data not shown). CPT1C transcripts were not detected by RT-PCR in either HFF or HeLa, but siCPT1C was associated with increased amastigote numbers and increased expression of CPT1A and CPT1B in HeLa (Figure 2.3A,D,F; yellow). The upregulation of CPT1B in response to siCPT1B and siCPT1C in HeLa, and siCPT1A and siCPT1B in HFF suggests a compensatory response to knockdown. Attempts to determine protein expression by Western blot failed.

**Stable knockdown of Cpt1a in C2C12 cells increases amastigote intracellular replication**

Variation in transient transfection efficiency and lack of knowledge about protein expression in HeLa and HFF lead us to generate a stable knockdown of Cpt1 in C2C12 mouse myoblasts, which was validated by functional assays. Stably expressed, short hairpin sequences directed
Figure 2.3. Targeting host CPT1 increases amastigote replication but upregulates alternate isoforms. (A) HeLa were reverse transfected with siRNA or a non-targeting control (siCtrl). Infection was assessed at 48 hpi by microscopy. Black line indicates median. Representative experiment shown. Kruskal-Wallis test with Dunn’s multiple comparisons test was applied. * p < 0.05, ** p < 0.01. (B) HFF were transfected with siRNA and infection was assessed by microscopy at 66 hpi. (C) HFF were transfected with siRNA and infected with CFSE-stained parasites. Amastigote proliferation was modeled based on CFSE dilution using flow cytometry (lower graphs) and the average number of amastigote divisions was calculated. Mean ± SD shown for two replicates from representative experiment. One-way ANOVA with Dunnett’s multiple comparisons test was applied. *** p < 0.001. Expression of CPT1A relative to GAPDH in uninfected HeLa (D) and HFF (E).
Figure 2.3 (Continued) Expression of \textit{CPT1B} relative to \textit{GAPDH} in uninfected HeLa (F) and HFF (G). RNA was collected at 18 hpi from uninfected cells from the transfections shown in (A, B). Mean with upper and lower limits based on 3 technical replicates shown.
against Cpt1a (shCpt1a) greatly reduced target expression (Figure 2.4A) without increasing Cpt1b expression (Figure 2.4B) relative to a control line with short hairpins directed against luciferase (shLuc). Palmitate was added to uninfected C2C12 to determine exogenous LCFA usage, and etomoxir was added to inhibit Cpt1 activity, revealing the contribution of FAO to mitochondrial respiration (Figure 2.4C-E). FAO was not a significant contributor to respiration in shLuc or shCpt1a, as basal respiration did not change with palmitate or etomoxir (Figure 2.4D). However, the spare respiratory capacity (SRC) of shLuc decreased with etomoxir, suggesting cells use endogenous fatty acids to fuel mitochondrial respiration during increased energy demands (Figure 2.4E). In contrast, shCpt1a had no spare respiratory capacity, indicating significant impairment of energy generation, as from FAO. Despite the impaired spare respiratory capacity, amastigote proliferation was increased in shCpt1a (Figure 2.4F,G).

2.4 Discussion

As the replicative stage within the mammalian host, T. cruzi amastigotes play an important role in sustaining life-long infections [1,5] and have been posited to contribute to maintenance of infection in the face of drug pressure [25,26]. Results from our previous RNAi screen pointed broadly toward a role for host fatty acid metabolism in supporting intracellular T. cruzi amastigote growth [9]. Suppression of FAO by knockdown of PDK4 or inhibition of peroxisomal metabolism of VLCFA-CoA was associated with decreased intracellular amastigote growth, while inhibition of host CPT1C was associated with increased intracellular growth [9]. Together the data suggested a role for host cytosolic LCFA-CoA pools in supporting amastigote replication (Figure 2.5), which may be exploited for new therapeutic intervention strategies.

Through microscopy and bioenergetics assays, we examined the capacity of intracellular amastigotes to access and utilize exogenous fatty acids. Here we used BODIPY-C16 fluorescence to reveal for the first time that amastigotes take up fatty acids in situ, although the exact form of the acquired fatty acid cannot be determined. Interestingly, the BODIPY staining
Figure 2.4. Stable knockdown of Cpt1a in C2C12 impairs spare respiratory capacity and increases amastigote replication. C2C12 lines stably expressing short hairpin RNA against luciferase (shLuc) or Cpt1a (shCpt1a) were generated. Expression of Cpt1a (A) and Cpt1b (B) relative to Gapdh. Graphs show mean with upper and lower limits based on technical triplicates from representative experiment. (C) Assessment of fatty acid oxidation in shCpt1a by Seahorse. Bovine serum albumin (BSA) or palmitate-BSA (C16) was provided as a substrate, etomoxir (40 µM) was added to inhibit Cpt1. Oligomycin (O), FCCP (F), and rotenone and antimycin A (R/A) were injected as indicated. Results were normalized to protein levels by Bradford assay. (D) Basal respiration remains unchanged. (E) shCpt1a lack spare respiratory capacity (SRC). Graphs show mean and SD. Two-way ANOVA with Tukey’s multiple comparisons test was applied. *** p < 0.001. (F) Infection of C2C12 was assessed by microscopy 48 hpi. Mann-Whitney test was applied. Black line indicates median. **** p < 0.0001. (G) C2C12 were infected with CFSE-stained parasites and the average number of amastigote divisions at 48 hpi was modeled based on CFSE dilution measured by flow cytometry. Graph shows mean and SD of two biological replicates from representative experiment. Student’s t-test was applied. * p < 0.05.
dissipated in intracellular amastigotes but not the host cell cytosol during a chase period, possibly due to expulsion of the full fatty acid or the BODIPY label following beta-oxidation, or a signal shift to far red which accompanies BODIPY-C16 accumulation in membranes [27]. As fatty acid elongases are upregulated in replicating intracellular amastigotes [15] and membrane synthesis is required for replication, additional studies of exogenous LCFA incorporation into new parasite membranes is warranted. Transcriptomic and proteomic profiling previously indicated that fatty acid oxidation is increased in intracellular amastigotes relative to extracellular trypomastigotes [15,16]. However, isolated amastigotes did not increase their OCR in response to palmitate in our experiments, corroborating previous findings [28]. Instead, we found that palmitoyl-CoA increased the OCR of isolated amastigotes. Combined with our microscopy data, we anticipate that amastigotes are accessing cytosolic palmitoyl-CoA and other LCFA-CoA, which they can oxidize for energy generation.

Using carbon depletion and RNAi, we analyzed the importance of LCFA-CoA in supporting T. cruzi intracellular growth in multiple mammalian cell types. Although lipid depletion alone did not significantly impair amastigote growth, exogenous palmitate was able to partially rescue intracellular T. cruzi growth under multiple carbon source depletion in both fibroblasts and cardiomyocytes. In contrast, knockdown of CPT1, predicted to increase host cytosolic LCFA-CoA pools, significantly increased T. cruzi amastigote replication in both transformed (HeLa) and non-transformed (HFF, C2C12) lines. The functional impairment of host FAO in shCpt1a and accompanying increase in amastigote replication suggest host Cpt1 activity limits amastigote replication, though confirmation that CPT1 protein levels are decreased in HeLa and HFF despite elevated CPT1B transcripts is still needed. In total, the effects seen here on amastigote replication due to host CPT1 knockdown or exogenous LCFA addback were small but statistically significant. However, knockout of the T. cruzi fatty acid transporter (R. Tarleton, personal communication) or enoyl-CoA hydratase 1 and 2 reduces intracellular parasite growth and significantly attenuates mammalian infection [17]. The contribution of multiple mammalian
Figure 2.5. Model of interaction between host LCFA-CoA and *T. cruzi*. Exogenous fatty acids can be taken up by the amastigote, which utilize palmitoyl-CoA for fatty acid oxidation. Knockdown of host *CPT1* increases amastigote replication, and previous work has indicated that peroxisomal fatty acid oxidation supports amastigote growth (Caradonna *et al.* 2013).
pathways to cytosolic LCFA-CoA pools, such as uptake of exogenous LCFA, peroxisomal beta-oxidation of VLCFA, and FAO (Figure 2.5), therefore obscures whether fatty acids are required for amastigote replication. Interestingly, *T. cruzi* infection upregulates FAO gene expression in the heart [8], potentially increasing intracellular amastigote access to fatty acids. Additional microscopy and proliferation assays utilizing a *T. cruzi* line with inducible knockout of the fatty acid transporter would help demonstrate whether cytosolic fatty acids are required for *T. cruzi* intracellular replication.

In summary, we demonstrated that intracellular amastigotes access exogenous fatty acids, which they can oxidize for energy generation. Our findings that exogenous palmitate and host *CPT1* knockdown aid *T. cruzi* intracellular replication in multiple mammalian cell types support the hypothesis that intracellular amastigotes draw on host cytosolic LCFA-CoA pools to fuel replication. However, targeting one metabolic pathway in the host to limit infection will likely be insufficient given that multiple pathways impact metabolite levels. Instead, targeting central nodes of parasite metabolism or multiple nodes of host metabolism may be required.

2.5 Materials and Methods

**Mammalian and parasite cell culture**

HeLa (ATCC CCL-2), human foreskin fibroblasts (HFF; ATCC), mouse skeletal muscle myoblasts (C2C12; ATCC CRL-1772), and African green monkey kidney epithelial cells (LLcMK2; ATCC CCL-7) were maintained at 37°C and 5% CO₂ in Dulbecco’s Modified Eagle Medium (DMEM; Hyclone) supplemented with 1 mM pyruvate, 25 mM glucose, 2 mM glutamine, 100 U/mL penicillin, 10 µg/mL streptomycin, 10 mM HEPES, and 10% fetal bovine serum (FBS) (D10). Induced pluripotent stem cell-derived cardiomyocytes (iPSC-CM; Cellular Dynamics CMC-100-010-001) were cultured in iCell Cardiomyocytes Maintenance Medium (Cellular Dynamics).
Trypanosoma cruzi Tulahuén strain parasites (ATCC PRA-330) were maintained by weekly passage in LLcMK2 cells in DMEM supplemented with 1mM pyruvate, 25 mM glucose, 2 mM glutamine, 100 U/mL penicillin, 10 µg/mL streptomycin, 10 mM HEPES, and 2% FBS (D2) as described [15]. Infected LLcMK2 supernatants were collected and trypomastigotes were pelleted at 2,000 g for 10 minutes and allowed to swim up from the pellet at 37°C, 5% CO₂ before use for infections.

For infections, mammalian cells were grown for 24-48 hours to achieve ~80% confluence, incubated with fresh trypomastigotes in D2, then washed 2-3 times in PBS (Gibco) to remove extracellular trypomastigotes. Monolayers were incubated in D2 or the indicated medium following infection.

Mammalian cell knockdown

HeLa reverse transfection: HeLa were reverse transfected with 50 nM of siRNA (ON-TARGETplus pool; Dharmacon) using Oligofectamine Reagent and Opti-MEM I Medium (Thermo Fisher Scientific) per manufacturer’s protocol at 5 x 10⁴ cells per well in 24 well plates. Medium was changed to D2 the following day.

HFF transfection: 5 x 10⁵ HFF were transfected with 200 nM siRNA in NHDF Nucleofector solution (Lonza) using the U-23 program of an Amaxa Nucleofector II (Lonza) per manufacturer’s protocol. Following transfection cells were resuspended in D10 and plated at 5 x 10⁴ cells per well in 24 well plates for RNA and cell microscopy or at 4.5 x 10⁵ cells in a 10 cm dish for CFSE proliferation assays.

C2C12 stable knockdown: The plasmid pSiren-RetroQ and double stranded oligonucleotides with short hairpin sequences targeting luciferase or Cpt1a were digested separately with EcoRI and BamHI for one hour at 37°C. The oligonucleotides were combined with linearized plasmid
and T4 DNA ligase (New England BioLabs) for ligation per manufacturer’s protocol. Following sequence confirmation, Phoenix packaging cells were transfected with the ligation product using TransIT-LT1 (Mirus Bio) per manufacturer’s protocol. Virion-containing media was obtained 2 days post transfection, passed through a 0.45 µm filter (VWR), and combined with 4 µg/mL polybrene to transduce C2C12 seeded the previous day at 1.5 x 10^5 per well in a 6 well plate. Transgenic C2C12 were selected with 2 µg/mL puromycin starting 2 days post-transduction. Knockdown was confirmed after 2 weeks.

shCpt1: GACGGATCCGCATGATTGCAAAGATCAATCTTCAAGAGAGATTGATCTTTGCAATCATGC
TTTTGATATCGAATTCGCC (underline indicates hairpin sequence and linker region)

Microscopy

HFF and iPSC-CM were plated at 1.5 x 10^5 per well on 12 mm round German glass coverslips (Electron Microscopy Services) in 6 well plates and infected with a multiplicity of infection of 10 (MOI 10). At 45 hours post infection (hpi), monolayers were washed with PBS and incubated in DMEM supplemented with 10 µM fatty acid free BSA (Sigma-Aldrich) and 1 µM BODIPY FL C16 (BODIPY-C16; Thermo Fisher Scientific) for 1 hour. Cells were washed with PBS and fixed with 4% paraformaldehyde (PFA; Electron Microscopy Sciences) or incubated in D2 for another 3 hours before fixation. Cells were stained with 2.5 µg/mL DAPI for 2 minutes and washed in PBS. Coverslips were mounted to slides in Mowiol mounting medium [29]. Images were captured using a Nikon TE300 and 60x objective.

Seahorse bioenergetics analysis

Isolated amastigote assessment: LLCMK2 were seeded at 1.5 x 10^6 in T75 flasks and infected with MOI 10 for 1 hour. At 48 hpi the monolayer was scraped, cells were collected from the supernatant, and amastigotes were released by repeated passage through a 28.5G needle (BD BioSciences) in Krebs-Henseleit Buffer (KHB; 111 mM sodium chloride, 4.7 mM potassium
chloride, 1.25 mM calcium chloride, 2 mM magnesium sulfate, and 1.2 mM sodium phosphate dibasic) supplemented with 2.5 mM glucose, 0.5 mM carnitine, and 5 mM HEPES. To remove large cellular debris, the supernatant was centrifuged at 60 g for 1 minute twice. To pellet amastigotes, the supernatant was centrifuged at 2100 g for 10 minutes. Wells of an XF®24 microplate were prepared with Cell-Tak (181.5 μg/mL in 0.1 M NaHCO₃, pH 8; Corning) per manufacturer’s protocol). Amastigotes were seeded at 2 x 10⁶ per well, and the microplate was centrifuged at 2100 g for 2 minutes to adhere amastigotes to the well. 50 µM palmitate, palmitoyl-CoA, or BSA (Sigma-Aldrich) was added immediately prior to the run. 1 µM rotenone and 1 µM antimycin A (Sigma-Aldrich) was injected during the run to inhibit mitochondrial respiration. Basal respiration was calculated as the difference between the baseline oxygen consumption rate (OCR) and that after rotenone and antimycin A injection.

_Mammalian cell assessment:_ C2C12 were seeded at 3.5 x 10⁴ per well in an XF®24 microplate. The medium was changed the following day to DMEM supplemented with 0.5 mM carnitine, 1 mM glutamine, 0.5 mM glucose, and 1% FBS. The following day, medium was replaced with KHB supplemented with 0.5 mM carnitine, 2.5 mM glucose, and 5 mM HEPES and cells were incubated at 37°C without CO₂ for 1 hour. Etomoxir (40 µM) was added and plates were incubated another 15 minutes at 37°C without CO₂. BSA (320 nM) or BSA conjugated to palmitate (Sigma-Aldrich) (320 nM BSA, 175 µM C16) was added immediately prior to the run. 1 µM oligomycin (O), 2 µM FCCP (F), and 1 µM rotenone and 1 µM antimycin A (Sigma-Aldrich) were injected sequentially during the run. Maximal respiration was calculated as the difference between max OCR after FCCP injection and that after rotenone and antimycin A injection. Spare respiratory capacity (SRC) was calculated as the percentage of maximal respiration divided by basal respiration.
Amastigote growth

*Microscopy counts:* HeLa and HFF were seeded at $5 \times 10^4$ per well on 12 mm round German glass coverslips in 24 well plates after transfection and infected 2 days later with MOI 5 for 1 hour. C2C12 were seeded at $4 \times 10^4$ per well and infected with MOI 10 for 1 hour, while iPSC-CM were seeded at $1 \times 10^5$ per well on 0.1% gelatin-coated coverslips and infected with MOI 1 for 30 minutes. At 48 hpi coverslips were fixed and stained with 2.5 µg/mL DAPI and mounted on slides as above. Slides were observed using a Nikon TE300 and the number of amastigotes per cell was counted for 100 cells per condition.

*Multiplexed infection assay:* HFF were seeded at $1.5 \times 10^3$ per well in 384 well plates and infected with MOI 5 for 1 hour. Following PBS washes, the medium was changed as indicated. At 66 hpi media was removed and fluorescence was read on an EnVision plate reader (PerkinElmer) following addition of 10 µL CellTiter-Fluor (Promega) to assess host cell number. Since a transgenic parasite line expressing beta-galactosidase was used, luminescence was read following addition of 10 µL Beta-Glo (Promega) to assess parasite number. The relative luminescence unit (RLU) was divided by the relative fluorescence unit (RFU) to determine the ratio of amastigotes to host cells.

*CFSE proliferation assay:* HFF were seeded at $5 \times 10^5$ in a 10 cm dish and infected with MOI 10 for 2 hours using CFSE-stained trypomastigotes. To stain, $5 \times 10^5$ trypomastigotes/mL were incubated with 1 µM CFSE (Thermo Fisher Scientific) at 37°C, 5% CO$_2$ for 15 minutes. Excess dye was quenched by adding D10 and trypomastigotes were centrifuged at 2100 g for 10 minutes and incubated in fresh D2 for 30 min at 37°C, 5% CO$_2$. At 18 and 48 hpi (pre-replication and during replication, respectively), cells were trypsinized, washed once in PBS, and lysed by repeated passage through a 28.5G needle (BD Biosciences). Lysate was fixed in 1% PFA and incubated on ice for 20 minutes. Samples were centrifuged at 300 g for 5 minutes to pellet host
debris, and the supernatant was centrifuged at 4000 g for 10 minutes to pellet amastigotes. Pellets were resuspended in PBS with 0.01% Triton-X-100 and 0.01 µg/mL DAPI for flow cytometry. Samples were run on a MACSQuant VYB (Miltenyi Biotec). Amastigotes were discriminated based on size and DAPI using FlowJo 7.6 and proliferation was modeled by setting the amastigote CFSE intensity at 18 hpi as generation 0 for all samples. For assays with C2C12, cells were seeded at 2.5 x 10^5 per 10 cm dish and infected with MOI 10.

**RT-PCR**

RNA was collected 3 days after cell transfection and isolated using an RNeasy kit (Qiagen) per manufacturer’s protocol. gDNA was degraded using a TURBO DNA-free Kit (Thermo Fisher Scientific) and cDNA was synthesized from 500 ng RNA using iScript Reverse Transcription Supermix (Bio-Rad) per manufacturer’s’ protocols. For RT-PCR, 20 ng cDNA was combined with TaqMan Supermix (Thermo Fisher Scientific) and GAPDH or gene of interest TaqMan probe in a 20 µL reaction per manufacturer’s protocol. The reaction was run at 95°C for 30 seconds, then cycled 45 times at 95°C for 15 seconds and 60°C for 1 minute and analyzed using the default Comparative Ct (ΔΔCt) settings of a StepOnePlus (Applied Biosystems).

**Statistical analysis**

Statistical analysis was performed using Prism 7 (GraphPad).

**2.6 References**


Chapter 3

Bioenergetic profiling of *Trypanosoma cruzi* life stages and amastigote metabolic adaptation using Seahorse extracellular flux technology

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**Author contributions:** SSS, CFAP, KLC, and BAB designed the experiments. SSS and CFAP performed all *T. cruzi* Seahorse experiments and analyzed the data. SSS performed all adaptation experiments and analyzed the data. KH, PP, EM, and NMES processed RNA, generated libraries, and analyzed the data. PCD performed propidium iodide staining of *T. cruzi* amastigotes. SSS and BAB wrote the manuscript with editorial input from PCD, CFAP, and KLC. This chapter includes unpublished work and a reprint of “Bioenergetic profiling of *Trypanosoma cruzi* life stages using Seahorse extracellular flux technology,” Mol Biochem Parasitol. Vol. 208, Shah-Simpson S, Pereira CFA, Dumoulin PC, Caradonna KL, Burleigh BA. 91–95, Copyright (2016), with permission from Elsevier.
3.1 Abstract

Energy metabolism is an attractive target for the development of new therapeutics against protozoan pathogens, including *Trypanosoma cruzi*, the causative agent of human Chagas disease. Despite emerging evidence that mitochondrial electron transport is essential for the growth of intracellular *T. cruzi* amastigotes in mammalian cells, fundamental knowledge of mitochondrial energy metabolism in this parasite life stage remains incomplete. Additionally, *T. cruzi* replicates within a variety of nucleated mammalian cells with divergent metabolic requirements, yet mechanisms of amastigote metabolic adaptation to differing intracellular nutrient microenvironments has not been explored. Here we evaluate the Seahorse XF®24 extracellular flux platform as an alternative method to assess mitochondrial bioenergetics in isolated *T. cruzi* parasites. We report optimized assay conditions used to perform mitochondrial stress tests with replicative life cycle stages of *T. cruzi* using the XF®24 instrument. To assess the parasite’s ability to respond to different host metabolic environments, we modulated host cell metabolism using media composition and found that amastigotes did not significantly alter their transcriptome or bioenergetics profile, though they had a significantly greater respiratory capacity than other *T. cruzi* life cycle stages. Our work indicates that amastigotes primarily adapt to intracellular environments by increasing metabolic capacity and plasticity during their intracellular stage rather than responding individually to alterations in host metabolism.

3.2 Introduction

The protozoan parasite *Trypanosoma cruzi*, estimated to infect 5.7-9.4 million individuals mainly in South and Central America [1,2], causes chronic Chagas disease in roughly 30% of infected individuals, comprised of cardiomyopathy, megacolon, or megaoesophagus syndromes [3]. Current drugs fail to improve clinical outcomes during the chronic phase of the disease, necessitating the development of new anti-trypanosomal therapies [4,5].
As the replicative stage in the mammalian host, *T. cruzi* intracellular amastigotes are an attractive target for limiting infection. The recent demonstration that inhibition of the *T. cruzi* electron transport chain reduces amastigote intracellular growth [6] indicates that targeting parasite metabolism may be an effective cure strategy. However, not much is known about the metabolism of intracellular amastigotes, though they are predicted to rely on fatty acids and amino acids for nutrients [7,8]. Interestingly, *T. cruzi* has a broad range of cells and hosts it is capable of infecting. Infection has been identified in over 42 mammalian species including opossums, rodents, and dogs [9–11], and in vitro, *T. cruzi* can invade almost any nucleated mammalian cell line [12]. During acute infection in rodents, *T. cruzi* intracellular amastigotes are found replicating in tissues throughout the body including within skin, kidneys, liver, and the reproductive tract, while during chronic infection parasites exhibit a tropism for the muscular layer of the gastrointestinal tract, skeletal muscle, and the heart [13–16], tissues which exhibit significant differences in metabolic flux even over the course of a day [17–19].

To understand how *T. cruzi* intracellular amastigotes adapt to different host metabolic environments, we examined the effect of reprogramming host cell metabolism on amastigote proliferation and bioenergetics. Manipulation of host metabolism using media composition, replacing high glucose containing medium with galactose containing medium, has previously been shown to push mammalian cells to rely less on glycolysis and more on oxidative phosphorylation for energy generation [20,21]. After establishing a method to evaluate *T. cruzi* bioenergetics, we determined that amastigotes exhibit higher respiratory capacities than other life cycle stages of *T. cruzi*, but do not substantially reprogram metabolism based on the host nutrient states tested. Additional examination of the transcriptome failed to identify signatures of metabolic adaptation. Together the data suggest that *T. cruzi* increases metabolic capacity during the replicative amastigote stage to take advantage of different microenvironments rather than altering metabolic capabilities in response to each environment.
3.3 Results

Bioenergetic profiling of *Trypanosoma cruzi* life stages using Seahorse extracellular flux technology

The kinetoplastid protozoan parasite, *Trypanosoma cruzi* has a complex life cycle in which it transitions between triatomine vectors and a range of mammalian hosts including humans, where it causes Chagas disease [3]. In mammalian hosts, the parasite cycles between the non-replicative, cell-invasive trypomastigote stage and the replicative, intracellular amastigote stage, while in the vector it proliferates as an epimastigote in the midgut. As *T. cruzi* cycles within and between hosts, alterations in its metabolic profile are predicted to aid in adaptation to environmental change [8,22]. Similar to other trypanosomatids, the first six enzymes of the glycolytic pathway in *T. cruzi* are compartmentalized within membrane-bound organelles known as glycosomes [23]. Despite evidence of a non-canonical tricarboxylic acid (TCA) cycle in *T. cruzi*, the TCA cycle enzymes are represented in all life cycle stages of the parasite, as are the mitochondrial electron transport chain complexes [23–25]. Parasite metabolism is an attractive therapeutic target, as biochemical differences between host and parasite metabolic processes can be exploited to control infection [26,27] and transmission [28]. With the recent demonstration that the mitochondrial respiratory chain can be selectively targeted in intracellular *T. cruzi* amastigotes to inhibit parasite growth [6], there is renewed interest in characterizing central carbon metabolism in these parasites, especially mammalian stages.

Electrodes measuring oxygen levels in cell suspension have been used successfully to derive fundamental information about mitochondrial respiration and substrate utilization in different *T. cruzi* life cycle stages [25,29]. Although sensitive, measurements are limited to a single sample at a time and generally require large numbers of parasites (∼1 x 10^8 per sample). Similarly, the Oxygraph-2K requires 5 x 10^7 parasites per sample and analyzes two samples at a time [30]. Alternatively, the MitoXpress-Xtra probe has been used in high-throughput assays with *T. cruzi* using fewer parasites (3 x 10^6 per sample) [6], but does not allow addition of
compounds of interest during the assay to measure acute effects on respiration. Seahorse extracellular flux technology provides the unique benefits of assaying multiple samples simultaneously using 24 or 96-well plates, allowing injection of multiple compounds during the assay, and interrogating glycolysis as well as mitochondrial respiration of cells within the same well [31]. Although extracellular flux analyzers were developed with mammalian cells in mind, the advantages associated with this method, as compared to the alternatives, motivated us to determine whether extracellular flux analyzers could be implemented in studies of *T. cruzi* energy metabolism.

The technology used in the Seahorse XF²4 (and XF²96) instrumentation has been described in detail [31]. Briefly, cells are plated in wells of a specialized microplate fitted with a cartridge containing two fluorophores that independently sense oxygen and pH in each well. The measurement cycle consists of: mix, wait, and measure periods for time intervals that are user determined. During the measure period, the top cartridge is lowered down such that individual microchambers are formed in each well. Fiber optic probes excite the fluorophores and read changes in oxygen and pH over time, translating readings into oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) respectively. OCR correlates with rates of respiration as cells consume oxygen to accept electrons from the electron transport chain (ETC). ECAR correlates with glycolytic rates in cells that excrete lactic acid as a product of glycolysis, such as mammalian cells. Four ports in the assay cartridge above each well allow injection of compounds directly into the wells at any point during the assay. This provides the opportunity to establish basal glycolytic and mitochondrial respiratory rates and determine substrate utilization preferences of cells under specified conditions. Additionally, sequential injections of specific inhibitors can be used to define bioenergetic profiles in different cell types or in the same cell type under different conditions. For example, the quantitative changes in OCR triggered by the injection of oligomycin, FCCP and rotenone/Antimycin A in sequence are used to calculate the amount of respiration linked to mitochondrial ATP-production and proton
leak, as well as mitochondrial coupling efficiency, maximal respiration, and spare respiratory capacity (reviewed in [32]). This is referred to as the 'mitochondrial stress test.'

Here we evaluated the potential to exploit the Seahorse XF\textsuperscript{e}24 extracellular flux analyzer to measure mitochondrial respiration and glycolysis in isolated \textit{T. cruzi} parasites. Initially, three different life stages of the parasite were used in these studies. Insect stage epimastigotes were maintained at mid-log phase in LIT medium and tissue-culture-derived trypomastigotes were harvested from the culture supernatants of infected LLcMK2 cells as described [22]. Intracellular \textit{T. cruzi} amastigotes were released from infected host cell monolayers 48-50 hours post-infection by syringe passage (28G needle) into XF Base Medium (DMEM-based medium; Seahorse Bioscience) supplemented with 1 mM sodium pyruvate, 2 mM glutamine, and 10 mM glucose (XBMS). The disrupted host cell suspension was centrifuged twice at 60 \textit{g} for 1 minute to remove large cellular debris, and amastigotes were recovered in the supernatant by centrifuging at 2100 \textit{g} for 10 minutes. Propidium iodide staining performed at the end of the amastigote isolation procedure demonstrates that isolated amastigotes maintain their plasma membrane integrity throughout the procedure (Figure 3.1A).

Given that existing mitochondrial energetics data was derived from parasites in suspension, pilot experiments were conducted with trypomastigote suspensions using the XF\textsuperscript{e}24. However, OCR and ECAR readings were extremely unstable, showing large variation between replicate measurement cycles (data not shown). This is likely due to both variability in the amount of parasites trapped in the microchamber for each measurement cycle and the inability of oxygen and pH to re-equilibrate due to the high concentration of suspended parasites (>5 x 10\textsuperscript{7} per well). The high concentration of parasites caused extremely low oxygen and pH readings that were reaching the limit of the probes' detection range. We conclude that this instrument is incompatible with \textit{T. cruzi} parasites in suspension and predict that this would generally hold for related trypanosomatids such as \textit{T. brucei} or \textit{Leishmania} species.
Figure 3.1. Optimization of isolation and assessment of three different T. cruzi life cycle stages in the Seahorse XF®24. (A) Isolated amastigotes were resuspended in PBS (filled) or permeabilized with 4% paraformaldehyde/PBS (dashed line) and incubated for 20 minutes on ice. Propidium iodide was added to a final concentration of 0.5 µg/mL. Amastigotes were discriminated based on size and surface staining (Fig. S1A). Data are representative of three biological replicates. (B) Epimastigotes were plated at the indicated concentrations per well. Data show at least three technical replicates and are representative of two biological replicates. (C) Amastigotes from infected (ama) and mock-infected (mock) cells were plated at 1 x 10⁶ per well (or corresponding dilution for mock). Data show at least 4 technical replicates and are representative of 3 biological replicates. (D-E) Trypomastigotes, amastigotes and epimastigotes were plated at 1 x 10⁶ per well. Measurements were done with 1 minute of mixing, 2 minutes of waiting, and 2 minutes of measuring to minimize disruption to the monolayer due to mixing and increase accuracy of the rate measurements. Parasite numbers in individual wells were normalized by qPCR of T. cruzi DNA as described [22] when comparing different parasite populations. Briefly, 2 pmol of primers amplifying the single copy oxysterol-binding protein gene (TriTrypDB ID: TcCLB.508211.10) were combined with 2% of total DNA isolated from each well, and the amount of DNA per well was calculated by comparing Ct values to a standard curve. Data show at least three technical replicates per life cycle stage and are representative of at least two biological replicates.
We then explored a method to gently adhere the parasites to the bottom of the well with Cell-Tak (Corning), which is routinely employed for XF® measurements in non-adherent mammalian cells [33]. After centrifugation of mid-log phase epimastigotes (1-2 x 10^7/mL) at 20 g for 5 minutes to remove aggregates, parasites were pelleted at 2100 g for 10 minutes and washed twice in XBMS to remove residual FBS, which interferes with binding to Cell-Tak. All centrifugation steps were carried out at 4°C in ice-cold medium except where indicated. Washed parasites were resuspended in XBMS warmed to 37°C, and 100 µL of a 4.5x parasite suspension was delivered into the wells of a XF®24 microplate prepared with Cell-Tak (181.5 µg/mL in 0.1 M NaHCO₃, pH 8), as per manufacturer's instructions. Of note, we found no difference in OCR when assaying epimastigotes at 30°C (the lowest temperature setting with an XF®24) versus 37°C, as previously described [34]. To create a parasite monolayer the plate was centrifuged briefly at 2100 g and an additional 350 µL of warm XBMS was added to each well. A linear OCR response was observed which correlated with the number of parasites plated per well (Figure 3.1B; R²=0.9626). With the exception of the lower parasite densities, where much of the parasite body was in contact with the matrix (<5 x 10^5 parasites/well), a relatively small portion of the parasite cell body or flagellum was in contact with the matrix, and epimastigotes were still moving in all plating concentrations at the end of an 80-minute assay. A density of 1 x 10^6 parasites/well was selected for further experiments since this concentration is within the linear range of the assay and also provides a high OCR signal. Before embarking on the comparative studies between different T. cruzi life cycle stages, we performed additional experiments to ensure that any remaining host cellular debris from amastigote isolations (which is visible by microscopy and flow cytometry; Appendix 2 Figure A2.1A) did not contribute to any of the measurements. The OCR and ECAR signals obtained for isolated amastigotes were compared to debris generated from syringe-passed, mock-infected host cell monolayers. Despite wells containing visible debris, no measurable OCR signal was detected and a relatively low ECAR signal was detected in only one of three biological replicates (Figure 3.1C).
A typical assay requires about 80 minutes to complete. We therefore examined the stability of OCR and ECAR readings in three parasites life cycle stages over this time period, isolated and plated in a microplate as described above. Amastigotes and epimastigotes remained energetically stable over the course of the assay (Figure 3.1D,E) with no detectable detachment from the matrix. In contrast, OCR and ECAR readings gradually declined in wells containing trypomastigotes (Figure 3.1D,E), in part due to the detachment of these highly motile parasites from the matrix with time (confirmed with microscope). Although it is possible to obtain reproducible metabolic readings from trypomastigotes in short-term assays [22], their detachment over time makes long-term assays such as the mitochondrial stress test difficult.

Focusing on epimastigotes and isolated intracellular amastigotes, we proceeded to optimize conditions for metabolic profiling in T. cruzi using the XF®24. In pilot experiments, we noted that the ECAR response mirrored the OCR response after injection of mitochondrial inhibitors FCCP (both increased) or rotenone/Antimycin A (both decreased) (data not shown). This unexpected pattern suggested that ECAR is primarily reporting mitochondrial activity in T. cruzi. Succinate, not lactate, is the major acidic end product of an extended form of glycolysis in T. cruzi epimastigotes [23], however, CO₂ produced by mitochondrial metabolism may be the major contributor to medium acidification in these experiments [35]. To determine the relative contribution of mitochondrial metabolism to ECAR, a mixture of 1 µM rotenone and 1 µM Antimycin A (AA) (Seahorse Bioscience) was injected into wells containing T. cruzi epimastigotes or amastigotes to shut down mitochondrial respiration by inhibiting complex I and III of the ETC, respectively (Figure 3.2A,B). The concentration of rotenone used here is low enough to avoid off-target effects previously reported and came pre-mixed with Antimycin A [36]. As expected this injection significantly reduced OCR in both parasite life cycle stages, but also significantly reduced ECAR (40-58%) (Figure 3.2A,B). Subsequent injection of 2-deoxyglucose (2-DG) (Sigma) to inhibit glycolytic flux further decreased ECAR in both amastigotes and epimastigotes (Figure 3.2B). We conclude that in the case of T. cruzi
Figure 3.2. Profiling mitochondrial function in *T. cruzi* amastigotes and epimastigotes. Compounds were diluted in XBMS to 10x the final concentration for injections. (A-B) 1 µM rotenone and 1 µM Antimycin A (AA) were injected followed by 100 mM 2-deoxyglucose (2-DG). Data show at least three technical replicates per life cycle stage and are representative of at least two biological replicates. (C-D) ATP-linked OCR was calculated as the difference between OCR at baseline and OCR after oligomycin injection. (E-F) Spare respiratory capacity (SRC) was calculated as the difference between OCR after FCCP injection and OCR at baseline. One-way ANOVA and Dunnett’s multiple comparison test was calculated relative to either 0 µM oligomycin injection or FCCP. * indicates p<0.01. Shaded bars indicate inhibitor concentrations chosen for the stress test. Each graph shows at least three technical replicates per concentration and is representative of two biological replicates. Original traces in Fig. S1E-H. As parasites for an individual experiment were plated from one starting population, data were not normalized by qPCR. (G-I) The mitochondrial stress test was applied to amastigotes and
Figure 3.2 (Continued) epimastigotes and the results of at least three biological replicates with at least three technical replicates per experiment are shown here. Measurements are reported as a ratio of basal OCR, which provides internal normalization and avoids the need for qPCR normalization. (H) The coupling efficiency was calculated as the ratio of ATP-linked OCR to basal OCR. (I) The spare respiratory capacity ratio was calculated as the ratio of SRC OCR to basal OCR. Welch’s t-test was used to determine statistical significance. Technical variability was similar for both amastigote and epimastigote samples with a coefficient of variation of 0.14 and 0.12, respectively.
parasites, ECAR reports the combined activities of glycolysis and mitochondrial respiration. As such, subtraction of the contribution of mitochondrially-derived CO$_2$ to ECAR would be required to quantify glycolytic activity in these and, potentially, in related parasites. OCR, however, is a faithful reporter of mitochondrial and non-mitochondrial respiration in *T. cruzi*.

We next focused on the optimization of parameters that would enable us to perform mitochondrial stress tests in the replicative stages of *T. cruzi*. For this, we titrated oligomycin and FCCP for use with *T. cruzi* amastigotes and epimastigotes. Oligomycin inhibits oxidative phosphorylation by targeting ATP synthase, and ATP-linked respiration can then be calculated as the difference between baseline OCR and OCR after oligomycin addition (Figure 3.2C,D) [32]. FCCP is an ionophore that ablates mitochondrial membrane potential, leading to an increase in respiration, which is uncoupled from ATP generation, as the cell attempts to restore the electrochemical gradient. Spare respiratory capacity (SRC) is calculated as the difference between maximal respiration after FCCP addition and baseline respiration (Figure 3.2E,F) [32]. Interestingly, the calculated SRC decreased significantly with increasing oligomycin concentration or exposure time for amastigotes, but not epimastigotes (Appendix 2, Figure A2.1B,D), suggesting that ATP produced by the ETC is required in amastigotes, but not epimastigotes, to maintain plasticity in responding to energetic stress [32]. For our stress test, we chose a concentration of oligomycin that generated a maximal drop in OCR for both life cycle stages, which will result in underreporting of amastigote SRC. Concentrations of FCCP above 3 µM in amastigotes reduced measures of SRC, potentially due to plasma membrane depolarization, an off-target effect (Figure 3.2E) [37]. In epimastigotes, 3 µM was sufficient to obtain maximal values, and higher concentrations did not reduce SRC as in amastigotes (Figure 3.2F). We therefore determined 2.5 µM oligomycin followed by 2 µM of FCCP to be optimal for amastigotes and 3 µM FCCP to be optimal for epimastigotes (Figure 3.2C-F).

To obtain a more complete profile of mitochondrial energetics in *T. cruzi* epimastigotes and amastigotes, we employed our optimized mitochondrial stress test (2.5 µM oligomycin, 2 or
3 µM FCCP, 1 µM rotenone and 1 µM Antimycin A) with intracellular-derived amastigotes and mid-log phase epimastigotes (Figure 3.2G). Of note, amastigotes exhibited greater biological variability (between preparations) than epimastigotes, which may reflect parameters of intracellular infection conditions that are not readily controlled. Regardless, epimastigotes and amastigotes have similar rates of basal metabolism under excess nutrient conditions (Figure 3.2A,G), in agreement with previous results [25]. With the caveat that a single condition was used for these experiments and mitochondrial metabolism will likely differ under alternate nutrient conditions, we observed that mitochondrial coupling efficiency was slightly higher in epimastigotes than amastigotes (Figure 3.2H), or that a greater proportion of mitochondrial respiration is linked to ATP generation in epimastigotes. The corollary is that amastigote mitochondria have more electron leakage, perhaps leading to more reactive oxygen species (ROS) generation than in epimastigotes, similar to what has been determined in bloodstream trypomastigotes [30]. The spare respiratory capacity ratio (SRC ratio) is indicative of a cell’s ability to meet new energy demands [32]. Although the SRC ratio consistently trended higher for isolated amastigotes as compared to epimastigotes, it did not reach statistical significance (Figure 3.2I). It is important to note that for this proof of principle study only one time point for both epimastigotes and amastigotes was assayed, and epimastigote metabolism is known to shift during growth [29]. Therefore, these results should be viewed only as a snapshot within the growth continuum rather than as the definitive metabolic states for these life cycle stages.

In summary, this study demonstrates that Seahorse XF® technology can be utilized to analyze Trypanosoma cruzi mitochondrial bioenergetics in multiple samples concurrently, using 3-100 fold fewer parasites than traditional methods [6,25,30]. We anticipate that our assay guidelines, optimized for the 24-well plate system (XF®24), will also be directly translatable to the 96-well plate reader (XF®96), and this format will further reduce the number of parasites required per sample. Creation of a parasite monolayer using Cell-Tak allows for repeated measurements while still permitting flagellar beating in adhered epimastigotes. Although we
cannot determine with this method whether epimastigote adherence impacts mitochondrial bioenergetics, epimastigotes are known to adhere to the culture flask and each other in vitro and insect midgut ex vivo [38], suggesting that our measurements are biologically relevant. However, the highly motile trypomastigote stage readily detached from the monolayer and performed poorly when adhered. Application of this system to other motile parasites such as Leishmania promastigotes or T. brucei trypomastigotes or procyclics will therefore have to be assessed individually. Assessment of metabolism in other non-motile stages such as Leishmania amastigotes is, however, feasible. Finally, we have demonstrated the utility of XF® technology in studying mitochondrial capabilities in the replicative stages of T. cruzi. However, the ability to derive accurate data for parasite glycolytic rates remains to be determined. By delivering a rapid readout in a multi-well plate format, the mitochondrial stress test would be a useful secondary screen of new anti-trypanosomals to quickly identify compounds that exert specific effects on parasite metabolism (e.g. at the level of ATP-coupled respiration like GNF7686 described by Khare et al. (2015)) and not host metabolism. As a discovery tool, this method can be used in combination with targeted perturbations (small molecule inhibitors or gene knockouts) to interrogate cellular processes that potentially impact the parasite ETC and oxidative phosphorylation. Additionally, bioenergetic profiling using the mitochondrial stress test can provide insight into how T. cruzi parasites respond and adapt to different environmental conditions and nutrient sources. The assay conditions optimized in this study will provide a useful tool to unlock current mysteries in T. cruzi metabolism, especially that of intracellular amastigotes, which has been understudied in relation to its importance in human disease.

Host metabolic reprogramming impacts amastigote replication without significantly altering amastigote metabolic capabilities

As T. cruzi replicates within a variety of different cell types, we sought to determine whether amastigotes respond to differences in host metabolism by altering their metabolic capabilities.
With mammalian cells, culturing cells in media with high concentrations of glucose (HG) results in high glycolytic rates, while culturing cells with galactose instead of glucose (Gal) pushes cells to rely more heavily on oxidative phosphorylation [20,21]. In HFF, culturing cells in Gal medium significantly increased OCR relative to ECAR (Figure 3.3A), but did not alter levels of reactive oxygen species (Appendix 2, Figure A2.2D). However, culturing cells in Gal medium caused a measurable, but small, decrease in amastigote replication as assessed by microscopy (Figure 2.2) and proliferation assay (Figure 3.3B).

To determine if amastigotes reprogram their metabolism in response to the host metabolic state, the transcriptome of intracellular amastigotes grown in Gal was compared to those grown in HG. Only five genes were differentially expressed between the amastigote populations, four surface proteins and one hypothetical protein (Appendix 2, Table A2.1), while in host cells, infection led to differential expression of over 200 genes (Appendix 2, Table A2.2), most of which are involved in immune response pathways upregulated in response to cellular infection [22,39,40]. In contrast, the differentiation of extracellular trypomastigotes into replicating amastigotes was associated with upregulation of multiple metabolic pathways including the mevalonate pathway, fatty acid synthesis and oxidation, glycolysis, the TCA cycle and oxidative phosphorylation (Appendix 1, Figure A1.2). However, large batch effects may have obscured real changes in metabolic gene expression when comparing media conditions (Appendix 2, Figure A2.3), as uninfected host cells had differential expression of 32 transcripts, which did not fall in expected metabolic pathways (Appendix 2, Table A2.3).

To determine if amastigotes demonstrate functional metabolic changes, despite the lack of a transcriptional response to the host metabolic state, amastigotes were isolated from HFF grown in HG or Gal medium and analyzed by mitochondrial stress test (Figure 3.3C). Changes in host metabolism were not associated with changes in basal respiratory rates (Figure 3.3D) or the coupling efficiency between the mitochondrial electron transport chain and ATP generation (Figure 3.3E), and ATP assays revealed no alteration in sensitivity to the complex III inhibitor
Figure 3.3. HFF metabolic reprogramming alters amastigote replication but does not significantly reprogram amastigote metabolism. (A) Assessment of HFF OCR and ECAR. Cells were assayed in the presence of multiple carbon substrates following 48 hours of adaptation in HG or Gal medium. The OCR:ECAR ratio is significantly increased in cells grown in Gal compared to HG. Raw OCR and ECAR measurements shown in (Appendix 2, Figure A.2A,B). Mean ± SD from one of 3 representative independent experiments. (B) Amastigote proliferation was assessed by CFSE dilution using flow cytometry 48 hpi. Plots show model imposed on data. Bar graph indicates the calculated average number of divisions amastigotes underwent. Student’s t-test was applied. * p < 0.05. Data from one of 3 representative independent experiments. (C) The OCR of amastigotes isolated at 48 hpi from cells grown in HG or Gal medium. Oligomycin (O), FCCP (F), rotenone and Antimycin A (R), and 2-deoxyglucose (2DG) were injected as indicated. Basal respiration (D) and ETC coupling efficiency (E) of isolated intracellular amastigotes. Student’s t-test was applied. Mean ± SD from one of 3 independent experiments. (F) Isolated intracellular amastigote sensitivity to
Antimycin A based on growth condition as analyzed by ATP content. Mean ± SD from one of 2 independent experiments. (G) Isolated intracellular amastigote response to 10 mM glucose and 2 mM glutamine assessed by Seahorse. Two-way RM ANOVA with Sidak’s multiple comparisons test applied. * p < 0.05. Mean ± SD of 4 independent experiments.
Antimycin A (Figure 3.3F). Analysis of amastigote substrate responses revealed a small, but statistically significant decrease in the ability of amastigotes isolated from HFF in Gal medium to respond to glucose, but no change in amastigote response to glutamine (Figure 3.3G). Similar results were seen in amastigotes isolated from LLcMK2 cells (Appendix 2, Figure A2.4).

3.4 Discussion

Mechanisms by which *T. cruzi* amastigotes survive and replicate within divergent host cell types are not well understood, despite the importance of amastigotes in driving pathogenic host responses [3,41]. As an obligate intracellular parasite, *T. cruzi* amastigotes must scavenge nutrients from the host cell when replicating [42], but information about their metabolism and nutrient requirements remains incomplete. Mammalian cells have been shown to reprogram their metabolism based on access to nutrients by altering expression of transporters and mitochondrial electron transport chain complexes [20,21,43,44]. Understanding whether *T. cruzi* responds to differing host metabolic states by reprogramming its metabolic capabilities is important when considering strategies to inhibit infection that target parasite metabolism.

To model different host metabolic states without host cell differences confounding results, we manipulated media composition, which had a small but measurable effect on amastigote replication, but no significant effect on the amastigote transcriptome. In contrast to mammalian cells, which increase glucose transport abilities during glucose deprivation [43,45], amastigotes derived from cells grown in Gal medium had slightly reduced responses to glucose, potentially due to hysteretic regulation of hexokinase in *T. cruzi* [46]. The decrease in glucose utilization may be linked to the lower amastigote replication rate in Gal conditions. However, amastigotes showed no other significant alterations in metabolic profile using the *T. cruzi*-optimized mitochondrial stress test, while Gal adaptation in mammalian cells is known to increase mitochondrial ETC activity and maximal respiratory capacity [21,44,47]. Interestingly, our transcriptome data indicates that multiple metabolic pathways are upregulated in replicating
amastigotes relative to extracellular trypomastigotes (Appendix 1, Figure A1.2), and functional follow-up showed that amastigotes have a greater respiratory capacity than extracellular trypomastigotes (Appendix 1, Figure A1.3) and insect stage epimastigotes (shown here). Together the data suggest that successful amastigote growth in different microenvironments arises from increased total metabolic capabilities and flexibility during the intracellular stage rather than programmed responses to differing host metabolic states, although additional studies of varying amastigote growth conditions are warranted. In vivo, metabolism of tissues like the liver and skeletal muscle varies over the course of the day based on feeding and fasting [18,19]. Employing a mechanism that allows for use of a range of nutrients by upregulating multiple pathways and dynamically altering flux may maximize efficiency for this intracellular parasite. Alternatively, amastigotes may actively modulate host metabolism to achieve a desired state.

In summary, we developed a method to analyze T. cruzi bioenergetics and determined that amastigotes do not significantly reprogram their metabolic capabilities to suit the environments encountered here, but increase their metabolic capacity during the intracellular stage. Active regulation of enzymatic activity may then provide the fine-tuning needed to respond to shifting substrate concentrations and dynamic host metabolic networks, enhancing the ability of T. cruzi to replicate in diverse host cells.

3.5 Methods

Mammalian and parasite cell culture

Human foreskin fibroblasts (NHDF-Neo; Lonza) and monkey kidney epithelial cells (LLcMK2; ATCC) were cultured at 5% CO₂ and 37°C in Dulbecco’s Modified Eagle Medium (DMEM; Hyclone) supplemented with 1 mM pyruvate, 25 mM glucose, 2 mM glutamine, 100 U/mL penicillin, 10 µg/mL streptomycin, 10 mM HEPES, and 10% FBS (D10).
The Tulahuén strain of *T. cruzi* was maintained by weekly passage in LLcMK2 cells in D2 (DMEM supplemented with 1 mM pyruvate, 25 mM glucose, 2 mM glutamine, 100 U/mL penicillin, 10 µg/mL streptomycin, 10 mM HEPES, and 2% FBS) as described [22]. Pure trypomastigotes were collected for infection by pelleting supernatant from infected LLcMK2 cells at 2000 g for 10 minutes and allowing trypomastigotes to swim up from the pellet at 37°C, 5% CO₂.

Mammalian cells were incubated with fresh trypomastigotes in D2 one day after plating. Following infection, monolayers were washed 2-3 times in PBS to remove extracellular trypomastigotes and incubated in D2 or the indicated medium.

**Bioenergetics analysis**

*Mammalian cell Seahorse assay:* NHDF were plated at 1.5 x 10⁴ per well on a 0.01% gelatin coated XF⁶²⁴ microplate. The medium was changed the next day to high glucose medium (HG: DMEM; Corning, supplemented with 1 mM pyruvate, 25 mM glucose, 2 mM glutamine, 100 U/mL penicillin, 10 µg/mL streptomycin, 5 mM HEPES, and 2% FBS) or galactose medium (Gal: DMEM; Corning, supplemented with 1 mM pyruvate, 10 mM galactose, 2 mM glutamine, 100 U/mL penicillin, 10 µg/mL streptomycin, 5 mM HEPES, and 2% FBS). The assay was run 48 hours later. All media was changed to XBMS and plates were incubated at 37°C without CO₂ for 1 hour. 1 µM rotenone and 1 µM Antimycin A, and 100 mM 2-DG were injected in. Measurements were done with 3 minutes of mixing, 2 minutes of waiting, and 3 minutes of measuring. Basal OCR was calculated as described above. Basal ECAR was calculated as the difference between the ECAR after 2-DG injection and that at baseline. To normalize results to protein, monolayers were incubated in 0.1 M sodium hydroxide for 10 minutes at room temperature, and 10 µL of lysate was used for comparison to a BSA standard curve using the Peirce 660nm Protein Assay (Thermo Fisher Scientific) per manufacturer’s protocol.
LLcMK2 cells were plated at 4 x 10^4 per well in HG or Gal medium after 3 days adaptation. The assay was run the following day in XBMS as above, with injections of 1 µM oligomycin, 2 µM FCCP, and 1µM rotenone and 1 µM Antimycin A.

*T. cruzi amastigote Seahorse assay:* Mammalian cells were adapted in HG or Gal medium for 3 days. HFF were seeded at 1.5 x 10^6 in T75 flasks, LLcMK2 were seeded at 2 x 10^6, and monolayers were infected with a multiplicity of infection (MOI) of 10 for 24 hours in respective medium. At 48 hpi amastigotes were isolated as described above (Shah-Simpson et al. 2016) in XBMS and the optimized mitochondrial stress test was applied [48]. For substrate responses, amastigotes were isolated in Krebs-Henseleit Buffer (KHB; 111 mM sodium chloride, 4.7 mM potassium chloride, 1.25 mM calcium chloride, 2 mM magnesium sulfate, and 1.2 mM sodium phosphate dibasic) and 10 mM glucose or 2 mM glutamine was injected, followed by compounds for the optimized mitochondrial stress test. Substrate response was calculated as the difference between max OCR after substrate injection and rotenone and antimycin A injection as a percentage of basal respiration. Results were normalized to parasite DNA as described [48].

**ATP assay:** Amastigotes were isolated in KHB supplemented with 2 mM glutamine from HFF infections as described above. 2 x 10^5 amastigotes were treated with the indicated amounts of Antimycin A for 24 hours at 37°C, 5% CO₂. ATP content was assessed by ATPlite (PerkinElmer) per manufacturer’s protocol using a Varioskan plate reader (Thermo Fisher Scientific).

**ROS analysis:** HFF were seeded at 5 x 10^3 in black 96 well plates and medium was changed to HG or Gal the next day. Cells were stained with 20 µM carboxy-H2DCFDA (Molecular Probes) in PBS at 37°C, 5% CO₂ for 30 minutes. Cells were washed with PBS, then incubated in phenol-free medium (HG or Gal) ± 1 mM N-acetyl cysteine (Sigma-Aldrich), the antioxidant, for 30 minutes. 25 µM Tert-butyl hydroperoxide solution (TBHP; Sigma-Aldrich) was added as a positive control to increase reactive oxygen species. Plate was read using a Varioskan plate
reader (Thermo Fisher Scientific) per manufacturer’s protocol at 48 hours after the initial medium switch.

**Amastigote proliferation**

HFF were preadapted for 3 days in HG or Gal medium, plated at 1.5 x 10^5 per well in 6 well plates in respective medium, and infected the next day with MOI 15 for 2 hours using CFSE-stained trypomastigotes. Briefly, 5 x 10^5 trypomastigotes/mL were incubated in 1 µM CFSE (Thermo Fisher Scientific) at 37°C, 5% CO₂ for 15 minutes. Excess label was quenched by addition of D2, and parasites were pelleted by centrifugation at 2100 g for 10 minutes. Stained trypomastigotes were resuspended in HG or Gal and incubated for 30 minutes before infection. At 18 and 48 hpi, amastigotes were collected. Briefly, cells were trypsinized, trypsin was quenched by addition of D10, and cells were pelleted at 300 g for 5 minutes. Cells were washed once in PBS and centrifuged again. Amastigotes were lysed out by passage through a 28.5G needle (BD Biosciences) and fixed in 1% paraformaldehyde (PFA) on ice for 20 minutes. The supernatant was centrifuged at 300 g for 5 minutes to remove large debris, then centrifuged again at 4000 g for 10 minutes to pellet amastigotes. Amastigotes were resuspended in PBS with 0.01% Triton-X-100 and 0.01 µg/mL DAPI for flow cytometry. Samples were run on a MACSQuant VYB (Miltenyi Biotec). Amastigotes were discriminated from debris based on size and DAPI using FlowJo 7.6. Proliferation was modeled by setting the 18 hpi amastigote CFSE intensity as peak 0 for all samples. For assays using LLcMK2, cells were seeded at 3 x 10^5 per well in 6 well plates and infected with MOI 10.

**RNA-Seq analysis**

HFF were preadapted for 3 days in HG or Gal medium, plated at 5 x 10^5 per 10 cm dish in respective medium, and mock-infected or *T. cruzi*-infected the next day with MOI 20 for 2 hours. RNA was collected at 48 hpi as described by Li et al. (2016) for library construction and
sequencing. Reads were processed and aligned, and data was normalized and assessed for differential gene expression as described [22].

Acknowledgements

We thank members of the Burleigh lab and Rebeca Manning-Cela for helpful discussion. S.S.S. was supported by the American Heart Association [15PRE22210008] and C.F.A.P. by Ciência sem fronteiras (CAPES).

3.6 References


Chapter 4

Modulation of host central carbon metabolism and *in situ* glucose uptake by intracellular *Trypanosoma cruzi* amastigotes

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**Author contributions:** SSS and BAB designed the experiments. SSS performed all Seahorse experiments, glucose uptake assays, lactate assays, and RT-qPCR experiments. GL generated transgenic HFF and *T. cruzi*, contributed all Western blots and their quantification, and prepared samples for flow cytometry in Appendix 3, Figure A3.3C. PCD performed flow cytometry for Figure 3B,D. SSS performed all other flow cytometry and analyzed the data. SSS and BAB wrote the manuscript, GL and PCD provided editorial input.
4.1 Abstract

Obligate intracellular pathogens satisfy their nutrient requirements by coupling to host metabolic processes, often modulating these pathways to facilitate access to key metabolites. Such metabolic dependencies represent potential targets for pathogen control, but remain largely uncharacterized for *Trypanosoma cruzi*, the causative agent of Chagas disease. Previously, host central carbon and energy metabolism were implicated as processes that are perturbed during *T. cruzi* infection, potentially supporting parasite replication. Here, we performed studies to elucidate the interplay between infection with intracellular *T. cruzi* amastigotes and host cellular energy metabolism. We demonstrate that *T. cruzi* infection mimics some aspects of glucose starvation in host cells, characterized by upregulation of hexose transporters, increased glucose uptake into infected cells, and increased mitochondrial respiration. Unexpectedly, the host cell respiratory capacity did not impact parasite growth *in vitro* but restriction of extracellular glucose impaired intracellular amastigote proliferation and sensitized the parasite to further growth inhibition by 2-deoxyglucose. These observations led us to consider whether intracellular *T. cruzi* amastigotes utilize glucose directly as a substrate to drive metabolism. Consistent with this prediction, *T. cruzi* amastigotes were shown to take up glucose with kinetics similar to those of extracellular trypomastigotes and subsequently metabolize it as demonstrated by $^{13}$C-glucose labeling and substrate utilization assays. Metabolic labeling of *T. cruzi*-infected cells further demonstrated the ability of intracellular parasites to access host hexose pools *in situ*. These findings are consistent with a model in which *T. cruzi* infection drives increased glucose uptake into host cells that is exploited by the intracellular parasites to fuel their replication in the host cytosol. Our findings challenge current views regarding available carbon sources for intracellular *T. cruzi* amastigotes and underscore the metabolic flexibility of this pathogen, a feature predicted to underlie successful colonization of tissues with distinct metabolic profiles in the mammalian host.
4.2 Introduction

Chagas disease is a vector-borne parasitic disease caused by the kinetoplastid protozoan parasite *Trypanosoma cruzi*. Acute *T. cruzi* infection is most often asymptomatic or characterized by flu-like symptoms, but can cause severe and fatal myocarditis in the first weeks following infection [1]. More typically, parasites establish a chronic infection that is controlled, but not eliminated, by host immune mechanisms [2]. A subset of these chronically infected individuals develop progressive disease characterized by serious cardiac and gastrointestinal disturbances [3], for which treatment options are limited [4]. *T. cruzi* exhibits a broad mammalian host range and colonizes multiple tissues and cell types within an infected host with a tropism in the chronic stage for highly metabolic tissues such as cardiac muscle, gastrointestinal smooth muscle, and adipose tissue [2,5–8]. Fundamental knowledge of the molecular mechanisms that govern successful intracellular colonization and replication by *T. cruzi* is currently lacking, yet functional insights into these processes are potentially exploitable in new therapeutic approaches.

*T. cruzi* is an obligate intracellular parasite that resides in the host cell cytosol and relies on cytosolic nutrient pools to fuel its replication. Thus, the ability of amastigotes to replicate in a variety of tissue types in mammalian hosts suggests a high degree of metabolic flexibility and the potential for intracellular amastigotes to modulate host metabolic pathways for their own gain. Parallel parasite and host transcriptomic profiling studies of *T. cruzi* infection revealed signatures of global metabolic adaptation in the parasite [9] and increased expression of transporters for metabolites, including glucose, in infected host cells [9,10]. Evidence of metabolic perturbation has also been documented at the tissue level in animal models of acute and chronic Chagas disease, where changes in host glycolytic and mitochondrial metabolism have been cited in skeletal muscle [11] and the heart [12–16]. Metabolomic profiling of acute cardiac infection by *T. cruzi* revealed increased glycolytic activity and decreased TCA cycle activity in the heart [17], despite dysregulation of glucose homeostasis resulting in decreased
adiponectin levels and hypoglycemia [8]. While the impact of host metabolic changes on infection outcomes is not well understood for T. cruzi, the regulation of fuel utilization balance and nucleotide and mitochondrial metabolism were recently identified in a genome scale functional screen as candidate host pathways linked to supporting intracellular T. cruzi amastigote growth [18]. Combined, the emerging data suggest a model in which changes in host metabolism induced at the cellular level by T. cruzi may fuel intracellular parasite growth and impact metabolic changes at the cellular, tissue, and organ levels.

To understand if changes in host transcriptomic signatures during infection result in functional consequences [9], we examined the impact of T. cruzi infection on bioenergetics in cultured human fibroblasts. Here, we show that T. cruzi infection is associated with increased glucose uptake, mitochondrial respiration, and mitochondrial content. Contrary to the view that T. cruzi amastigotes have a limited capacity for glucose uptake [19,20], we observed that isolated amastigotes transport and metabolize exogenous glucose, which supports optimal parasite replication. We hypothesized that amastigotes may be utilizing glucose directly from the cytosol and inadvertently driving an increase in host mitochondrial respiration, which we find is superfluous to amastigote proliferation. Indeed, metabolic labeling studies revealed that intracellular T. cruzi amastigotes can access glucose in situ from their mammalian host cells, demonstrating the previously unrecognized potential for glucose to serve as a direct carbon source for intracellular amastigotes.

4.3 Results

T. cruzi infection modulates host glucose and mitochondrial metabolism

Host central carbon and energy metabolism are modulated as part of the global response to T. cruzi infection [12–17], but the functional consequences for the intracellular parasite life cycle are unknown. Using a human fibroblast model for which host transcriptomic data are available [9,10], we sought to confirm predictions of altered host glucose metabolism in
response to *T. cruzi* infection. Here, we demonstrate that host glucose transporter expression (Figure 4.1A and Appendix 3 Figure A3.1A-B) and hexose uptake (Figure 4.1B) are significantly increased in *T. cruzi*-infected cells. The increase in [³H]-2-DG uptake was proportional to the level of infection (Figure 4.1B; Appendix 3 Figure A3.1C). Furthermore, specific inhibition of mammalian glucose transporters using cytochalasin B [21,22] blocked the increase (Figure 4.1C), confirming the role of host glucose transporters in this host response to parasite infection. Despite increased glucose uptake into parasite-infected monolayers, we did not observe a corresponding increase in lactate secretion (Figure 4.1D), which often correlates with increased glycolytic flux in mammalian cells [23]. However, lactate production may not change if pyruvate generated by glycolysis is consumed to drive the TCA cycle and mitochondrial respiration or if glucose enters the pentose phosphate pathway or is scavenged by the parasite.

Mitochondrial respiration was monitored in control and *T. cruzi*-infected HFF monolayers by measuring the oxygen consumption rate (OCR) in real-time using a Seahorse XF²4 extracellular flux analyzer. The OCR was consistently 2-4 fold higher in *T. cruzi*-infected fibroblast monolayers as compared to uninfected control cells (e.g. Figure 4.1E). We anticipated that changes in the OCR may reflect a combination of host and parasite respiration, and therefore sought to discriminate between these individual contributions to the total OCR. To this end, we exploited the endochin-like quinolone 300 (ELQ300), a small molecule inhibitor of cytochrome *bc₁* in protozoan parasites [24,25], to specifically ablate the parasite OCR. 1 µM ELQ300 proved an effective inhibitor of *T. cruzi* respiration by reducing the basal OCR of isolated amastigotes by >90% (Appendix 3, Figure A3.2A). Selectivity for *T. cruzi* over HFF mitochondrial respiration was also demonstrated (Appendix 3, Figure A3.2B), in agreement with previous reports [24,25]. To confirm that ELQ300 effectively blocks amastigote respiration in *situ*, we exploited a mitochondrial complex III-deficient fibroblast cell line (CIII mutant) [26,27] with a significantly lower mitochondrial respiratory rate than control cell lines (Appendix 3, Figure A3.2C-E). Treatment of *T. cruzi*-infected CIII mutant fibroblasts with 1 µM ELQ300
Figure 4.1. *T. cruzi* infection modulates host glucose and mitochondrial metabolism. (A) Relative SLC2A12 mRNA expression in uninfected and *T. cruzi*-infected HFF monolayers at indicated hours post infection (hpi). Mean ± standard deviation (SD) from 3 independent experiments. Two-way RM ANOVA with Sidak's multiple comparisons test was applied ** p < 0.01. (B) Uptake of $[^3]H$-2-deoxyglucose ($[^3]H$-2-DG) into uninfected or *T. cruzi*-infected HFF monolayers (48 hpi) in which infection was established with varying multiplicity of infection. Mean ± SD for 3 technical replicates from a representative experiment. One-way ANOVA with post-test for linear trend was applied. p < 0.0001. (C) Cytochalasin B (10 µM) blocks glucose uptake into *T. cruzi*-infected monolayers. Mean ± SD shown for 3 technical replicates from a representative experiment. Two-way ANOVA with Tukey's multiple comparisons test was applied. ** p < 0.01, **** p < 0.0001. (D) Extracellular lactate measured in culture supernatants of uninfected and *T. cruzi*-infected HFF monolayers (48 hpi). Mean ± SD shown for 3 technical replicates from a representative experiment. Student's t-test was applied. (E) Oxygen consumption rate (OCR) in uninfected and *T. cruzi*-infected HFF monolayers (48hpi) before and after injection of oligomycin (O), FCCP (F), and rotenone and antimycin A (R/A). Mean ± SD shown for 3 technical replicates from a representative experiment. (F) *T. cruzi*-infected HFF monolayers were treated with 1 µM ELQ300 to selectively remove amastigote respiration (amastigote contribution) from the total OCR signal (+*T. cruzi*, ±ELQ300). Increased host respiration (host contribution) during *T. cruzi* infection (+*T. cruzi*, +ELQ300). Mean ± SD shown for 3 technical replicates from a representative experiment. Two-way ANOVA with Tukey's multiple comparisons test was applied. * p < 0.05, *** p < 0.001, **** p < 0.0001.
almost completely abrogated the OCR increase in infected monolayers as compared to uninfected monolayers (Appendix 3, Figure A3.2F). Experiments performed in parallel with respiration-competent, control human fibroblasts show that following inhibition of amastigote respiration by treatment with 1 µM ELQ300, a substantial residual OCR signal remains that is attributable to increased host cell mitochondrial respiration associated with T. cruzi infection (Appendix 3, Figure A3.2G,H). Similar results were obtained with T. cruzi-infected HFF (Figure 4.1F). Simultaneous extracellular acidification rate (ECAR) measurements, which correlate with glycolytic rate, reveal an increase with T. cruzi infection that is also abrogated by ELQ300 (Appendix 3, Figure A3.1D), suggesting that the majority of the ECAR increase is not from lactate production, but media acidification linked to amastigote respiration. Thus, the use of ELQ300 to selectively target T. cruzi respiration in the context of intracellular infection revealed the robust metabolic activity of T. cruzi amastigotes in situ as well as an increased respiratory capacity of parasite-infected host cell monolayers.

Increased reliance on mitochondrial respiration for ATP generation occurs in mammalian cells in response to a number of environmental changes including glucose deprivation [28–32]. As flux through the glycolytic pathway slows, cells compensate by increasing glucose transporter expression at the plasma membrane [28,33], but increasingly rely on other fuels, such as glutamine and fatty acids, to drive ATP generation via mitochondrial respiration. These events are often accompanied by increased mitochondrial volume in cells [28,29,34]. Because the global host cell response to T. cruzi infection mirrors aspects of a glucose stress response (Figure 4.1B,F), we explored the possibility that parasite infection might also drive increased mitochondrial abundance in infected host cells. To this end, we generated a fibroblast cell line stably expressing mCherry in the mitochondrial matrix (mito-mCherry) as a tool to monitor quantitative changes in mitochondrial volume by flow cytometry, as demonstrated with the use of valproic acid (Appendix 3, Figure A3.3A), a known inducer of mitochondrial biogenesis [35]. Consistent with the literature [28,29,34], glucose deprivation triggered an increase in
mitochondrial content across the fibroblast population, with a 30% increase in mCherry geometric mean fluorescence intensity (GMFI) (Figure 4.2A). To determine the impact of *T. cruzi* infection on host cell mitochondrial abundance, HFF mito-mCherry cells were infected with GFP-expressing *T. cruzi* parasites to allow discrimination of parasite-infected cells in the host cell population (Appendix 3, Figure A3.3B). We found that the intensity of the mito-mCherry signal increased (40-75%) specifically in host cells harboring intracellular parasites (Figure 4.2B-D; GFP+) with no observed increase in cells lacking intracellular parasites derived from the same infected cell culture compared to non-infected controls (Figure 4.2B-D, GFP-). Western blot analysis of endogenous host cell mitochondrial marker proteins, ATP5B and TOMM20 (Figure 4.2E) and analysis of ATP5B by flow cytometry (Appendix 3, Figure A3.3C) support the conclusion that host cell mitochondrial content increases in *T. cruzi*-infected cells. These observations are consistent with the measured increase in mitochondrial respiratory activity and potentially result from the alterations in glucose uptake driven by infection.

**Intracellular *T. cruzi* replication is supported by exogenous glucose but does not require host mitochondrial respiration**

To assess whether the metabolic changes observed in *T. cruzi*-infected host cells impact parasite replication, we examined the replicative capacity of intracellular amastigotes under conditions of metabolic perturbation. To our surprise, *T. cruzi* amastigote proliferation was not perceptibly altered in the CIII mutant cells with impaired mitochondrial respiration (Figure 4.3A). In contrast, glucose withdrawal from the infected culture medium was associated with a significant reduction in intracellular amastigote proliferation (Figure 4.3B). Perturbation of glucose homeostasis with 2-deoxyglucose (2-DG), a non-metabolizable glucose analogue that competes for plasma membrane hexose transporters and inhibits hexokinase activity [36], resulted in a dose-dependent decrease in either intracellular *T. cruzi* amastigote numbers or replication (Figure 4.3C). Limiting extracellular glucose sensitized amastigotes to 2-DG,
Figure 4.2. *T. cruzi* infection increases mitochondrial content specifically in parasitized cells. (A) Flow cytometric detection of mitochondrial-mCherry levels in HFF grown in the absence (0 mM) or presence (25 mM) of glucose or treated with 10 mM valproic acid for 48 h to trigger mitochondrial biogenesis. (B) Host mitochondrial-mCherry expression in parasitized HFF (+*T. cruzi*, GFP+) and uninfected fibroblasts (+*T. cruzi*, GFP-) from the same culture. Geometric mean fluorescence intensity (GMFI) of mitochondrial mCherry signal for each condition in HFF (C) and C2C12 (D). Mean ± SD for 2 biological replicates from a representative experiment. Two-way ANOVA with Sidak’s multiple comparisons test was applied. * p < 0.05. ** p < 0.01. (E) Western blot analysis of total lysates prepared from uninfected or *T. cruzi*-infected HFF cultures at 48 hpi indicate increased levels of mitochondrial markers mCherry, ATP5B and TOMM20 relative to vimentin (VIM). Blots are representative of three independent experiments.
presumably by increasing its effective concentration within the host cell. In the absence of extracellular glucose, 2-DG treatment (2mM; Figure 4.3C, dotted line) resulted in a near complete block of intracellular amastigote proliferation, suggesting a slowing of amastigote growth as opposed to lethality (Figure 4.3D). Under these conditions, we expect that 2-DG targets glucose metabolism in the intracellular amastigote (Figure 4.4A,B) as well as in the host cell. Inhibition of hexokinase or glycosome function in *T. cruzi* amastigotes drastically reduces intracellular amastigote numbers [37–39], suggesting that glucose flux through parasite hexokinase is required for *T. cruzi* amastigote replication. However, the possibility that *T. cruzi* amastigotes require intermediates generated by host glycolysis that are limited by the action of 2-DG cannot be excluded.

**Transport and catabolism of extracellular glucose by *T. cruzi* amastigotes**

Given mixed literature reports, including one that *T. cruzi* amastigotes have a limited capacity for glucose uptake [20,40], we sought to determine whether exogenous glucose can fuel energy metabolism in isolated *T. cruzi* amastigotes. By performing extracellular flux measurements employing glutamine as a positive control [40], we show that isolated *T. cruzi* amastigotes responded to glucose with significant increases in OCR (Figure 4.4A) and ECAR (Figure 4.4B) that were quenched by the injection of 2-DG. Metabolite profiling performed following incubation of isolated *T. cruzi* amastigotes with [13C]-U-glucose revealed incorporation of 13C into glycolytic and TCA cycle intermediates, and into several biosynthetic pathways (Table 4.1) confirming the capacity for glucose uptake and metabolism by this parasite life stage.

To determine whether *T. cruzi* amastigotes utilize a facilitated transport mechanism for hexose uptake, the kinetics of [3H]-2-DG uptake by freshly isolated intracellular amastigotes were compared to those of extracellular trypomastigotes, a life cycle stage of *T. cruzi* for which hexose transporter expression is abundant [9,20]. The initial rates of hexose transport (V₀) measured for isolated amastigotes and trypomastigotes were found to be comparable, with a
Figure 4.3. Intracellular *T. cruzi* replication is sensitive to exogenous glucose but not host mitochondrial electron transport chain activity. (A) Proliferation of *T. cruzi* amastigotes in human dermal fibroblasts with ETC complex III deficiency (CIII mutant) or two independent control fibroblast lines (Control 1 and 2) derived from flow cytometric data (as detailed in Methods). Data are normalized to represent the percentage of initial amastigotes (18 hpi) that divided the indicated number of times by 48 hpi. (A,B,D) Graphs show one of at least two independent experiments. Dotted lines represent average number of amastigote divisions achieved by 48 hpi in each condition. (B) Proliferation of *T. cruzi* amastigotes in HFF cultured in medium with varying glucose concentrations. (C) Dose-dependent inhibition of intracellular *T. cruzi* (amastigote) or host cell (HFF) growth by 2-deoxyglucose (2-DG) following cell culture in medium with varying glucose concentrations. Relative number assessed by CellTiter-Fluor fluorescence (HFF) and Beta-glo luminescence (amastigote) at 66 hpi. Dashed line denotes 2 mM 2-DG (concentration used in D). Mean ± SD of 4 independent experiments shown with nonlinear fit using log(inhibitor) vs. response with variable slope. (D) Amastigote proliferation in fibroblasts is arrested in the presence of 2mM 2-DG under conditions of glucose depletion.
similar $K_M$ ($87.0 \pm 21.7$ vs. $81.2 \pm 3.7 \mu M$) and $V_{\text{max}}$ ($857.0 \pm 76$ vs. $666.5 \pm 36.1$ pmol 2-DG/mg protein/min) (Figure 4.4C). Next, we sought to determine whether the capacity for glucose uptake and metabolism by $T. cruzi$ amastigotes is relevant in the context of an intracellular infection. $T. cruzi$-infected HFF monolayers (48 hpi) were incubated with $[^3H]$-2-DG prior to isolation of intracellular amastigotes from mammalian host cells. As shown, we detected $[^3H]$-counts from isolated amastigotes which were significantly diminished when the infected monolayer was treated with cytochalasin B to block host glucose transporters (Figure 4.4D). To establish that $[^3H]$-2-DG was not merely surface bound to amastigotes after isolation from infected cells, we permeabilized isolated amastigotes with alamethicin (Appendix 3, Figure A3.4) and detected a $>70\%$ drop in signal, demonstrating internal incorporation of label into amastigotes in situ (Figure 4.4E). Next we evaluated the ability of individual substrates to maintain ATP levels in isolated amastigotes over a 24-hour period. Glucose was able to sustain amastigote ATP pools at a similar level to multiple carbon sources (Figure 4.4F), highlighting the potential for glucose to serve as a major carbon source for intracellular amastigotes.

4.4 Discussion

As an obligate intracellular parasite that replicates in the cytosol of mammalian host cells, $T. cruzi$ amastigotes must draw from cytosolic nutrients and metabolites to satisfy their metabolic requirements. The host carbons utilized by intracellular $T. cruzi$ amastigotes in situ have not been directly identified, but on the basis of expression data [9,41], functional transport studies [20] and metabolic assays conducted with isolated amastigotes [40,42], it has been proposed that fatty acids and amino acids are the most likely host carbons used to fuel metabolism in these cytosolic parasites. Hexose sugars have been largely discounted as a potential carbon source for intracellular $T. cruzi$ amastigotes [19,20,41]. This hypothesis is partly due to the perception that intracellular glucose is a negligible commodity in the mammalian cell cytoplasm [19]. However, the use of fluorescent glucose sensors that reveal a significant and
Figure 4.4. Acquisition and metabolism of glucose by intracellular *T. cruzi* amastigotes. Isolated *T. cruzi* amastigotes utilize exogenous substrates as determined by increased (A) oxygen consumption rate (OCR) and (B) extracellular acidification rate (ECAR). After establishing baseline rates, the substrates (subs) glucose, glutamine (5 mM) or buffer were injected, followed by 2-DG (100mM) to rapidly inhibit glycolysis, and rotenone and antimycin A (R/A) to shut down mitochondrial respiration. Mean ± SD of ≥3 replicate wells shown. Data representative of 2 independent experiments. (C) Initial rate ($V_0$) of $[^3H]$-2-DG uptake by isolated
Figure 4.4 (Continued) T. cruzi amastigotes or trypomastigotes plotted for a range of substrate concentrations. Mean ± SD of two independent experiments with technical duplicates shown for each lifecycle stage. Inset shows Lineweaver-Burk plot. (D) Intracellular T. cruzi amastigotes access exogenous hexose in situ. T. cruzi-infected monolayers were incubated with 10 µCi [³H]-2-DG in the absence or presence of cytochalasin B (15 µM) for 20 minutes prior to isolation of intracellular amastigotes for scintillation counts. Graph shows mean ± SD of two independent experiments. Paired t-test was applied. ** p < 0.01. (E) [³H]-2-DG is internalized by intracellular amastigotes. Following isolation from monolayers incubated with [³H]-2-DG, treatment of amastigotes with 0.05 mg/mL alamethicin released internalized, non-bound substrate. (F) ATP levels measured in intracellular-derived amastigotes 24 hours after incubation in with the indicated carbon substrate relative to initial ATP levels of freshly isolated parasites. Mean ± SD of technical triplicates are presented from a representative experiments. One-way ANOVA with Dunnett's multiple comparison test was applied. *** p < 0.001, **** p < 0.0001.
Table 4.1. Amastigotes incorporate exogenous glucose into multiple metabolic pathways.

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>% label incorporation ± SD</th>
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<tbody>
<tr>
<td><strong>Glycolysis</strong></td>
<td></td>
</tr>
<tr>
<td>glucose-6-phosphate</td>
<td>40.51 ± 10.29</td>
</tr>
<tr>
<td>fructose-6-phosphate</td>
<td>97.49 ± 0.40</td>
</tr>
<tr>
<td>fructose-1,6-bisphosphate</td>
<td>95.15 ± 0.84</td>
</tr>
<tr>
<td>glyceraldehyde-3-</td>
<td>85.94 ± 1.08</td>
</tr>
<tr>
<td>3-phosphoglycerate</td>
<td>92.90 ± 1.88</td>
</tr>
<tr>
<td>phosphoenolpyruvate</td>
<td>93.23 ± 0.98</td>
</tr>
<tr>
<td><strong>TCA</strong></td>
<td></td>
</tr>
<tr>
<td>acetyl-CoA</td>
<td>78.16 ± 4.85</td>
</tr>
<tr>
<td>citrate</td>
<td>83.52 ± 1.97</td>
</tr>
<tr>
<td>a-ketoglutarate</td>
<td>8.89 ± 2.82</td>
</tr>
<tr>
<td>succinate</td>
<td>74.95 ± 0.91</td>
</tr>
<tr>
<td>fumarate</td>
<td>75.46 ± 0.77</td>
</tr>
<tr>
<td>malate</td>
<td>70.79 ± 0.84</td>
</tr>
<tr>
<td>oxaloacetate</td>
<td>67.36 ± 3.44</td>
</tr>
<tr>
<td><strong>PPP</strong></td>
<td></td>
</tr>
<tr>
<td>ribose-5-phosphate</td>
<td>63.11 ± 4.45</td>
</tr>
<tr>
<td>sedoheptulose-7-</td>
<td>89.29 ± 2.66</td>
</tr>
<tr>
<td>erythrose-4-phosphate</td>
<td>94.43 ± 1.55</td>
</tr>
<tr>
<td><strong>Amino Acids</strong></td>
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<tr>
<td>aspartate</td>
<td>63.88 ± 1.40</td>
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<tr>
<td>alanine</td>
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<tr>
<td>asparagine</td>
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<tr>
<td>D-glucosamine-6-</td>
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<tr>
<td>glucosamine</td>
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<tr>
<td>glutamate</td>
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<tr>
<td>glutamine</td>
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<tr>
<td>mevalonate</td>
<td>79.47 ± 11.47</td>
</tr>
<tr>
<td>N-acetyl-glutamate</td>
<td>33.92 ± 18.40</td>
</tr>
<tr>
<td>N-acetyl-glutamine</td>
<td>49.95 ± 22.71</td>
</tr>
</tbody>
</table>

Isolated intracellular amastigotes were incubated with $[^{13}\text{C}]$-U-glucose (6 mM) for 3 hours and the relative label incorporation into a specific metabolite pool was calculated. Mean ± SD of technical triplicates shown for a subset of metabolites representing glycolytic, tricarboxylic acid (TCA), pentose phosphate (PPP) pathways and amino acids.
dynamic cytosolic free glucose pool in multiple human cell lines [43,44] and demonstration that other cytosolic pathogens utilize glucose as a major carbon substrate [45] help to revise perceptions regarding glucose as a potential carbon source for cytosolic pathogens like T. cruzi.

Results of the present study provide the first demonstration that T. cruzi amastigotes access glucose from host intracellular pools, likely via a facilitated transport mechanism similar to that observed in the extracellular trypomastigote stage of the parasite. While this finding contradicts an earlier report showing negligible hexose transporter expression and glucose uptake by isolated intracellular T. cruzi amastigotes [20], the choice of an avirulent parasite strain in that particular study raises the intriguing possibility that flexibility with regard to fuel utilization might impact parasite virulence in vivo. Following uptake into amastigotes, glucose is metabolized through glycolysis and the TCA cycle to drive mitochondrial respiration, consistent with previous reports [40,46,47]. A key element of the current study is the exploitation of ELQ300, an inhibitor of protozoan cytochrome bc1 [24,25], to specifically block T. cruzi respiration in infected host cells. This selective inhibitor allowed us to discriminate host and parasite respiratory activity to reveal the robust metabolic activity of T. cruzi amastigotes. While we anticipate that a number of host-derived carbons, including amino acids and fatty acids [41], fuel mitochondrial respiration in these intracellular parasites, our data indicate that glucose is among the carbons utilized to drive energy metabolism in situ. Moreover, as glucose can sustain ATP levels in isolated T. cruzi amastigotes, we conclude that it can serve as an important fuel source for these intracellular parasites.

In the present study, we identify changes in host cellular metabolism during T. cruzi infection comprising of an increase in glucose uptake, mitochondrial abundance, and mitochondrial respiration that are suggestive of a host compensatory response to increased metabolic demands. Similar general increases in mitochondrial content and respiration and glucose uptake are associated with glucose deprivation [28–30,34,48]. Amastigote scavenging from host cytosolic glucose pools may therefore contribute to the increased glucose demand
seen in host cells, though changes in host metabolism are likely multifactorial. Here, fibroblast mitochondrial respiration failed to impact parasite growth rates, though increased mitochondrial respiration in macrophages is associated with parasite clearance [49]. Conversely, elevated mitochondrial respiration in macrophages during *Leishmania* infection is associated with increased parasite growth [50]. The impact of host metabolism on infection outcomes is therefore somewhat specific to the combination of host and pathogen being investigated. Additionally, changes in cellular metabolism during infection do not necessarily reflect those in whole tissue. *T. cruzi* infection increases cardiac glucose uptake in mice [17], but is associated with dysfunctional mitochondrial respiration of the heart and skeletal muscle [11–17]. More work is needed to understand whether host mitochondrial respiration increases in infected cells *in vivo* despite tissue-wide mitochondrial dysregulation, and if this phenotype is protective for the parasites.

In summary, we determined that *T. cruzi* infection modulates host cell metabolism, increasing mitochondrial respiration and enhancing uptake of glucose, which can be scavenged by the intracellular amastigote from the host cytosol for utilization in energy generating and biosynthetic processes. Evidence that exogenous glucose supports amastigote viability and replication points to glucose as a previously unappreciated carbon source for this intracellular parasite, facilitating colonization and long-term survival in metabolically diverse cell and tissue types.

### 4.5 Materials and methods

**Parasite and mammalian cell culture**

Mammalian cell lines: human foreskin fibroblast (provided by S. Lourido, MIT and NHDF-Neo from Lonza), mouse skeletal muscle myoblast (C2C12; ATCC CRL-1772), African green monkey kidney epithelial (LLcMK2; ATCC CCL-7) were propagated at 37°C and 5% CO₂ in Dulbecco’s Modified Eagle Medium (DMEM; HyClone) supplemented with 1mM pyruvate, 25
mM glucose, 2 mM glutamine, 100 U/ml penicillin, 10 µg/ml streptomycin and 10% fetal bovine serum (FBS) (D-10). Human dermal fibroblasts (Control 1: F-CR2631; Control 2: F-CR2571; CIII mutant: F-MT2614; GMD-MDbank) were propagated as above with 50 µg/mL uridine (Sigma-Aldrich). *Trypanosoma cruzi* Tulahuén strain parasites (ATCC PRA-330) were maintained by weekly passage in LLCMK2 cells in DMEM supplemented with 1 mM pyruvate, 25 mM glucose, 2 mM glutamine, 100 U/ml penicillin, 10 µg/ml streptomycin and 2% FBS (D-2) at 37°C, 5% CO₂ as previously described [9]. Motile extracellular trypomastigotes were collected from infected LLCMK2 supernatants, pelleted at 2,000 g for 10 min and allowed to swim up from the pellet for a minimum of 2 h at 37°C, 5% CO₂ before collection and use for infections. For all experimental *T. cruzi* infections, mammalian cells were grown in D-10 for 24 h to achieve ~80% confluence prior to incubation with freshly isolated trypomastigotes in D-2, followed by multiple washes in PBS (Gibco) to remove remaining extracellular trypomastigotes and further incubation in D-2 to allow intracellular development and growth of *T. cruzi* amastigotes for the indicated time periods. *T. cruzi* epimastigotes were maintained axenically in liver infusion tryptose (LIT; 1% liver infusion broth, 68.4 mM sodium chloride, 5.4 mM potassium chloride, 5.5 mM glucose, 0.5% bacto-tryptose, 56.4 mM sodium phosphate dibasic, 0.002% hemin, 10% FBS, and 100 U/mL penicillin, and 100 µg/mL streptomycin) at 27°C.

**Construction of transgenic mammalian and parasite lines**

Mammalian cell lines expressing mCherry targeted to the mitochondrial matrix were generated by retroviral transduction of HFF and C2C12 with a construct containing the sequence encoding the first 25 amino acids of the mouse Cox8a protein fused to mCherry [51,52]. Briefly, 5 x 10⁵ Phoenix packaging cells were plated in a 100 mm tissue culture dish and transfected the following day with 10 µg of the plasmid pLNCX2 containing the chimeric sequence (kindly provided by C-H Lee, HSPH) using TransIT-LT1 (Mirus Bio) per manufacturer's protocol. Virion-containing medium obtained 2 days post-transfection was passed through a 0.45 µm filter, and
stored at -80°C. Mammalian cells were seeded 1.5 x 10^5 per well in a 6 well plate, and virion-containing medium with 4 µg/mL polybrene was added the following day. Transgenic fibroblasts were selected with 400 µg/mL G418 (Sigma-Aldrich) starting two days post-transduction and confirmed by microscopy and flow cytometry after 2 weeks.

_Trypanosoma cruzi_ Tulahuén strain parasites (from M. Perrin, Tufts University) were transfected with pROCK-GFP-NEO for constitutive expression of GFP from the tubulin locus [53]. Axenically-grown _T. cruzi_ epimastigotes were transfected as described [54] with minor alterations. Briefly, 10 µg of linearized plasmid was transected into 4 x 10^7 epimastigotes in 100 µL of Tb BSF-buffer using the U-33 program of an Amaxa Nucleofector II (Lonza). Cells were subsequently transferred to 5 mL LIT and incubated at 27°C overnight, then cloned in 96-well plates. After 3 weeks, clones were screened by flow cytometry, and GFP expressing parasites were confirmed by microscopy and PCR amplification of a 655 bp GFP sequence (forward: 5′-TTCACTGAGTGTCC-3′; reverse: 5′-AGTTCATCCCATGCCC-3′) and 772 bp Neo′ sequence (forward: 5′-ATGGGATCGGCCAT-3′; reverse: 5′-TCAGAAGAACTCGTCAAG-3′) using Taq DNA polymerase (GenScript) per manufacturer protocol. To generate trypomastigotes, 1 mL of stationary phase epimastigotes was added to a T75 flask of confluent LLCMK₂ cells. The medium was changed to fresh D2 every day, and after 1 week newly emerging trypomastigotes were collected and used to start new mammalian stage cultures. Constitutive GFP expression in the parasite population was confirmed by routine fluorescence microscopy (Nikon TE-300).

**Isolation of intracellular _T. cruzi_ amastigotes**

HFF were plated at 1.5 x 10^6 in T75 flasks and infected with MOI of 10 for 18-24 h. At 48 hpi, monolayers were scraped and amastigotes were released from disrupted host cells by syringe passage (28½G needle; BD) into the indicated ice-cold buffer. Amastigotes were purified from debris by passage through a PD-10 desalting column (GE Healthcare Life Sciences), and fractions containing clean amastigotes were centrifuged at 4000 g for 10 minutes at 4°C to pellet
amastigotes, which were resuspended in warm (37°C) buffer as needed for indicated applications.

Quantitative PCR assays

*RT-PCR*: HFF were plated at 1.5 x 10⁵ per well in 6 well plates and infected with *T. cruzi* at an MOI of 40 for 1 hour. Total RNA was isolated at the indicated times using the RNeasy kit (Qiagen) with gDNA degradation using the RNase-free DNase set (Qiagen). cDNA was synthesized from 500 ng RNA using iScript Reverse Transcription Supermix (Bio-Rad) per manufacturer protocol. For RT-PCR, 20 ng of cDNA was combined with human GAPDH TaqMan probe (VIC-MGB_PL), the indicated FAM-MGB TaqMan probe against SLC2A1 (ENSG00000117394), SLC2A3 (ENSG00000059804), or SLC2A12 (ENSG00000146411), and TaqMan Supermix (Thermo Fisher Scientific) in a 20 µL reaction per manufacturer protocol. The reaction was run at 95°C for 10 minutes, then cycled 45 times at 95°C for 15 seconds then 60°C for 1 minute and analyzed using the default Comparative Ct (ΔΔCt) settings of a StepOnePlus (Applied Biosystems).

*Genomic DNA PCR*: HFF were plated and infected as for mammalian glucose uptake assays (described below). At 48 hpi genomic DNA was isolated using the DNeasy kit (Qiagen) and eluted in water. 1 µL of sample was combined with 10 µL of iTaq Universal SYBR Green Supermix (Bio-Rad) and 5 µM of each human TNF primer (forward: 5'-TAAGATCCCTCGGACCCAGT-3'; reverse: 5'-GCAACAGCCGAAATCTCAC-3') in a 20 µL reaction, run as above, and analyzed using the default Standard Curve (absolute quantitation) settings of a StepOnePlus.

Glucose transport assays

All transport assays were performed using 1,2-³H(N)-2-deoxyglucose, (³H-2-DG; PerkinElmer). *Mammalian cells*: HFF were plated at 1 x 10⁶ per well in 12 well plates and incubated with T.
cruzi trypomastigotes at the indicated MOI or MOI of 40 for 1 hour. At 48 hpi monolayers were washed twice with Krebs-Ringer bicarbonate HEPES buffer (KRBH; 120 mM sodium chloride, 4 mM potassium phosphate monobasic, 1 mM magnesium sulfate, 0.75 mM calcium chloride, 30 mM HEPES, and 10 mM sodium bicarbonate) and incubated in 400 µL fresh KRBH at 37°C for 10 minutes. Transport assays were started by the addition of 0.5 µCi ³H-2-DG and a final concentration of 55.5 µM unlabeled 2-DG and incubated at 37°C for 20 minutes. The reaction was stopped by addition of 35 µM cytochalasin B (MP Biomedicals) and monolayers were washed 5 times to remove residual 2-DG. All cells were lysed in 500 µL of 0.1 M sodium hydroxide at room temperature for 10 minutes, and 400 µL was added to 5 mL of Ecolite(+) (MP Biomedicals) for scintillation counting in a Beckman LS6500 scintillation counter. Background counts were removed by subtracting the measurements from samples where ³H-2-DG and cytochalasin B were added simultaneously before immediate washing.

In situ amastigotes: HFF were seeded at 1.5 x 10⁶ in T75 flasks and infected with MOI of 10 for 18 h. The assay was conducted as above using 10 µCi of ³H-2-DG and 83.3 µM unlabeled 2-DG to start transport. To inhibit mammalian cell glucose transport, cytochalasin B was added with 2-DG to a final concentration of 15 µM. The reaction was stopped as above and monolayers were washed 5 times rapidly to remove exogenous ³H-2-DG. Amastigotes were isolated from host cell monolayers in Krebs-Henseleit Buffer (KHB; 111 mM sodium chloride, 4.7 mM potassium chloride, 1.25 mM calcium chloride, 2 mM magnesium sulfate, and 1.2 mM sodium phosphate dibasic) then lysed for scintillation counts and protein normalization. To verify that the intracellular parasites had taken up ³H-2-DG label from host cell, amastigotes were isolated following radiolabeling of infected monolayers as above, split equally into two tubes and incubated with vehicle alone (1% DMSO) or 0.05 mg/mL alamethicin (VWR) in 500 µL for 15 minutes at room temperature while shaking to permeabilize the parasite plasma membrane. An additional 500 µL of buffer was added, and amastigotes were pelleted at 4000 g for 10 minutes at 4°C and lysed in sodium hydroxide for scintillation counts.
**Isolated parasites:** For parasite transport assays, trypomastigotes were washed twice and assayed in KHB and amastigotes were isolated and assayed in KHB or cytobuffer (10 mM sodium chloride, 140 mM potassium chloride, 2 mM magnesium chloride, 2 µM calcium chloride, and 10 mM HEPES at pH 7.4) meant to mimic intracellular ion concentrations [55]. As determined subsequently, amastigote glucose transport capacity was similar in cytobuffer and KHB. Parasites were aliquoted at 5 x 10^6 in 100 µL and 100 µL of loading solution (0.5 µCi ³H-2-DG in 2x the indicated final 2-DG concentration) was added. After 1 minute, 800 µL of ice-cold buffer supplemented with 25 mM glucose was added and the tube was placed on ice to stop transport. Parasites were washed twice by centrifugation at 4000 g for 10 minutes at 4°C and resuspension in ice-cold buffer with 25 mM glucose, then lysed in sodium hydroxide following centrifugation. 400 µL were used for scintillation counts and 10 µL were used for protein normalization by comparison to an IgG standard curve using the Peirce 660nm Protein Assay Reagent (Thermo Fisher Scientific) per manufacturer protocol. Background counts were removed by subtracting the measurements of samples where transport was prevented by adding ice-cold buffer with 25 mM glucose and ³H-2-DG simultaneously. To calculate the number of moles of 2-DG/counts per minute (cpm), the total concentration of 2-DG in each loading solution was multiplied by the volume and divided by the cpm of 100 µL of each loading solution. V_o was calculated by multiplying that number by the measurement of cpm/mg protein for each sample.

**Lactate determination**

The wells of an XF®24 cell culture microplate (Agilent Technologies) were coated with 0.1% gelatin and incubated at 37°C for 1 hour before gelatin was aspirated and HFF were plated at 1.5 x 10^4 in 250 µL. Cells were infected with MOI of 50 for 1 hour then placed in 250 µL D-2. At 48 hpi media was changed to Seahorse XF base medium without phenol red (DMEM-based medium; Agilent Technologies), supplemented with 2 mM glucose and 10 mM glutamine, by
rinsing cells twice with 1 mL medium, then replacing it with 500 µL medium. After 1 hour of incubation at 37°C, the supernatant was collected and 2 µL of sample was assayed by lactate assay kit I (Biovision) per manufacturer protocol.

**Seahorse bioenergetics analysis**

*Mammalian cells:* HFF were plated and infected as in the lactate assay. Seahorse XF Base Medium was supplemented with 1 mM sodium pyruvate, 2 mM glutamine, and 10 mM glucose (XBMS; Agilent Technologies). Cells were rinsed with XBMS as above, then incubated in 400 µL at 37°C without CO₂ for 45 minutes. ELQ300 (generously provided by M. Riscoe, OHSU), or equivalent vehicle was added directly to cell monolayers to a final concentration of 1 µM in 450 µL and the plate was incubated at 37°C without CO₂ for 15 minutes prior to initiation of assay. Compounds from the Seahorse XF Cell Mito Stress Test (Agilent Technologies) were resuspended in XMBS per manufacturer protocol, then diluted to 10x final concentrations and loaded into the sensor cartridge ports for injection. Final concentrations were 1 µM oligomycin (O), which inhibits ATP synthase, 2.5 µM FCCP (F), which uncouples ETC activity from ATP synthesis, and 1 µM each of rotenone and antimycin A (R/A), which inhibit ETC complex I and III respectively. The Seahorse XF®24 was programed to mix for 3 minutes, wait 2 minutes, then make measurements for 3 minutes. Basal respiration was calculated as the difference between OCR at baseline and that after R/A injection. Spare respiratory capacity (SRC) was calculated as the difference between OCR after FCCP injection and that at baseline. Protein concentrations were calculated where indicated by lysing monolayers in 0.1 M sodium hydroxide and comparing protein abundance to an IgG standard curve by Bradford assay (Bio-Rad).

*Isolated amastigotes:* Amastigotes were purified from host cells as above, then adhered to microplates with Cell-Tak (Corning) as described [42]. For the ELQ300 titration experiment (S2 Fig. A), amastigotes were isolated and assayed in XBMS and pretreated with the indicated
amount of ELQ300. Oligomycin, FCCP, and rotenone and antimycin A were injected sequentially, and measurements were taken as previously described [42]. For substrate response assays (Fig. 3A-B), amastigotes were isolated and assayed in KHB. During the assay, 2.5 mM glucose or glutamine, 100 mM 2-DG, and 1 µM each of rotenone and antimycin A was injected at the times indicated.

**Mitochondrial content by flow cytometry**

HFF or C2C12 stably expressing mCherry in the mitochondria were plated at 1.5 x 10^5 per well and infected with MOI of 20-40 GFP-expressing parasites for 18 h or treated with the indicated concentration of valproic acid starting from the time of infection. All centrifugation steps were carried out at room temperature at 350 g for 5 minutes. At 48 or 66 hpi, infected cells were trypsinized and centrifuged, then resuspended in 4% paraformaldehyde in PBS to fix on ice for 20 minutes. Cells were recentrifuged and the pellet was permeabilized in PBS + 0.1% Triton X-100 (PBST) with 0.5 µg/mL DAPI. Samples were run on a MACSQuant VYB and data was analyzed using FlowJo 7.6. Cells were discriminated based on size and DAPI staining. Infected cells were identified based on GFP expression (S3 Fig. B), and mCherry expression was examined for all populations. At least 10,000 events were collected per condition.

Flow cytometric analysis of an endogenous mitochondrial marker, was carried out using antibodies to ATP5B (Abcam) to stain infected and uninfected HFF (without mCherry) as above. Following permeabilization for 20 min at room temperature, cells were centrifuged and resuspended in blocking solution (1% bovine serum albumin in PBS) and incubated for 30 minutes at room temperature. After centrifugation, cells were incubated for 30 minutes at room temperature with mouse anti-ATP5B (Abcam) at 1:2,000 in blocking solution then washed twice in PBST and incubated in 1:2,500 AlexaFluor 594 Goat anti-Mouse IgG (Thermo Fisher Scientific) in blocking solution for 30 minutes, washed as with primary antibody and resuspended in PBST with 0.5 µg/mL DAPI for flow cytometry analysis as above.
**Western blot**

HFF were seeded at $1.5 \times 10^6$ in T75 flasks and infected with a MOI of 10 for 2 hours. At 48 hpi, cells were scraped, pelleted by centrifugation and resuspended in Laemmli SDS-PAGE sample buffer at $2 \times 10^4$ cells/µL. Cell lysates were treated with 0.5 µL benzonase to digest DNA, incubated on ice 30 minutes, then at 95°C for 10 minutes before clarification by centrifugation at 15,000 $g$ for 10 minutes. Due to the presence of parasites in infected cells compared to mock-infected cells, we could not load gels based on protein quantification. Instead, $2 \times 10^5$ cells (~30 µg of uninfected cells) were loaded in wells of a 4-15% Mini-Protean TGX Precast Gel (Bio-Rad) and the Western gel and wet transfer to a PVDF membrane was done per manufacturer protocol. All incubation and wash steps were done while shaking. The membrane was blocked in 1:1 SeaBlock:PBS (Thermo Fisher Scientific) for 1 hour at room temperature, incubated overnight at 4°C with the corresponding antibodies in 1:1 SeaBlock:PBS-0.1% Tween-20, then washed three times in PBS-0.1% Tween-20 prior to incubation with secondary antibodies in 1:1 SeaBlock:PBS-0.1% Tween-20 for 30 min at room temperature. The membrane was washed 3 times briefly in PBS-0.1% Tween-20 and 3 times in PBS for 10 minutes before imaging using an Odyssey Imaging System (Li-cor). The intensity of the signal for each antibody was assessed by Image Studio software (version 5.2). Lack of cross-reactivity of the antibodies with amastigote proteins was verified using amastigotes lysate following amastigote purification. Vimentin was used as a loading control. Primary antibodies: TOMM20 (F-10, Santa Cruz; 1:1,000), ATP5B (3D5, Abcam; 1:1,000), mCherry (ab167453, Abcam; 1:2,000), vimentin (5741, Cell Signaling; 1:2,000). Secondary antibodies: Dylight 800 anti-rabbit (Invitrogen, 1:10,000), Dylight 800 anti-mouse (Invitrogen, 1:5,000), Dylight 700 anti-rabbit (Invitrogen, 1:20,000).

**T. cruzi amastigote proliferation assay**

To determine the number of divisions that an intracellular *T. cruzi* amastigote has undergone in a defined period of time, a modified flow cytometry protocol, based on (Caradonna et al. 2013)
was performed. Briefly, HFF were plated at 1.5 x 10^5 per well in 6 well plates and infected with MOI of 15 for 2 hours using CFSE-stained trypomastigotes. For staining, 5 x 10^6 trypomastigotes/mL were stained with 1 µM CFSE (Thermo Fisher Scientific) in PBS by incubating at 37°C for 15 minutes. Extra dye was quenched by addition of D-10, and trypomastigotes were pelleted by centrifugation at 2100 g for 10 minutes and incubated in fresh D-10 at 37°C for 30 minutes before infection. At 18 (pre-replication) and 48 (replicative phase) hpi, infected monolayers were trypsinized, washed once in PBS, and cells were lysed to release amastigotes by passing the supernatant 10 times through a 28 1/2 G needle. Lysate was fixed by adding paraformaldehyde (Electron Microscopy Sciences) to a final concentration of 1% and incubating 20 minutes on ice. Samples were centrifuged at 300 g for 5 minutes to pellet away host debris, and the supernatant centrifuged at 4000 g for 10 minutes to pellet amastigotes. Pellets were resuspended in PBS with 0.1% Triton X-100 and 0.01 µg/mL DAPI for analysis by flow cytometry. Amastigotes were run on a MACSQuant VYB (Miltenyi Biotec) or LSRII (BD Biosciences) and at least 10,000 events were collected per condition. Data was analyzed using FlowJo 7.6, and amastigotes were discriminated based on size and DAPI staining. Proliferation was modeled using FlowJo 7.6, and CFSE intensity at 18 hpi was set as peak 0 for all samples.

**Multiplexed *T. cruzi* infection assay**

Host cell and intracellular *T. cruzi* amastigote numbers were assessed as described with minor modifications [18]. Briefly, HFF were plated at 1.5 x 10^3 per well in 384 well plates and infected with MOI 1.25 for 2 hours before incubation in phenol-free media at the indicated glucose concentration. At 18 hpi, the indicated concentration of 2-DG was added, and at 66 hpi media was removed and host cell number and parasite number were assessed using 10 µL of CellTiter-Fluor (Promega) and 10 µL of Beta-Glo (Promega) per well, respectively.
Metabolite profiling

Isolated amastigotes were resuspended in cytobuffer at 2 x 10⁷ parasites/mL and incubated for 3 hours at 37°C with 6 mM U-13C-glucose (Cambridge Isotope Laboratories) or unlabeled glucose (Sigma Aldrich). For metabolite extraction, amastigotes were rapidly cooled to 4°C in a dry-ice ethanol bath with gentle agitation as described [56], then centrifuged at 3200 g for 10 minutes at 4°C and resuspended in 80% (v/v) methanol:water to extract metabolites from the pellet as described [57]. Samples were run in technical triplicate and metabolites detected by the Beth Israel Deaconess Medical Center Mass Spectrometry Facility as described [58–60]. The percent of label incorporation was calculated for each replicate as the peak area of all ¹³C-labeled variants of a metabolite divided by the sum of both the labeled- and unlabeled-metabolite peak areas. Background was subtracted by averaging the percent label incorporation of unlabeled-replicates and subtracting that value from each labeled replicate.

ATP assay

Amastigotes were isolated in KHB and incubated at 4 x 10⁵ amastigotes/mL in 5 mM glucose, 5 mM glutamine, or 1 mM pyruvate as indicated at 37°C. At 0 and 24 hours amastigote ATP content was assessed by ATPlite (PerkinElmer) per manufacturer’s protocol.

Statistical analysis

All figures presented show mean values with standard deviation of data from one of at least two independent experiments with at least three technical replicates per condition. Statistical analysis was performed using Prism 7 (GraphPad).

Acknowledgments

We would like to thank M. Riscoe, I. Forquer, and S. Doggett (OHSU) for providing ELQ300, and J. Asara (BIDMC Mass Spectrometry Facility) for metabolite identification. We are grateful
to C-H Lee and N. Knudsen (HSPH) for technical advice regarding transport assays and for providing the mCherry pLNCX2 plasmid and delivery system, and to A. Silber (University of São Paulo) for help with parasite transport assay design and analysis. We would also like to thank S. Nolan and I. Coppens (Johns Hopkins) for advice regarding parasite isolation. Cell line and DNA Bank of Genetic Movement Disorders and Mitochondrial Diseases (GMD-MDbank), member of the Telethon Network of Genetic Biobanks (project no. GTB12001), funded by Telethon Italy, and EuroBioBank network, provided us with patient dermal fibroblast lines.

4.6 References


Chapter 5

Discussion
*T. cruzi* is an obligate intracellular parasite that replicates by binary fission within a wide range of nucleated mammalian cells [1,2]. Despite the importance of amastigote replication in sustaining infection and the potential to control infection through targeting parasite metabolism [3,4], nutrient sources supporting this life cycle stage and their utilization are not well established. Functional studies and proteomics have suggested that amino acids and fatty acids are the main energy substrates of *T. cruzi* amastigotes [5–8], while data surrounding glucose utilization conflicts [8,9]. In addition, knowledge of amastigote adaptation to different host metabolic conditions has remained unexplored, even though parasite persistence in tissues is associated with disease progression [10–12]. We therefore asked how intracellular amastigote metabolism connects with host metabolic networks and the impact on parasite growth, specifically examining amastigote intracellular nutrient access and utilization and the ability of amastigotes to reprogram host or parasite metabolism.

### 5.1 *T. cruzi* amastigote metabolic adaptation

#### 5.1.1 Amastigote metabolic programming

Despite replicating in metabolically distinct tissues such as the heart and adipose tissue [10,13], *T. cruzi* adaptation to distinct intracellular environments had not been previously explored. In mammalian cells, cellular metabolism is modulated in response to the nutrient environment by alterations in gene expression and post-translational modifications [14–17], so we tested the hypothesis that *T. cruzi* amastigotes reprogram metabolism in response to their environment. Our analysis of transcriptomic data collected across the *T. cruzi* life cycle revealed that *T. cruzi* upregulates multiple metabolic pathways during the replicative amastigote stage compared to the trypomastigote stage, including glycolysis, fatty acid oxidation, glutamate dehydrogenase, and the TCA cycle and electron transport chain, suggesting that metabolic capacity and plasticity is increased [18]. Similarly, extracellular flux analysis demonstrated that amastigotes have increased respiratory capacity and glutamine oxidation compared to trypomastigotes [18].
Replacing glucose with galactose in the medium resulted in a slight decrease in parasite replication, showing that amastigote replication rates are dependent on the environment despite the potential to use multiple different substrates. However, culturing infected monolayers in different media did not result in substantial changes to the amastigote transcriptome, though batch effects may have obscured true alterations. Extracellular flux analysis of isolated intracellular amastigotes also revealed no change in basal respiration, respiratory capacity, coupling efficiency, or glutamine response. However amastigotes isolated from cells grown in glucose-free medium showed slightly lower responses to glucose, potentially reflecting the hysteretic behavior of *T. cruzi* hexokinase, which sustains glucose phosphorylation rates for a time even after substrate concentrations have altered [19]. While testing of additional parasite strains and growth conditions is needed, the data suggest that intracellular amastigotes do not transcriptionally regulate their metabolism based on host cell milieu, but increase metabolic plasticity and capacity relative to other life cycle stages as a general mechanism for surviving in multiple different intracellular environments. Since amastigotes were purified from their host cells for analysis, differences in metabolic flux *in situ* could not be measured here. Future study of mitochondrial respiration in infected monolayers using ELQ300 to differentiate between host and parasite contributions will therefore yield interesting additional information about whether amastigote respiratory rates differ based on environment, which can potentially be correlated with amastigote replication rates.

### 5.1.2 Amastigote modulation of host cellular metabolism

*T. cruzi* infection induces significant systemic changes in host metabolic regulation and metabolism [20–23], likely driven by a combination of parasite- and immune-mediated factors. At a cellular level, the transcriptome of fibroblasts demonstrates that expression of a number of small molecule transporters significantly alters over one round of infection with *T. cruzi* [18,24]. Based on the increase in glucose transporter expression in fibroblasts and the increase in
glucose uptake and dysregulation of mitochondrial respiration seen in infected cardiac tissue [18,20,23,24], we explored the ability of *T. cruzi* infection to modulate host cellular metabolism to support parasite replication.

Radiolabeled glucose transport assays confirmed that glucose uptake is increased in infected fibroblasts. Additionally, host or parasite glucose metabolism supports parasite replication as depletion of exogenous glucose and addition of 2-DG significantly impaired amastigote replication. Lactate assays, labeling experiments, and extracellular flux analysis further revealed that host lactate production and ECAR were unaltered by infection, but that amastigotes access host cytosolic glucose pools, which they can use for energy generation or biosynthesis. Taken together, the data suggest that *T. cruzi* infection increases host transporter expression to drive glucose uptake, which intracellular amastigotes utilize to support replication via energy generation and biosynthesis of required metabolites. Glucose deprivation is known to increase glucose transport capabilities in mammalian cells [16,25]. Whether *T. cruzi* increases host glucose transport by essentially starving the host of glucose can be tested by comparing fluorescence of a cytosolic FRET glucose sensor in infected and uninfected cells [26].

A study of glucose homeostasis during Chagas disease revealed that mice become hypoglycemic during acute *T. cruzi* infection, which the authors posited might be partially due to parasite glucose utilization [22]. Though they were likely thinking of extracellular trypomastigote forms, our data indicating that intracellular infection increases host glucose uptake, and evidence that cardiac glucose uptake is increased during acute infection [20], suggest that amastigote glucose usage may also contribute to hypoglycemia during acute disease. During chronic disease, when both extracellular trypomastigotes and intracellular amastigotes are less abundant, data on blood glucose levels conflicts [27].

Treatment of infected monolayers with the small molecule ELQ300 to inhibit parasite respiration during extracellular flux analysis revealed that host mitochondrial respiration is increased due to infection. However host mitochondrial respiration does not seem to impact *T.
cruzi intracellular growth, as parasite replication was unaltered in fibroblasts with impaired mitochondrial respiration. Additional work in the lab has shown that the amastigote flagellum maintains a close physical association (<20 nm) with host mitochondria (G. Lentini, personal communication), while we show here that mitochondrial content increases specifically in infected cells. As kinetoplastid organisms are thought to use their flagella in environmental sensing and signaling [28], we hypothesize that amastigotes use their flagellum to sense the host cell apoptotic or nutritional state via the host mitochondria. Furthermore, the increase in mitochondrial content and respiration during T. cruzi infection may be driven by the flagellum-mitochondria interaction, though attempts to disrupt the physical interaction have remained unsuccessful to date. Proximity-labeling experiments however have identified a few amastigote proteins that may be involved in mediating this interaction (G. Lentini, personal communication), and on-going work to generate T. cruzi knockouts will hopefully yield interesting information about their function.

5.2 Amastigote substrate utilization, host metabolic redundancy, and implications for drug development

5.2.1 Fatty acids

Proteomic data predicted that fatty acid oxidation is increased in amastigotes relative to other life cycle stages [6]. Here our experiments with BODIPY-labeled fatty acids revealed that amastigotes access exogenous fatty acids via the host. Interestingly, isolated amastigotes increased mitochondrial respiration in response to palmitoyl-CoA, but not palmitate. Previous work with isolated amastigotes similarly showed no alteration in respiratory rates in response to palmitate, and radiolabeled analysis revealed very low levels of palmitate oxidation [8]. Together the data suggest that amastigotes access and utilize fatty acyl-CoA but not fatty acid, which should be further confirmed by a reduction in BODIPY-labeling of amastigotes following chemical inhibition of long chain fatty acyl-CoA synthetases.
Targeting host CPT1 with RNA interference increased amastigote replication in multiple host cell backgrounds, though the level of functional knockdown remains unclear in two of the three lines used. However, when combined with the information that knockdown of host peroxisomal fatty acid oxidation or PDK4 results in decreased parasite replication [29], the data suggest that amastigote access to host cytosolic LCFA-CoA supports intracellular replication across multiple tissues.

In this study, amastigotes only exhibited small changes in replication in response to infection. However, since multiple host pathways generate and utilize cytosolic LCFA-CoA (Figure 2.5), host pathways acting redundantly to provide amastigotes with the substrate may obscure an amastigote requirement for LCFA-CoA during replication, which is supported by the evidence that knockout of either the fatty acid transporter or enoyl-CoA hydratase in T. cruzi significantly impairs amastigote replication (R. Tarleton, personal communication) [30].

In mice, T. cruzi induces increased lipolysis in adipose tissue [13,21] and increases lipid droplet formation in infected macrophages [31], which may represent additional mechanisms of increasing fatty acid availability for intracellular amastigotes. Furthermore, work from our lab shows that amastigote triglyceride composition mirrors that of host triglycerides, which is not seen in other lipid classes and suggests that T. cruzi amastigotes scavenge host triglycerides (J. Martin and F. Gazos-Lopes, personal communication). Additional examination of whether host pathways such as fatty acid uptake, synthesis, and VLCFA-CoA oxidation act redundantly to support parasite intracellular replication is needed to establish whether host LCFA-CoA are truly essential for parasite intracellular replication. Metabolomic experiments examining the fate of scavenged LCFA-CoA versus triglycerides may further reveal how T. cruzi regulates use of lipids in energy generation versus membrane synthesis.
5.2.2 Glucose

Previous study of amastigote glucose utilization in different parasite strains yielded discordant results [8,9]. While a study using Sonyia strain parasites showed isolated amastigotes oxidize glucose, another study using the non-virulent CL14 strain, demonstrated that isolated amastigotes lack the hexose transporter and fail to transport glucose, though late stage intracellular forms do [8,9]. In our labeling experiments and extracellular flux analysis, we find that Tulahuén strain amastigotes can access glucose through the host cytosol during active replication, which they metabolize and use in energy generation and multiple biosynthetic pathways, similar to the replicative bloodstream-form of *T. brucei* [32]. Transcriptomic data also shows glycolytic genes are downregulated during initial intracellular differentiation from trypomastigotes to amastigotes and upregulated during active amastigote replication [18]. Together the data suggest that amastigote glucose uptake varies over the course of intracellular infection, and may be tied to replication and/or parasite strain. In addition, removal of exogenous glucose reduced parasite replication. Chemical inhibition of *T. cruzi* hexokinase or disruption of PEX14-PEX5 protein-protein interactions, which is required for glycosomal protein targeting, has been shown to almost completely inhibit amastigote replication [33–35], suggesting parasite glycosomal function may be required for intracellular growth.

5.2.3 Targeting parasite metabolism for drug development

Our data indicate that *T. cruzi* increases its own metabolic capacities and plasticity while simultaneously modifying host metabolism as part of its adaptation for intracellular growth. Manipulation of media composition or knockdown of a single host pathway resulted in relatively small alterations in amastigote replication, suggesting fatty acids and glucose are not essential. However, knockout of the fatty acid transporter or enoyl-CoA hydratase in *T. cruzi* results in abrogated infection in mice, with decreased amastigote replication (R. Tarleton, personal communication) [30], while selective inhibition of amastigote hexokinase with bisphosphonates
also blocks amastigote replication [35,36]. As multiple host pathways feed in and out of metabolite pools, host cells may be able to largely maintain cytosolic substrate levels, obscuring true amastigote substrate requirements. Generating inducible knockout of T. cruzi transporters or key nodes of metabolism will be important for determining how plastic amastigote metabolism really is and whether certain nutrients are actually required for growth. Traditional methods of generating T. cruzi knockouts have required genetic manipulation of the epimastigote stage, followed by selection and then differentiation to the mammalian stages and are complicated by the parasite’s diploid genome. However, the recent adaptation of the CRISPR-Cas9 system for T. cruzi epimastigotes and trypomastigotes [37,38] suggests that generating knockouts will soon be faster and reduce complications from knocking out genes that are essential during differentiation or growth of epimastigotes.

If glucose and LCFA-CoA are found to be essential for amastigote replication, drugs targeting parasite transporters or central nodes of metabolism may be particularly effective in inhibiting parasite growth. Recently, isotretinoin, an acne medication, was shown to inhibit T. cruzi polyamine transport and decrease trypomastigote release from infected monolayers [4], suggesting parasite transporters may be good targets for limiting parasite load. However, trypanosomatids often have multi-copy genes where gene duplication has given rise to transcript variants. The T. cruzi hexose transporter alone appears as eight tandem copies in the genome and produces two different mRNAs [39]. Any compounds must therefore be able to inhibit all functional paralogs. In addition to inhibiting polyamine transport, isotretinoin reduced proline and lysine transport, all mediated by different members of the TcAAAP amino acid transporter family [4]. Additional compounds capable of selectively targeting parasite transporters over mammalian transporters may also be found.

5.2.4 Inhibition of T. cruzi amastigote metabolism: Static or cidal?

T. cruzi enzymes like hexokinase and cytochrome b are attractive targets given their key roles in
parasite metabolism and structural differences from mammalian homologs [3,35,36]. GNF7686, has been shown to inhibit T. cruzi mitochondrial respiration via cytochrome b and drastically reduce the number of intracellular amastigotes seen after 48 hours of treatment, though some amastigotes still persist [3]. Similarly, 2-DG significantly reduced the numbers of intracellular amastigotes by the end of 48 hours of treatment in our work, but did not eliminate them. As compounds are added at the beginning of amastigote replication, the reduction in parasite numbers could be due to death of intracellular amastigotes or inhibition of amastigote growth. CFSE proliferation assays show an almost complete inhibition of parasite replication with 2-DG treatment, while intracellular amastigotes are still visible by microscopy, suggesting inhibition of replication is the main mechanism of action.

Additional work by our lab combining parasite growth assays with cell cycle analysis has shown that nutrient limitation or treatment with benznidazole reduces amastigote replication and increases the number of parasites in the G0/G1 phase (P. Dumoulin, personal communication). Moreover, nutrient addback or compound washout increases replication rates and reduces the number of amastigotes in G0/G1, which suggests amastigotes are actively regulating their cell cycle in response to their environment and hints at the ability of amastigotes to become quiescent where they are more protected from nutrient deprivation or drug insults (P. Dumoulin, personal communication). Many pathogenic fungi and bacteria are known to enter a quiescent state in which they are more resistant to immune system and drug pressures [40,41], while the existence of a quiescent amastigote state has been posited based on the ability of T. cruzi to persist in the face of drug treatment during chronic disease [42].

Understanding whether T. cruzi amastigotes enter a quiescent state, how quiescence is regulated, and how that protects the parasite from other challenges, will be important for identifying new therapeutic targets and designing drug treatment strategies. For instance, would drugs targeting parasite metabolism inhibit replication but induce a quiescent state that prevents parasite clearance, allowing for later resurgence? Currently, most in vitro studies start
compound treatment of *T. cruzi*-infected cultures within 24 hours of infection and examine amastigote growth over one cycle of infection [3,43,44]. Examining amastigote responses to drug treatment and washout using prolonged infection assays will help determine if targeting parasite metabolic pathways such as glycolysis and the ETC can induce amastigote death in addition to inhibiting replication. Without cidal activity or permanently blocking differentiation of amastigotes to trypomastigotes, drugs targeting parasite metabolism are unlikely to result in parasitological cure of indeterminate or chronic infection if parasites secreted in tissues can recrudesce upon removal of drug pressure.

In conclusion, we identified two carbon substrates that *T. cruzi* intracellular amastigotes access in situ and utilize for energy generation and biosynthetic processes. In addition, we found that *T. cruzi* upregulates multiple metabolic pathways as the intracellular amastigote, increasing its metabolic capacity, and that *T. cruzi* infection upregulates host glucose transport, increasing amastigote access to this important substrate, as mechanisms of adaptation for intracellular survival and growth.

5.3 References


Appendix 1

Transcriptome remodeling in *Trypanosoma cruzi* and human cells during intracellular infection

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**Contributions:** NMES and BAB conceived and designed the experiments. YL, KLC, JC, PP, DMN, and SSS performed the experiments. SSS performed experiments in Figure 3B-E. YL, MRT, KO, ATB, HCB, NMES, and BAB analyzed the data. YL, SSS, KC, NMES, and BAB wrote the paper. This appendix is a reprint of “Transcriptome remodeling in *Trypanosoma cruzi* and human cells during intracellular infection,” PLoS Pathog. Y. Li, S. Shah-Simpson, K. Okrah, A.T. Belew, J. Choi, K.L. Caradonna, et al., e1005511, (2016).
Abstract

Intracellular colonization and persistent infection by the kinetoplastid protozoan parasite, Trypanosoma cruzi, underlie the pathogenesis of human Chagas disease. To obtain global insights into the T. cruzi infective process, transcriptome dynamics were simultaneously captured in the parasite and host cells in an infection time course of human fibroblasts. Extensive remodeling of the T. cruzi transcriptome was observed during the early establishment of intracellular infection, coincident with a major developmental transition in the parasite. Contrasting this early response, few additional changes in steady state mRNA levels were detected once mature T. cruzi amastigotes were formed. Our findings suggest that transcriptome remodeling is required to establish a modified template to guide developmental transitions in the parasite, whereas homeostatic functions are regulated independently of transcriptomic changes, similar to that reported in related trypanosomatids. Despite complex mechanisms for regulation of phenotypic expression in T. cruzi, transcriptomic signatures derived from distinct developmental stages mirror known or projected characteristics of T. cruzi biology. Focusing on energy metabolism, we were able to validate predictions forecast in the mRNA expression profiles. We demonstrate measurable differences in the bioenergetic properties of the different mammalian-infective stages of T. cruzi and present additional findings that underscore the importance of mitochondrial electron transport in T. cruzi amastigote growth and survival. Consequences of T. cruzi colonization for the host include dynamic expression of immune response genes and cell cycle regulators with upregulation of host cholesterol and lipid synthesis pathways, which may serve to fuel intracellular T. cruzi growth. Thus, in addition to the biological inferences gained from gene ontology and functional enrichment analysis of differentially expressed genes in parasite and host, our comprehensive, high resolution transcriptomic dataset provides a substantially more detailed interpretation of T. cruzi infection biology and offers a basis for future drug and vaccine discovery efforts.

Author summary
In-depth knowledge of the functional processes governing host colonization and transmission of pathogenic microorganisms is essential for the advancement of effective intervention strategies. This study focuses on *Trypanosoma cruzi*, the vector-borne protozoan parasite responsible for human Chagas disease and the leading cause of infectious myocarditis worldwide. To gain global insights into the biology of this parasite and its interaction with mammalian host cells, we have exploited a deep-sequencing approach to generate comprehensive, high-resolution transcriptomic maps for mammalian-infective stages of *T. cruzi* with the simultaneous interrogation of the human host cell transcriptome across an infection time course. We demonstrate that the establishment of intracellular *T. cruzi* infection in mammalian host cells is accompanied by extensive remodeling of the parasite and host cell transcriptomes. Despite the lack of transcriptional control mechanisms in trypanosomatids, our analyses identified functionally-enriched processes within sets of developmentally-regulated transcripts in *T. cruzi* that align with known or predicted biological features of the parasite. The novel insights into the biology of intracellular *T. cruzi* infection and the regulation of amastigote development gained in this study establish a unique foundation for functional network analyses that will be instrumental in providing functional links between parasite dependencies and host functional pathways that have the potential to be exploited for intervention.

**Introduction**

The kinetoplastid protozoan parasite *Trypanosoma cruzi* is the etiologic agent of human Chagas disease. This parasite has a complex life cycle that involves hematophagous triatomine insects as vectors for transmission and a broad range of mammalian hosts including extensive domestic animal and sylvatic reservoirs [1]. Epimastigote forms of the parasite proliferate in the midgut of the insect vector and give rise to non-dividing, mammalian-infective metacyclic trypomastigotes that are shed in the feces of blood-feeding triatomine bugs and initiate infection in the vertebrate host. *T. cruzi* trypomastigotes actively penetrate a wide range of nucleated cell
types, become enveloped in an acidified lysosome-like compartment [2] where they receive signals to differentiate into amastigotes [3]. Differentiating parasites gradually escape the lysosomal vacuole [4] and proliferate as amastigotes in the host cell cytosol for 3-5 days (Figure 6.1A) before differentiating back into trypomastigotes (referred to as tissue or tissue culture trypomastigotes to distinguish these from metacyclic trypomastigotes), which are released into the extracellular space/medium upon host cell lysis. Motile trypomastigotes disseminate infection via the lymphatics and bloodstream to distal sites where they undergo further cycles of intracellular multiplication, egress and reinvasion. Thus, at several key points in its life cycle, T. cruzi undergoes developmental reprogramming to adapt to different hosts and variable niches within hosts, however the mechanisms governing these adaptive processes are not well defined.

Cellular differentiation is controlled at multiple levels including, for most eukaryotic cells, initiation of gene transcription (e.g. [5, 6]). In trypanosomatids discriminatory mechanisms for the initiation of transcription at individual loci is largely absent. Most protein-coding genes lack promoters and are transcribed as long polycistronic units that are processed into individual mRNAs [7-10]. Consequently, trypanosomes rely on post-transcriptional processes such as mRNA stability, translational efficiency and post-translational modification to coordinate developmental transitions and other adaptive responses encountered throughout their complex life cycles [11-15]. Despite the recent emphasis on mRNA translation efficiency as a primary regulator of protein abundance in trypanosomatids [13, 16-18] and across eukaryotes more generally [19], there is strong evidence for the existence of post-transcriptionally generated mRNA regulons in Trypanosoma brucei and Leishmania that coordinate major developmental shifts in these organisms [20-23].

As with other eukaryotes, mRNA stability and translational efficiency are influenced by trans-acting factors (RNA-binding proteins: RBPs) that interact with cis-acting regulatory elements in the untranslated regions of trypanosomatid mRNAs (recently reviewed in [15, 24]).
Because trans-acting factors regulate multiple mRNAs in a combinatorial fashion [25, 26], it has been challenging to identify cis-acting and trans-acting elements that are associated with the expression of functionally-regulated trypanosomatid genes [27]. However, a growing number of examples link candidate RBP expression levels with the modulation of mRNA subsets (e.g. [15]). Indeed, an entire cellular differentiation program was shown to be triggered by the over expression of a single RBP in African trypanosomes [28]. Functional cis-acting elements have been identified in a number of T. cruzi transcripts and associated with the regulation of expression in this organism [29, 30] including sets of developmentally-regulated [30-33] and functionally-related [34] genes. Although suggestive of the existence of mRNA regulons in T. cruzi, high-resolution transcriptomic data are needed to relate dynamic changes in parasite gene expression to functional adaptation on a global scale. Here, we exploit deep sequencing and informatics approaches to construct high-resolution transcriptome maps for three main T. cruzi lifecycle stages and include the simultaneous capture of parasite and host transcriptional responses during an intracellular infection of human fibroblasts by T. cruzi. With this approach, we gain deeper insights into the biology of T. cruzi with an emphasis on intracellular infection and conclude that transcriptome remodeling is required to alter the ‘blueprint’ upon which major developmental transitions are based.

Results/Discussion

Simultaneous capture of T. cruzi and human host cell transcriptomes by RNA-seq.

To capture the global transcriptomic response associated with the establishment and maintenance of intracellular T. cruzi infection, RNA was isolated from low passage primary human foreskin fibroblasts (HFF) infected with tissue culture-derived T. cruzi Y strain trypomastigotes, and from mock-infected cells, at 4, 6, 12, 24, 48 and 72 hours post-infection (hpi) (Figure A1.1A). RNA was also generated from extracellular trypomastigotes and from axenically cultured log-phase T. cruzi epimastigotes for comparative purposes. Two to four
independent biological replicates were sequenced for each condition generating 2.7 billion high quality reads from 35 samples (S1 Table) that were subsequently processed through our RNA-Seq and data analysis pipeline (S1 Fig). Sequence reads generated from T. cruzi-infected cell samples were resolved by mapping pre-processed reads against T. cruzi [35] and human hg19 reference genomes using the Tophat aligner program [36] (S2 and S3 Table). The well-documented differences in transcriptional regulation between trypanosomes and humans [7, 8, 10] were reflected in the distributions of the log2-transformed and size-factor-normalized gene counts for both species (Figure A1.1B and S2 Fig). As expected, the fraction of total reads mapping to the T. cruzi genome from the mixed host-parasite read pool increased over time as intracellular amastigote replication ensued (Figure A1.1A). It is worth noting that due to the stringency imposed during mapping (≤ 2 mismatches allowed/read) and the necessity to map T. cruzi Y strain sequences against a heterologous (CL Brener Esmeraldo) genome [35], the depth of coverage of the T. cruzi transcriptome at each stage (S1 Table) is most certainly underestimated. Despite this limitation, the demonstrated ability to resolve parasite and human sequences from a mixed read pool, and to obtain a high level of coverage of the T. cruzi transcriptome, bodes well for future transcriptomic analyses of the T. cruzi infection process in vitro and in vivo, particularly as whole genome sequence information for additional T. cruzi strains become available (e.g. [37, 38]).

The overall reproducibility and experimental variation between similarly generated independent samples was evaluated with Pearson correlation (S3 Fig) and median pairwise correlation analyses for T. cruzi (S4 Fig) and human (S5 Fig) samples. For T. cruzi, biological replicates corresponding to each of the parasite developmental stages were highly correlated (S3A and S4 Fig), with the intracellular stages (from 4-72 hpi) exhibiting greater similarity to each other than to either of the extracellular stages (trypomastigotes and epimastigotes) (S3A Fig). The human transcriptome samples also displayed a high level of correlation between biological replicates (S3B and S5 Fig). One exception (“4hr2”, HPGL0111), identified as an
outlier in a more systematic median pairwise correlation analysis (S6 Fig), was removed from downstream analysis (S3B and S5 Fig).

To investigate general trends in the data while identifying and quantifying batch effects, principal component analysis (PCA) was carried out (Figure A1.1C) as well as hierarchical clustering of all parasite (S7A Fig) and human (S7B Fig) samples. PCA plots reveal a high degree of similarity between biological replicates for both the T. cruzi and human samples (Figure A1.1C). For T. cruzi, the extracellular parasite stages (trypomastigotes and epimastigotes) were well separated from each other and displayed very tight clustering within each group (Figure A1.1C; T. cruzi). The intracellular stages grouped according to their maturation status, with nascent amastigotes (4 and 6 hr) clustering together and well separated from the mature replicative amastigote stages (24, 48, 72 hr) with the 12 hr amastigotes in between (Figure A1.1C; T. cruzi). A similar trend is observed for the human data (Figure A1.1C; Human). Parasite-infected HFF samples are well separated from uninfected samples (PC2) and the early infection time points (4-12 hpi) clustered together (Figure A1.1C; Human). The later infection time points were more loosely clustered with outliers observed at 48 and 72 hpi (Figure A1.1C; Human). Notably, the transcriptome of uninfected fibroblasts changed considerably with time in culture (Figure A1.1C; Human PC1) underscoring the necessity to include mock-infected controls for each infection time course for direct comparison, as we have done here. Consistent with the PCA results, the unsupervised hierarchical clustering of T. cruzi samples (S7 Fig) labeled both by biological group and experimental batch date segregated trypomastigote, epimastigote and intracellular amastigote samples into distinct clusters. The partitioning of immature (4, 6, 12 hpi) and mature (24, 48 and 72 hpi) intracellular developmental stages of T. cruzi is also evident (S7A Fig). A similar partitioning of infected human cell samples into early (4, 6 and 12 hpi), mid (24 and 48 hpi) and late (72 hpi) time points suggest distinct phases of the host cell response to parasite infection (S7B Fig).
Figure A1.1. Simultaneous interrogation of parasite and host transcriptomes. (A) Intracellular *T. cruzi* life cycle and sample collection scheme. Extracellular *T. cruzi* trypomastigotes actively penetrate mammalian cells where they receive cues to differentiate into amastigote forms that replicate in the host cell cytoplasm for 3-5 days, beginning at ~22 hpi with a doubling time of ~12 hr. Amastigote division ceases on day 4 or 5 post-infection and parasites differentiate back into trypomastigotes that rupture the host cell to initiate a new cellular infection cycle. For RNA-Seq analysis, total RNA was isolated from axenic *T. cruzi* epimastigotes (insect vector stage), extracellular trypomastigotes and from amastigote-containing human fibroblast monolayers at 6 time points spanning 4-72 hpi. Pie charts indicate the proportion of mapped sequence reads assigned to the parasite (pink) or human (grey). (B) Distribution of global gene expression levels in a representative subset of *T. cruzi* and human samples. Box plots showing comparisons of the distribution of per-gene counts (log2 counts per million with an offset of 1) normalized for sequencing library size. The ends of the whiskers represent the lowest datum still within 1.5 interquartile range (IQR) of the lower quartile, and the highest datum still within 1.5 IQR of the upper quartile. Genes with extremely high or low expression levels are shown as open circles above and below the whiskers, respectively. (C) Principal component analysis plots of global transcriptome profiles. Principal component analysis (PCA) plot for RNA-Seq data with *T. cruzi* and human samples plotted separately. The two first principal components (PC1 and PC2) are plotted with the proportion of variance explained by each component next to the axes labels. Each sample is represented by a dot and the color label corresponding to the sample group, such as the number of hours (hr) post-infection.
To extract biologically meaningful *inferences* from our expression data, lists of differentially expressed genes (DEGs) were constructed from pairwise comparisons of parasite or human expression data (S4 Table). As a large number of genes survived this initial cut-off (q-value < 0.05) a second filter (log$_2$fold-change $\geq$1.0) was imposed to identify gene expression changes with the highest potential impact (S4 Table) for *T. cruzi* developmental stages (S5 Table) and human fibroblasts at different stages of *T. cruzi* infection with matched controls (S7 Table). Because of frequent gene duplication and the presence of several expanded gene families in the *T. cruzi* genome, the parasite DEG data (S5 Table) was filtered to remove all but a single representative of each paralogous group (S6 Table). DEG information for *T. cruzi* (S6 Table) and human fibroblasts (S7 Table) were used for downstream Gene Ontology (GO) enrichment analysis [39, 40] and K-means clustering [41] (S1 Fig). Highlights from this collective analysis are presented and discussed in the context of the relevant biology of *T. cruzi*-host cell interactions in the following sections.

**Transcriptomic signatures mirror biological features of *T. cruzi* developmental stages.**

Steady-state transcriptome data was generated for three distinct *T. cruzi* life stages with a more comprehensive analysis of intracellular amastigote development in human fibroblasts (Figure A1.1A). Focusing on the most disparate samples first, i.e. those derived from distinct developmental stages of *T. cruzi*: epimastigotes, trypomastigotes and intracellular amastigotes (24 hpi), we observed $\geq$2 fold differences in steady state transcript abundance for $\sim$2000 to $\sim$3500 parasite genes (S4 and S5 Table) or between $\sim$1500 - 2600 after correcting for paralogues (S4 Table). Thus, as a conservative estimate, we find stage-regulated changes in transcript abundance to occur for $\sim$15-30% of the predicted protein-coding genes in the *T. cruzi* genome contrasting with a previous estimate of $>$50% derived from comparative microarray hybridization analysis of *T. cruzi* life stages [42].
Due to the nature of polycistronic transcription in *T. cruzi* [7-9] few parasite transcripts are expected to exhibit strict stage-specificity i.e. detectable in one life stage and undetectable in others (Figure A1.1B). Despite this, we find a subset of *T. cruzi* genes (between ~135 and 350) to be over-represented at the transcript level in a single life cycle stage as compared to the other two developmental stages compared in this study (S8 Table). Adding weight to this approach, we observed the expected stage-selective expression of d- and b-amastins in amastigotes and epimastigotes respectively (S8 Table). With the high proportion of hypothetical genes (>50% in each gene list; S8 Table) we were not able to identify significantly enriched GO terms associated with parasite stage-enriched genes. Nonetheless, some general observations offer insight into lifestyle differences among these different parasite stages. For example, trypomastigotes uniquely express a number of protein kinases and an intermediate filament-binding trichohyalin-like protein [43], which may reflect specialized capacities linked to host cell recognition, signaling and invasion by this parasite life stage [44-46]. Epimastigotes express twice as many genes in a stage-selective manner as the other parasite life stages (S8 Table), many of which encode metabolic enzymes (e.g. pentose phosphate pathway, amino acid metabolism) or proteins involved in nutrient acquisition (e.g. transporters of hexose sugars, nucleosides, folate/pteridine and amino acids). In contrast, amastigotes display elevated expression of cation transporters including (TcCLB.509197.39) which is upregulated early in amastigote development (by 6 hpi) with mRNA abundance reaching very high levels by 12 hpi (log$_2$ fold-change 4.2; ~20-fold increase) to become the most highly expressed amastigote gene at 72 hpi (~35-fold increase) (S5 Table) as confirmed by qRT-PCR (S9 Fig). Another cation transporter gene (TcCLB.507527.50) that displays preferential expression in amastigotes at 24 hpi (S5 and S8 Table) bears homology to the ferrous iron transporter characterized in *Leishmania amazonensis* [47], the expression of which is intimately coupled to amastigote development in this parasite. Thus, selective cation transporter expression, and potentially the
need for iron uptake, may be a common feature of amastigote development and intracellular maintenance in both *Leishmania* and *T. cruzi*.

By virtue of their critical role in regulating gene expression in trypanosomatids [15], RNA-binding proteins (RBPs) represent another class of genes for which parasite stage-selective expression information is of interest. Only the RBPs that exhibit differential expression during the intracellular infection process are highlighted (S10A Fig). *T. cruzi* trypomastigotes selectively express two RBPs, RBP5 (TcCLB.511127.10) and RBP6 (TcCLB.506693.30) (S8 Table). While RBP5 has yet to be characterized, RBP6 has emerged as a critical regulator of metacyclogenesis in *T. brucei* [28]. Therefore, stage-specific expression of the orthologous gene in *T. cruzi* trypomastigotes suggests that TcRBP6 may exert important stage-specific functions in the non-dividing, cell-invasive forms of this parasite as well. While several RBPs exhibit transient and relatively low level increases in expression in developing amastigotes, a notable exception to this trend is TcCLB.504005.6 (S5 Table), for which mRNA abundance tracks with increasing intracellular amastigote numbers (S10 Fig). It is tempting to speculate that this particular RBP may participate in regulating processes critical to late stage amastigote growth or the next phase of the infection cycle that involves amastigote to trypomastigote conversion starting at ~96-120 hpi. Genome-scale dynamic mRNA expression data for RNA-binding proteins provides new and valuable information regarding the life cycle stage at which these important *trans*-acting mRNA regulatory factors are likely to act.

**Extensive transcriptome remodeling in *T. cruzi* accompanies adaptation to life inside the mammalian host cell.**

To gain global insights into the intracellular *T. cruzi* infection process, a focused transcriptomic analysis was performed to capture dynamic changes in *T. cruzi* mRNA abundance as the parasite established intracellular residence in mammalian host cells (Figure A1.1A). Extensive remodeling of the *T. cruzi* transcriptome was observed within the first 4 hours of trypomastigote
invasion of human fibroblasts (2790 DEGs, S4 and S5 Table), corresponding to the dramatic shift in environment and initiation of the amastigote differentiation program. More modest changes in *T. cruzi* transcript abundance occurred as amastigote development and maturation progressed over the next 20 hours of the infection cycle (644 DEGs in the 4-24 hpi interval) (S4 and S5 Table). Then, once the amastigotes entered into replicative phase of the intracellular infection cycle (~22 hpi), few additional changes in the steady state transcriptome were detected (43 DEGs emerge in the 24-72 hpi interval; S4 and S5 Table). While it is conceivable that a failure to detect additional DEGs at this stage is due to the masking of subtle transcriptome dynamics in asynchronously replicating amastigote populations, an alternative interpretation of this observation is that widespread transcriptome remodeling is only required to launch the *T. cruzi* amastigote differentiation program. Upon completion of the developmental switch, other important aspects of amastigote biology, such as nutrient acquisition and cell cycle regulation, are likely controlled by mechanisms other than mRNA stability. While we lack definitive data for this prediction, our observations align well with documented global gene expression patterns in related trypanosomatids, *T. brucei* [12, 48] and *Leishmania donovani* [11, 13, 23], where mRNA stability is cited as playing a more prominent role in early parasite development and both translation efficiency and post-translational modification acting as the main regulatory processes that control homeostatic functions [11, 13, 16-18, 23, 48].

**Signatures of early *T. cruzi* amastigote development in mammalian cells.**

If transcriptome remodeling is required to generate a new blueprint to guide developmental transitions in *T. cruzi*, it follows that associated changes in morphology and functionality should be reflected in corresponding transcriptomic signatures. The capture of dynamic changes in parasite mRNA abundance over a time course of infection in human fibroblasts provides an opportunity to derive biological inferences based on differential expression patterns and to compare these with known aspects of *T. cruzi* amastigote biology. Focusing first on the early
phase of intracellular infection by *T. cruzi* where the greatest number of DEGs was detected, we observe several expected features of the trypomastigote to amastigote transition in the transcriptome changes. These include: (1) rapid downregulation of transcripts encoding major polymorphic surface protein classes (trans-sialidases, mucins, MASPs and gp63) (S4 and S5 Table Trypo vs Ama4), some of which have been implicated in host recognition, signaling and immune evasion [45, 49-51]; (2) reduced expression of genes involved in flagellar assembly and motility (within 4 hpi) (S5 and S9 Table) coincident with dramatic shortening of the single *T. cruzi* flagellum; and (3) increased abundance of transcripts encoding the amastigote-specific surface protein, d-amastin (S5 Table) [31, 52]. Consistent with the plasma membrane remodeling that is expected during trypomastigote to amastigote differentiation, we also observe significantly increased transcript levels for GPI-inositol deacylase (*TcCLB.510289.40*) [53], membrane-bound/secreted phospholipase A1 (*TcCLB.509011.90*; S5 Table) and a surface-localized phosphatidylinositol-phospholipase C (PI-PLC) (*TcCLB.504149.160*) [55] (S5 Table).

Although central to parasite plasma membrane remodeling during differentiation [56], amastigote surface lipases are also well positioned to facilitate breakdown of the parasitophorous vacuole alongside the activity of a secreted hemolysin [57], analogous to the mechanism of vacuole egress by the intracellular bacterial pathogen, *Listeria monocytogenes* [58]. Moreover, once *T. cruzi* amastigotes become cytosolically-localized in mammalian cells there are ample opportunities for parasite surface and secreted/released products to interact with host molecules and to modulate host functions during the course of infection. In this regard, *T. cruzi* amastigote phospholipase A1 can be considered a parasite-derived effector protein given that its expression is associated with perturbations in host cell phospholipid metabolism [59] and the activation of host protein kinase C [60].

In addition to remodeling at the plasma membrane during the early trypomastigote to amastigote transition, indicators of signaling pathway retooling also emerge in the transcriptome data (S5 Table; Trypo vs Ama4). For example, a number of predicted protein kinases and
phosphatases are differentially expressed in the parasite shortly after trypomastigote entry into mammalian host cells (S5 Table; Trypo vs Ama4) including the previously characterized farnesylated protein tyrosine phosphatases (TcCLB.506743.130; TcCLB.506743.110) [61]. Consistent with the recognized role of cyclic AMP in T. cruzi differentiation processes [62], we also observe developmental regulation of central components of the cAMP-dependent signaling pathway, such as receptor-type adenylate cyclases (eg. TcCLB.511043.60; TcCLB.428999.20; TcCLB.507467.10), cAMP-dependent protein kinase A (TcCLB.509805.10; TcCLB.506227.150) and cAMP-dependent phosphodiesterases (eg. TcCLB.508277.100; TcCLB.506625.80) (S5 Table; Trypo vs Ama4). In the broader context of sensory detection and signal transduction, it is worth noting that ‘ciliary and flagellar motility’ emerges as an enriched GO term associated with mature T. cruzi amastigotes (>48 hpi) when compared to immature amastigote stages (S9 Table; eg. Ama4 vs Ama48). This signature is driven by the increase in mRNA abundance for several flagellum-associated protein coding genes in mature amastigotes after their initial decline during the initial stages of amastigote development (S10B Fig). The expression of the calcium-sensing FCaBP family members [63] in intracellular T. cruzi amastigotes suggests that the minimal amastigote flagellum may engage in sensory functions in the intracellular life stages, as suggested for Leishmania amastigotes [64]. As little is known regarding the mechanism(s) by which T. cruzi parasites detect and integrate sensory information, particularly for the intracellular mammalian-infective stages, the availability of high-resolution mRNA expression data for T. cruzi intracellular stages (S5 and S8 Table) opens the door to discovery of additional parasite molecules that function in a sensory or signaling capacity including the many predicted protein kinases and phosphatases encoded in the T. cruzi genome [65] that have yet to be characterized.

Consistent with a period of rapid remodeling during early amastigote development in which proteins and membranes are expected to undergo extensive turnover, Gene Ontology enrichment analysis identified ribosomal RNA processing (GO:0006364), protein translation
(GO:0006412) and protein folding (GO:0006457) as significantly enriched GO terms associated with nascent and developing amastigotes (S9 Table; Trypo vs Ama4-Ama12). Despite the fact that nascent intracellular amastigotes will not undergo a first round of replication for another ~20 hours, preparation for the eventual cell doubling is already evident in the transcriptome of early amastigote stages. Consistent with the projected increase in nucleic acid synthesis, nucleoside transporters (TcCLB.506203.10; TcCLB.506773.50) and enzymes involved in pyrimidine synthesis (eg. orotidine-5-phosphate decarboxylase; TcCLB.508373.29) and purine salvage are upregulated in immature amastigotes (S5 Table). Genes encoding enzymes in the guanine branch of the purine salvage pathway [66] are selectively upregulated in intracellular amastigotes as compared to trypomastigotes (eg. guanine deaminase: TcCLB.504431.100; inosine 5' monophosphate dehydrogenase (IMPDH) TcCLB.507211.40; TcCLB.511351.9; XPRT; GMP synthase: TcCLB.508085.10; GMP reductase; TcCLB.506519.130). In contrast, enzymes associated with the adenine branch are preferentially expressed in trypomastigotes over amastigotes: eg hypoxanthine-guanine phosphoribosyltransferase: TcCLB.506457.30; adenine phosphoribosyltransferase: TcCLB.508207.74; adenylosuccinate synthetase: TcCLB.508731.60). This observation raises the possibility that flux through the purine salvage pathway is tuned to the different environments encountered by *T. cruzi* life stages.

The anticipated demand for lipid precursors to support membrane synthesis in *T. cruzi* amastigotes is mirrored by the enrichment of GO functions associated with isoprenoid (GO:0008299), sterol (GO:0006696) and fatty acid (GO:0006633) synthesis at early stages of amastigote development (S9 Table). Specifically, several enzymes in the mevalonate pathway are upregulated (Figure A1.2A) as are the first two enzymes in the fatty acid synthesis/elongation pathway [67] ELO1 (TcCLB.506661.30) and ELO2 (TcCLB.506661.20) (Figure A1.2B and S5 Table). Combined, these observations indicate that intracellular *T. cruzi* amastigotes generate sterols and fatty acids *de novo* to support replication and membrane homeostasis. Despite its biosynthetic capacity, *T. cruzi* may opt to scavenge some lipids or
Figure A1.2. Temporal expression of metabolic pathway genes in mammalian-infective stages of *T. cruzi*. Relative mRNA expression of selected genes in intracellular *T. cruzi* amastigote stages (4-72 hpi) compared to extracellular trypomastigotes (T). Genes in the following metabolic pathways are highlighted: (A) **Mevalonate pathway**: mevalonate kinase (TcCLB.436521.9), mevalonate diphosphate decarboxylase (TcCLB.507993.330), squalene monooxygenase (TcCLB.509589.20), farnesyl pyrophosphate synthase (TcCLB.508323.9), 3-hydroxy-3-methylglutaryl-CoA reductase (TcCLB.509167.20). (B) **Fatty Acid Synthesis**: fatty acid elongase 1 (ELO1) (TcCLB.506661.30), fatty acid elongase 2 (ELO2) (TcCLB.506661.20), fatty acid elongase 3 (ELO3) (TcCLB.506661.10). (C) **Glycolysis**: glyceraldehyde-3-phosphate dehydrogenase (TcCLB.506943.60); hexokinase (TcCLB.508951.20), triosephosphate isomerase (TcCLB.508647.200), phosphoglycerate kinase (TcCLB.511419.40), aldolase (TcCLB.504163.40). (D) **Tricarboxylic Acid / Oxidative Phosphorylation (TCA/Ox-PHOS)**: cytochrome b5 (TcCLB.506773.44), ATPase subunit 9 (TcCLB.503579.70), cytochrome c oxidase subunit V (TcCLB.510565.30), succinate dehydrogenase 11
Figure A1.2 (Continued) (TcCLB.504035.84), succinate dehydrogenase 6 (TcCLB.507091.30), cytochrome c oxidase Figure 6.2 (Continued) subunit VII (TcCLB.509233.150), cytochrome c oxidase subunit VI (TcCLB.511145.10), cytochrome c oxidase subunit IV (TcCLB.506529.360).

(E) Fatty Acid Oxidation: 3-ketoacyl-CoA thiolase (TcCLB.510507.20), enoyl-CoA hydratase/isomerase (TcCLB.511529.170); enoyl-CoA hydratase, mitochondrial (TcCLB.508185.10) enoyl-CoA hydratase/isomerase (TcCLB.510997.40). (F) Glutamate Dehydrogenases (DH): NADP+-GlutDH (TcCLB.507875.20), NAD+ GlutDH (TcCLB.509445.39). All values are reported as log2 fold-change of difference between expression at in the trypomastigote stage and intracellular stages as reported in S5 Table.
precursors from its host cell as seen with other parasites [68-70]. It is currently unclear if or how
*T. cruzi* amastigotes balance *de novo* synthesis of macromolecular precursors with uptake from
the host cell.

**Metabolic adaptation during intracellular *T. cruzi* development.**

Intermediary metabolism has been of interest to biochemists in the *T. cruzi* field for more than
fifty years (e.g. [71, 72]). *T. cruzi*, like its trypanosomad relatives, has a partially
compartmentalized glycolytic pathway [73] and a non-canonical TCA cycle is predicted [74]. All
major *T. cruzi* life stages exhibit the capacity for oxidative phosphorylation [75, 76]. Although
developmental differences in energy metabolism have been documented for *T. cruzi* (e.g. [77,
78]), the specific impact of mammalian host cell colonization on parasite and host bioenergetics
remains a poorly understood aspect of the host-parasite relationship. Here, we report dynamic
changes in the expression of *T. cruzi* genes involved in energy metabolism as trypomastigotes
establish intracellular infection in mammalian cells (Figure A1.3A). Highlighted are glycolytic
enzymes (Figure A1.2C) and components of the mitochondrial electron transport chain (Figure
6.2D) that exhibit biphasic responses during infection. *T. cruzi* transcripts encoding glycolytic
enzymes were rapidly repressed in nascent amastigotes as compared to trypomastigotes (4-12
hpi) consistent with an earlier report that intracellular amastigotes do not take up hexose sugars
[79]. However, transcript levels corresponding to a subset of glycolytic genes rebound in mature
*T. cruzi* amastigotes (Figure A1.2C), with the emergence of ‘Glycolysis’ as an enriched GO term
(GO:0006096) at this stage (>24 hpi) as compared to immature amastigotes (eg. Ama4) (S9 Table)
suggesting that replicative amastigote stages likely retain some capacity for glycolysis
inside the mammalian host cell.

Genes encoding enzymes involved in mitochondrial oxidative phosphorylation are also
upregulated in the intracellular replicative stages of *T. cruzi* as compared to trypomastigotes
(Figure A1.2D; Figure A1.3A) suggesting that the respiratory capacity differs for these two
Figure A1.3. Validation of predicted metabolic features of *T. cruzi* developmental stages. (A) Heatmap of expression values of annotated *T. cruzi* genes predicted to function in intermediary metabolism with a focus on glycolysis, TCA cycle and Ox-PHOS. (B) Calculated basal respiratory capacity (C) ATP-linked respiration and (D) spare-respiratory capacity of extracellular trypomastigotes and isolated intracellular amastigotes (60 hpi) as pmol of oxygen consumed per min (oxygen consumption rate; OCR) normalized to *T. cruzi* DNA (ng) per well. (E) ATP content measured in isolated trypomastigotes and amastigotes in KHB buffer without a consumable carbon source at time points indicated. (F) OCR response to glutamine (10 mM) and ELQ271 (10 µM) in isolated *T. cruzi* trypomastigotes and amastigotes. (G) Dose-dependent inhibition of *T. cruzi* amastigote growth in HFF following addition of ELQ271 at 18 hpi and relative infection measured at 72 hpi. Host cell viability and growth (inset) is unaffected by the compound over the course of the assay. Graphs shown are representative of 3 independent experiments.
parasite life stages. A mitochondrial stress test was performed to test this prediction. In line with early comparative studies of mitochondrial respiratory capacity in *T. cruzi* life cycle stages [75, 80], basal respiration (Figure A1.3B) and ATP-coupled respiration (Figure A1.3C) were found to be similar for trypomastigotes and amastigotes. In contrast, the bioenergetic properties of these two parasite life stages diverged significantly at the level of mitochondrial spare respiratory capacity (SRC) (Figure A1.3D), an indicator of the potential of a cell to respond to sudden increases in energy demand [81]. In repeated measurements, isolated intracellular amastigotes displayed measurable mitochondrial reserve capacity whereas trypomastigotes had none (Figure A1.3D). Furthermore, trypomastigotes failed to maintain ATP levels in the absence of exogenous carbon, whereas homeostatic mechanisms to preserve cellular ATP levels were evident in amastigotes (Figure A1.3E). We hypothesize that mitochondrial reserve capacity may be important for *T. cruzi* amastigotes in the context of cell/tissue infection to provide a buffer against environmental stressors such as oxidative stress (e.g. [82]). A key short-term regulator of spare respiratory capacity in cells is cytochrome c oxidase (mitochondrial complex IV) [83]. Several cytochrome c oxidase subunits are more highly expressed at the transcript level in mature amastigotes as compared to trypomastigotes (Figure A1.2D and S5 Table). Differences in expression of this enzyme complex may contribute to observed homeostatic differences and mitochondrial SRC between these life stages. In the related organisms, *T. brucei* and *Leishmania*, cytochrome c oxidase subunit expression correlates with mitochondrial respiration rates, ATP production, parasite replication and virulence [84-86]. In this light, it would be interesting to probe the relationship between cytochrome c oxidase activity, mitochondrial spare respiratory capacity and *T. cruzi* strain-dependent differences in host infectivity and virulence.

The main carbon sources that fuel energy production in intracellular *T. cruzi* amastigotes are not definitively known. It is assumed that glucose is limiting in the host cell cytosol and that intracellular amastigotes rely on uptake of amino acids and fatty acids for energy [42, 87].
transcriptomic data generally support this projected trend with the anticipated increase in expression of fatty acid oxidation genes (Figure A1.2E) and of several amino acid permeases (S5 and S10 and S8 Fig cluster 5) during amastigote development in mammalian cells. In addition, glutamate dehydrogenase (GlutDH) was found to be highly expressed in replicative T. cruzi amastigote stages as compared to trypomastigotes (Figure A1.2F and S5 Table). GlutDH exerts an important anapleurotic function by converting glutamate to a-ketoglutarate that feeds into the TCA cycle to replenish intermediates diverted to biosynthetic functions. T. cruzi has two different glutamate dehydrogenase activities: one NAD+-linked [88] and the other NADP+-linked [89]. Both activities are expressed in epimastigotes [90]; also reflected in our mRNA expression analysis (S5 Table). However, only the NADP+-linked enzyme (TcCLB.507875.20) is upregulated in intracellular amastigotes (Figure A1.2F). The significance of this finding is unknown but suggests a degree of specialization for these enzymes in the two main replicative T. cruzi life stages. Consistent with higher GlutDH expression in T. cruzi amastigotes, we find that exogenous glutamine drove higher oxygen consumption rates (OCR) in isolated amastigotes as compared to trypomastigotes (Figure A1.3F). Oxidative phosphorylation is inhibited in both parasite life cycle stages by ELQ271 (Figure A1.3F), an endochin-like quinolone that selectively inhibits mitochondrial complex III activity of apicomplexan parasites over mammalian cells [91-93]. We further show that intracellular T. cruzi amastigote growth is inhibited by ELQ271 in a dose-dependent manner (Figure A1.3G), whereas growth/viability of human fibroblast host cells is not compromised (Figure A1.3G; insert) as expected [91-93]. Although it is well-established that mitochondrial respiration in T. cruzi is sensitive to the mammalian complex III inhibitor, antimycin A [75] this is the first demonstration of sensitivity of a kinetoplastid protozoan to endochin-like quinolones. These data agree with the recent demonstration that electron transport is a targetable process in intracellular T. cruzi [94] and support the concept that mitochondrial respiratory chain activity may be essential for T. cruzi amastigote proliferation in mammalian cells.
As an obligate intracellular *T. cruzi* life cycle stage, amastigotes must tap into the nutritional resources of their mammalian host cells in order to survive. The ability of *T. cruzi* to colonize a wide variety of mammalian cell types suggests a high degree of metabolic flexibility and the capacity for rapid adaptation. With the exception of the essential nutrients that *T. cruzi* is incapable of synthesizing (e.g., purines, pterins) we have little knowledge of what cytosolically-localized *T. cruzi* amastigotes extract from their host cells or how energy metabolism is balanced in this parasite life cycle stage. While isotopic tracer experiments are required to make any definitive statements regarding nutrient uptake and utilization by intracellular *T. cruzi* amastigotes, the dynamic changes observed for core metabolic processes at the transcriptome level is indicative of metabolic remodeling during *T. cruzi* amastigote development. Similar metabolic retooling has been described in related kinetoplastid protozoan parasites [95-99]. Going forward, it will be critical to understand how *T. cruzi* amastigote metabolism is wired, how it is coupled to host metabolic pathways, the degree of flexibility that exists within these connections and how this can change in the context of the different cell types that *T. cruzi* colonizes in the human host.

**Host cell response to *T. cruzi* infection.**

Transcriptomic changes induced in mammalian host cells by *T. cruzi* have been reported in a variety of host cell types and under different experimental conditions [100-110]. Because *T. cruzi* is capable of infecting most nucleated mammalian cell types, there has been little consistency among these experiments, complicating direct comparison of host transcriptional response data. Here we opted to use human foreskin fibroblasts (HFF) as the model host cell type for *T. cruzi* infection to facilitate comparisons to microarray hybridization studies previously conducted by our group [100, 107]. As outlined in the Methods section, our experimental approach permitted the capture of both parasite and host transcriptome response information across an infection time course in vitro (Figure A1.1A). RNA-Seq libraries generated in parallel
for mock-infected HFF cultures provided the appropriate controls for each infection time point. As anticipated, some of the previously documented features of the global host transcriptional response to *T. cruzi* infection [100, 107] were recapitulated in the present analysis (S7 Table) as discussed below. One notable difference, however, relates to the detection of ~450 differentially expressed genes in *T. cruzi*-infected fibroblasts within the first 4 hpi of infection (S4 Table) contrasting sharply with the minimal response previously observed at early parasite infection time points [100]. The enhanced detection capability is likely due to the increased dynamic range and sensitivity achieved with the deep sequencing approach used here.

The Gene Ontology enrichment categories associated with the early transcriptome response in *T. cruzi* infected fibroblasts (4-6 hpi), while numerous (S10 Table), can be distilled into two main categories: host cell cycle progression and immune response. Among the 288 fibroblast genes that are upregulated >2-fold following parasite infection at 4 hpi (S4 and S7 Table) a significant enrichment in functions related to cell cycle progression, mitosis and cell division are observed eg. GO:0000278 (S10 Table; upregulated). Plotting the mRNA expression dynamics for several cell cycle regulators (Figure A1.4A) shows this trend continuing until 24 hpi, after which the expression of host cell cycle genes declines precipitously (Figure A1.4A, S8B Fig cluster 2, and S12 Table) such that 'mitotic cell cycle' becomes an enriched biological process associated with downregulated host genes (S10 Table; downregulated). Overall, these observations coincide with our previous finding that *T. cruzi* infection pushes host cells toward S-phase in the first 24 hr of the infection cycle, with a subsequent block imposed on host cell cytokinesis at later time points [107].

An innate immune response to *T. cruzi* infection was also evident in the early transcriptome signature of infected fibroblasts (S10 Table; upregulated; e.g. GO:0002376) with the upregulation of pro-inflammatory cytokine and chemokine genes (Figure A1.4B and S7 Table) as well as type I interferon inducible genes (Figure A1.4C and S7 Table) with different dynamics (Figure A1.4C). Cytokine/chemokine gene expression peaks at 24 hpi (Figure A1.4B)
Figure A1.4. Dynamic host response signatures in *T. cruzi*-infected human fibroblasts.
Expression patterns for selected genes in the most strongly modulated pathways in *T. cruzi* infected HFF. Genes in the following categories are highlighted. (C) Mitotic Cell cycle: AURKA (ENSG00000087586); CDC6 (ENSG00000094804); CDC20 (ENSG00000117399); CDC25A (ENSG00000164045); CDK1 (ENSG00000170312); CCNA2 (ENSG00000145386); NEK2 (ENSG00000117650); ORC1L (ENSG00000085840); PLK1 (ENSG00000137807); KIF23 (ENSG00000178207). (B) Cytokines/Chemokines: IL-8 (ENSG00000169429); IL-6 (ENSG00000136244); CCL8 (ENSG00000108700); CCL2 (ENSG00000108691); CXCL10 (ENSG00000169245). (C) Type I Interferon: OAS (ENSG00000089127); IFNB (ENSG00000171855); ISG44 (ENSG00000137959); GBP1 (ENSG000001177228); ISG15 (ENSG00000187608). (D) Mevalonate/Sterol biosynthesis: HMGCR (ENSG0000013161); HMGCS1 (ENSG00000112972); DHCR7 (ENSG00000172893); FDP5 (ENSG00000160752); FDFT1 (ENSG00000079459); HSD17B7 (ENSG00000132196); LSS (ENSG00000160285); MVD (ENSG00000167508); SQLE (ENSG0000010549); MSMO1 (ENSG00000152802); SC5D (ENSG00000109929). All values are reported as log2 fold-change of the difference in expression of infected and matched uninfected controls at each time point as listed in S7 Table.
whereas the type I IFN response (ie. genes that are expressed downstream of type I IFN receptor activation) increases gradually over the infection time course (Figure A1.4C) to become the dominant host transcriptomic signature by 72 hpi (S10 Table; upregulated). Differences in the expression profiles of these distinct immune response pathways is presumably related to differences in the mechanism of pathway activation by T. cruzi [111-116] and regulatory processes related to signal amplification [107]. Pro-inflammatory cytokine activation via Toll-like receptor (TLR) and cell-intrinsic response pathways is required for host protection against T. cruzi [111, 113-116]. In contrast, the type I IFN response does not require TLRs for activation in response to T. cruzi [112] and is associated with exacerbation of T. cruzi infection under instances of high parasite load to the detriment of the host [117], similar to the impact of type I IFNs on the host in a number of other non-viral pathogen infection models (reviewed in [118]). Although not required for host protection, type I IFN (IFNA6) and several IFN-inducible genes (IFI44L, STAT1 and GBP1) emerged in an unbiased RNAi screen conducted in HeLa cells [119] as positively effecting the T. cruzi infection process. This finding raises the unexplored possibility that the host type I IFN response triggered by T. cruzi may be beneficial to the parasite under certain circumstances.

Corresponding with increasing intracellular parasite burden is the elevated expression of host genes related to metabolism. Included in this list are several classes of solute transporter (S7 Table) and enzymes involved in lipid biosynthesis (S8B Fig; cluster 4). In fact, most of the genes in the mevalonate pathway are upregulated ≥2-fold between 24-72 hpi (Figure A1.4D) as is SREBP2 (ENSG00000198911), an important regulator cholesterol homeostasis in mammalian cells [120]. It is tempting to speculate that elevated sterol biosynthesis fuels membrane synthesis in infected host cells to accommodate a steadily increasing intracellular parasite load and/or provides a pool of sterol intermediates to be scavenged by intracellular amastigotes. However, given that cholesterol biosynthesis is an intensely oxygen-consuming process [121], it is possible that the main function of this late host response to T. cruzi is
defense against oxidative stress. While the presence of replicating parasites in the host cell cytoplasm is expected to dramatically impact energy homeostasis in the host cell, energy metabolism does not emerge as an enriched GO function at any time in the intracellular infection cycle (S12 Table). Thus, the cellular response to such perturbations is predicted to occur at the post-transcriptional and post-translational levels.

The ability to simultaneously capture host cell and parasite transcriptomes with high resolution and sensitivity sets the stage for the generation of host-parasite interaction networks. The integration of transcriptome data with other types of expression data and with functional information will be instrumental in modeling the critical aspects of the parasite-host interaction and aid in the identification of targetable processes therein. As a limited exercise, we performed an intersection of datasets containing host genes that are upregulated in response to *T. cruzi* infection (24 hpi; S7 Table) with those previously shown to impact *T. cruzi* growth in a genome-scale RNAi screen [119]. Within this subset of genes (S13 Table) is *GCH1*, which encodes GTP-cyclohydrase 1, the rate-limiting enzyme in tetrahydrobiopterin (BH₄) synthesis. In previous work we demonstrated that siRNA-mediated knockdown of *GCH1* expression in host cells impaired intracellular *T. cruzi* amastigote growth in a manner that was rescued by the addition of dihydrobiopterin [119]. Here we find that *GCH1* (ENSG00000131979) is upregulated in *T. cruzi*-infected fibroblasts (S7 Table; ≥12 hpi) at the same time that its negative regulator (*GCHI feedback regulator; ENSG00000137880) is repressed (S7 Table). Additionally, the *T. cruzi* gene encoding pterin-4-alpha-carbinolamine dehydratase (PCDB1: TcCLB.503613.40), an enzyme involved in biopterin recycling, was also found to be rapidly upregulated in developing intracellular amastigotes (S5 Table; 4 hpi). Together, these observations are consistent with a predicted need to increase flux through the host BH₄ synthesis pathway to fuel the growth of intracellular *T. cruzi*, a pterin auxotroph [122]. With this example, it is possible to see how threads of a functional host-parasite network can emerge from data integration, an important goal of host-pathogen transcriptomic studies going forward.
Conclusion

A key feature of our work is the demonstrated ability to parse out human and parasite sequence reads from complex pools generated from *T. cruzi*-infected cells and to obtain high coverage of both the parasite and host transcriptomes to enable downstream analyses with high statistical confidence. As emphasized here, the simultaneous capture of dynamic changes in host and parasite gene expression over an infection time course provides immediate and new insights into the biology of *T. cruzi* infection and serves as a unique resource for the construction of high-resolution maps of parasite-host interactions. Moreover, the transcriptome dynamics observed during a major life stage transition in *T. cruzi* revealed marked similarities between this parasite and its trypanosomatid relatives with respect to the ordering of processes that control global gene expression during differentiation. Specifically, our data support previous observations in *T. brucei* [12, 48] and *Leishmania donovani* [11, 13, 23, 123] indicating that regulation of mRNA levels exerts the greatest impact during the initial phases of a developmental transition in these parasites, whereas downstream mechanisms such as translational efficiency and post-translational modification dominate in the subsequent phases of development and maintenance. While regulation of gene expression in *T. cruzi* is understood to be multi-layered and complex (eg. [124, 125]), with translational efficiency playing a key regulatory role [18], our findings argue for the value of transcriptome data to derive meaningful biological inferences related to parasite biology. Extrapolation of this finding to the many hypothetical genes encoded in the *T. cruzi* genome, for which we now have dynamic mRNA expression data, has exciting implications for biological discovery in the trypanosome field. Finally, integration of transcriptome information, with emerging proteomic (eg. [87, 126-133]), functional [119] and metabolic data will, in the near future, create novel opportunities to pinpoint critical processes that govern successful pathogen colonization in the host with links to Chagas disease pathogenesis and increase the potential to identify novel targets for this important neglected disease.
Methods

Parasite maintenance and mammalian cell infection

*Trypanosoma cruzi* Y strain [134] was cultivated by weekly passage in LLCMK₂ cells (ATCC #CCL-7) in Dulbecco’s modified Eagle medium (DMEM) with 2% fetal bovine serum (FBS), 2 mM L-glutamine, 10 mM HEPES and penicillin-streptomycin maintained at 37°C and 5% CO₂. *T. cruzi* trypomastigotes released into the supernatants of infected LLCMK₂ cells were collected, pelleted by centrifugation (1000g, 10 min) and collected from the supernatant after swimming up from the pellet over a 2-4 hour incubation at 37°C, 5% CO₂. Human foreskin fibroblasts (HFF) (ATCC #CRL-2522) were seeded onto 10 cm² plates or T-25 flasks in complete DMEM (as above, with 10% FBS) and grown to 80% confluence (48 hr) before infection. Briefly, HFF monolayers were washed with DMEM-2%FBS (DMEM-2) and either incubated with medium (mock) or *T. cruzi* trypomastigotes for 2h before washing 5 times with PBS and incubation in DMEM-2 at 37°C and 5% CO₂. At the indicated time points (4-72 hpi), cells were rinsed with ice-cold PBS and lysed directly in Trizol for RNA isolation. *T. cruzi* epimastigotes, maintained at mid-log phase in liver infusion tryptose medium at 27°C, or purified extracellular trypomastigotes (>95% pure) were also used for RNA isolation. To obtain intracellular *T. cruzi* amastigotes for metabolic studies, infected LLCMK₂ (60 hpi) were washed with ice-cold Krebs-Henseleit Buffer (KHB) containing 0.5mM glucose, and scraped into 1ml ice-cold KHB + 0.5mM glucose with a cell scraper. Dislodged cells were collected in 9ml final volume of KHB + 0.5mM glucose in a 50ml tube, and centrifuged at 2100g for 10 minutes at 4°C. Pelleted cells were resuspended in 1ml of KHB + 0.5mM glucose, transferred to Eppendorf tubes, vortexed for 45s and passed through a 1ml syringe with a 28.5G needle 20 times to release amastigotes. Unbroken cells and debris were pelleted at 100g for 1 minute and the amastigote-enriched supernatant was pelleted at 1000g for 10 minutes at room temperature. Amastigotes were resuspended in warm (37°C) KHB + 0.5mM glucose at 2x10⁷ amastigotes per ml.
RNA isolation and library construction for simultaneous transcriptome profiling of parasite and host cells.

RNA was isolated in Trizol reagent, as per manufacturer, and the quality determined using an Agilent 2100 bioanalyzer and quantified by qPCR using a KAPA Biosystems library quantification kit. Standard Illumina protocols were used for mRNA-Seq sample preparation. RNA-Seq libraries were constructed from polyA-enriched mRNAs generated from eight *T. cruzi* developmental stages: epimastigotes, trypomastigotes, and intracellular amastigotes at 4, 6, 12, 24, 48 and 72 hrs post-infection (hpi) of HFF. Libraries were also constructed from mock-infected HFF at the same time points. For each condition, 2-4 independent biological replicates were sequenced on an Illumina HiSeq1000. A total of 2.7 billion reads from 35 samples were generated from 101 bp paired-ends.

**Sequence read processing, alignment, abundance estimation and data normalization.**

The quality of the raw reads was evaluated using the FastQC tool [http://www.bioinformatics.babraham.ac.uk/projects/fastqc/] and one nucleotide was trimmed using the FASTX toolkit (Hannon Lab, CSHL) when the mean of the quality score fell below 30 in the last position (see analysis pipeline S1 Table). Tophat v2.0.8 [36] was used to align all reads to the reference human genome sequence (hg19, GRCh37) and independently to the of the *T. cruzi* CL Brener reference genome (v. 4.1) [35] Esmeraldo haplotype obtained from the TriTrypDB database (www.tritrypdb.org). Alignment settings allowed 2 mismatches per read and the default -g/--max-multihits parameter of -g=20 was used for alignments to the human genome. A parameter of -g=1 was used for *T. cruzi* to allow reads to map to a single locus in this organism where multi-gene families are abundant. The read counts per coding sequence (CDS) were determined using HTSeq [http://www-huber.embl.de/users/anders/HTSeq/] as listed *T. cruzi* (S2 Table) and human (S3 Table) genes.
Data quality assessment by statistical sampling and visualization.

Weakly expressed genes, defined as having less than 1 read per million in ‘n’ of the samples, where ‘n’ is the size of the smallest group of replicates [135] (here n=2 and 3 for the T. cruzi and human samples, respectively) were removed from subsequent analyses. Pearson correlation and standardized median correlation analyses, box plots, Principal Component Analysis (PCA) and Euclidean distances-based hierarchical clustering approaches were used to evaluate replicates and the relationships between samples across time points and to visualize sample-sample distances. All components of our statistical pipeline, named cbcbSEQ, can be accessed on GitHub (https://github.com/kokrah/cbcbSEQ/). Samples that did not pass the following quality assessment procedure were removed: for each sample we computed the median pairwise correlation (mpc) to all other samples in the dataset (S5 Fig). A standard outlier identification method was then applied to remove samples with low correlation to the other samples: samples were removed if their median pairwise correlation (mpc) is less than Q1(mpc) – 1.5 IQR(mpc) where Q1(mpc) and IQR(mpc) are the first quartile and inter-quartile range of the median pairwise correlation across all samples respectively. HPGL0111 was removed from subsequent analyses as a result.

Differential Expression Analysis.

A quantile normalization scheme was applied to all samples [1]. Following log2 transformation of the data, LIMMA [136] was employed for differential expression analyses. LIMMA utilizes a standard variance moderated across all genes using a Bayesian model and produces p-values with greater degrees of freedom [137]. When appropriate, the Voom module was used to transform the data based on observational level weights derived from the mean-variance relationship. Experimental batch effects were adjusted by including experimental batch as a covariate in our statistical model. Type I error introduced by multiple testing was corrected with q-value [138]. A contrast matrix was used within LIMMA. To control for expression profile changes in human cells that occur naturally over time in cell culture, normalized-log2-
transformed expression values for each gene in ‘uninfected’ was subtracted from ‘infected’ in the paired uninfected/infected HFF samples at each time point. Differentially expressed genes were defined as genes with q-value < 0.05.

Filtering T. cruzi DEG lists to remove paralogous genes.

Each list of differentially expressed T. cruzi genes generated from pairwise comparisons (S5 Table) was submitted to a search against itself using FASTA36 [139]. Groups of genes were counted as paralogous when observed with an e-value ≤0.0001 and percentage identity ≥80%. The first lexically listed gene in each group was taken as a representative for the group shown in S6 Table. It should be noted that while a large proportion of paralogous genes have been collapsed, a number of truncated (partial) genes as well as genes encoding large hypervariable regions (i.e. MASP) can only be manually removed, a process more prone to error due to reliance on manual curation.

K-means clustering and functional enrichment analysis

K-means clustering analysis was performed to identify genes with similar expression profiles across different developmental stages of T. cruzi or human host cells with the R function “kmeans” and using the Hartigan-Wong algorithm. Quantile-normalized and batch effect-adjusted expression values were used for clustering and Euclidean distance was computed as the distance metric; 8 partitions were used to generate the clusters following the method of [41, 140]. Lists of significantly regulated genes resulting from differential expression or clustering analyses were classified into GO functional categories and tested for enrichment using GOSeq, which applies Wallenius approximation to correct the bias of over-detection of differential expression for long and highly expressed transcripts [40]. False discovery rate (FDR) was controlled using the Benjamini and Hochberg’s procedure [141].

Metabolic analyses

Mitochondrial respiratory capacity was measured using an XF®24 extracellular flux analyzer (Seahorse Biosciences). Briefly, XF®24 assay plates were pre-coated with 30 µl of 7.7% Cell
Tak (Corning) in 100 mM sodium bicarbonate, pH 8 for 30 minutes, then the wells were washed three times with 0.5 ml warm Krebs-Henseleit Buffer (KHB) before plating parasites. Isolated T. cruzi (Tulahuen strain) [142] trypomastigotes and amastigotes were resuspended in either XF Base Medium (Seahorse Biosciences) + 10mM glucose, 2mM glutamine, and 1mM sodium pyruvate or KHB + 0.5mM glucose at 2x10^7 parasites/ml. 2x10^6 parasites in 100 µl were delivered to each well of a Cell-Tak pre-coated Seahorse XF®24 assay plate and immediately centrifuged at 2056g for 2 minutes. The volume of medium in each well was adjusted to a total volume of 450 µl/well of plating medium. To determine basal respiratory capacity, ATP-coupled respiration, and spare respiratory capacity, drugs from the XF Cell Mito Stress Test Kit (Seahorse Biosciences) were resuspended in warm media and injected at 10x their final well concentrations of 2.5µM oligomycin, 3µM carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP), and a mixture of 1µM antimycin A and rotenone, in order. Results were normalized to parasite DNA based on a quantitative PCR assay of the single copy T. cruzi gene: OSBP (TcCLB.508211.10). At the end of the run, the media was replaced with 200 µl PBS and the plate was frozen at -20°C. DNA was isolated after thawing the plate using the DNeasy Blood and Tissue Kit (Qiagen). For qPCR, 10 µl of iTaq™ Universal SYBR Green Supermix (BioRad) was combined with 0.33 µl isolated DNA, 1 pmol each of primers to amplify a single copy T. cruzi gene OSBP (F: 5'-CAT CAC CTA CGG CCA CAA GA-3', R: 5'-TGC AGT GGA TAC GCA TAC GG-3'), and water for a 20 µl reaction volume. The reaction was run at 95°C for 5 minutes, then cycled 45 times at 95°C for 15 seconds followed by 60°C for 60 seconds. The amount of DNA per Seahorse plate well was calculated by comparing Ct values to a standard curve generated by 1:2 dilutions of 10 ng of T. cruzi DNA. To calculate basal respiratory capacity, the normalized oxygen consumption rates (OCR) after antimycin A and rotenone addition was subtracted from the baseline OCR. For ATP-coupled respiration, normalized OCR after oligomycin injection was subtracted from baseline OCR. Spare respiratory capacity was calculated as the difference between normalized OCR after FCCP.
injection and control wells at the same timepoint without any drug injections. Glutamine (Gibco, Life Technologies) and ELQ271 (generously supplied by M. Riscoe; OHSU) were resuspended in warm medium and injected at 10x their final well concentrations of 2mM and 10µM, respectively.

**Total ATP determination.**
To measure total ATP levels, *T. cruzi* trypomastigotes and amastigotes were isolated (as above) and resuspended in Krebs-Henseleit Buffer (KHB) at 4x10⁶ parasites/ml. Trypomastigotes and amastigotes were plated at 4x10⁵ parasites in 100µl in a separate white 96-well plates (Corning) for each time point and incubated at 37°C. Parasites were lysed and ATP levels were determined using the ATPlite assay kit (PerkinElmer), measuring luminescence on a Varioskan Flash plate reader (ThermoScientific).

**Drug susceptibility assay**
HFF were seeded at 1500 cells/well in 384 well black bottom plates (Corning). At 24h post plating, cells were infected with β-galactosidase expressing *T. cruzi* Tulahuen strain (moi 5) for 2hr, washed twice, and left in DMEM (2% FCS, 2 mM glutamine, 1 mM pyruvate). At 18hpi cells were treated with 0.3-10 µM of ELQ271. At 72 hpi HFF viability was measured in a fluorescence-based readout (CellTiter-Fluor, Promega) and *T. cruzi* was measured by luminescence-based readout (Beta-Glo reagent, Promega) using an Envision Plate Reader (PerkinElmer) as described [2]. The relative infection (RLU/RFU) was calculated and normalized to untreated control fitted by non-linear regression to high (=100%) and low (=0%) values using GraphPad Prism software.

**Quantitative RT-PCR**
RNA was isolated from purified *T. cruzi* trypomastigotes and from infected monolayers (at the indicated timepoints post infection) following cell lysis in Trizol reagent and purification using the PureLink RNA Mini Kit (Ambion). DNase-digested (Turbo DNase, Ambion) total RNA (1 µg) was converted to cDNA using the iScript (Bio-Rad) cDNA synthesis kit according to manufacturer's
instructions. Specific primer pairs to amplify genes of interest in quantitative-RT-PCR reactions were selected on their ability to form single peak in melting curve analysis and verified by sequencing of PCR products. Forward (F) and reverse (R) primer pairs for are listed below in a 5’ to 3’ orientation. Cation transporter (TriTrypDB: TcCLB.509197.39) F: GAGTGTACATGCTTGAAGTG and R: CGTTAAAAATAAGAGAAAAATG; Glutamate dehydrogenase (TriTrypDB: TcCLB.507875.20) F: GAGTACTGCCAGGATTCTC and R: CAAAGCCAAGAAACTTAAG; Fatty acid elongase (TriTrypDB: TcCLB.506661.30) F: GAGGCAACCTGCACATT TAAC and R: GTGTCCATCAACTCAGGAATCT; Fatty acid desaturase (TriTrypDB: TcCLB.511073.10) F: AAGGAACGTGAAGAATCTC and R: AACGGACTTCTCCAGATC; Hypothetical protein, conserved (TriTrypDB: TcCLB.509767.170) F: ATGAAGCTTGCGTTCTCT and R: GGTCACAATAGGCCAGTC; Ribosomal RNA large subunit gamma M1 (TriTrypDB: TcCLB.411483.20) F: TGTGAAATGCGAAACAC and R: CCCAGGTTTTTGCTTTAATG. Relative mRNA transcript abundance was quantified by SYBR green (iTaq Universal SYBR Green Supermix, Bio-Rad) PCR using a StepOnePlus Real-Time PCR Systems (Applied Biosystems). Large subunit ribosomal RNA gamma (M1): TcCLB.411483.20) used as endogenous control.

Data accession

RNA-Seq data are available at the National Center for Biotechnology (NCBI) Sequence Read Archive (SRA) (http://www.ncbi.nlm.nih.gov/bioproject) under Bioproject PRJNA251582 (accession numbers ranging from SRR1346026-SRR1346052) and Bioproject PRJNA251583 (accession numbers ranging from SRR1346053-SRR1346059). Individual accession numbers are also shown in S1 Table.

Acknowledgements.

We thank members of the Burleigh and El-Sayed labs for support and helpful discussions and Rebeca Manning-Cela for critical reading of the manuscript.
All supporting information can be found at https://doi.org/10.1371/journal.ppat.1005511.

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Figure A2.1. Supplemental material for MBP article. (A) Isolated amastigotes were incubated with an anti- *T. cruzi* rabbit immune serum (green) or pre-immune rabbit serum.
Figure A2.1 (Continued) (black) followed by an anti-rabbit Alexa 488 secondary. Amastigotes were first gated based on forward and side scatter characteristics followed by their ability to stain using the anti- \textit{T. cruzi} immune serum. (B-C) Final concentrations for oligomycin are indicated. SRC was calculated following oligomycin titration and 3 \( \mu \text{M} \) FCCP injection. Data represent at least three technical replicates per condition and are representative of two biological replicates. One-way ANOVA and Dunnett’s multiple comparison test were calculated relative to 0 \( \mu \text{M} \) oligomycin injection. * indicates \( p<0.05 \).

(D) 2.5 \( \mu \text{M} \) oligomycin was added and after either 3 or 6 “mix, wait, measure” cycles, 2 \( \mu \text{M} \) FCCP was added and SRC was calculated for both amastigotes and epimastigotes. Sidak’s multiple comparison test was calculated. Data show five technical replicates and are representative of two biological replicates. (E) OCR over time for amastigote oligomycin titration shown in Fig. 2C. (F) OCR over time for epimastigote oligomycin titration shown in Fig. 2D. (G) OCR over time for amastigote FCCP titration shown in Fig. 2E. (H) OCR over time for epimastigote FCCP titration shown in Figure 3.2F.
Figure A2.2. Gal medium increases mammalian cell mitochondrial respiration without impacting reactive oxygen species generation. OCR (A) and ECAR (B) of HFF assayed in the presence of multiple carbon substrates following 2 days of adaptation in HG or Gal medium. (C) The OCR:ECAR ratio of HFF. Mean ± SD from one of 3 representative independent experiments. Student’s t-test was applied. * p < 0.05. (D) Reactive oxygen species (ROS) levels of HFF culture in HG or Gal assessed by carboxy-H2DCFDA relative fluorescence units (RFU). Tert-butyl hydroperoxide (TBHP) was added as a positive control for ROS induction. N-acetyl cysteine (NAC) was added as a negative control to scavenge ROS. Two-way ANOVA with Sidak’s multiple comparisons test was applied. ** p < 0.01. Mean ± SD of one of 2 representative independent experiments.
Table A2.1. Differential gene expression in amastigotes isolated from Gal versus HG.

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<tr>
<th>Gene ID</th>
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<th>pval</th>
<th>a pval</th>
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LogFC - log fold change of gene in Gal relative to HG, pval - p value, a pval - adjusted p value.
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Figure A2.3. Large portion of variability explained by batch. (A) Principal component analysis of amastigote transcriptome. (B) Principal component analysis of fibroblasts.
### Table A2.3 Differential gene expression in fibroblasts grown in Gal versus HG.

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</table>
Figure A2.4. LLcMK2 metabolic reprogramming alters amastigote replication but does not significantly reprogram amastigote metabolism. (A) Assessment of LLcMK2 OCR and ECAR. Cells were assayed in the presence of multiple carbon substrates following 48 hours of adaptation in HG or Gal medium. Mean ± SD from one of 2 representative independent experiments. (B) Amastigote proliferation was assessed by CFSE dilution using flow cytometry 48 hpi. Student’s t-test was applied. ** p < 0.01. Data from one of 2 representative independent experiments. (C) The OCR of amastigotes isolated at 48 hpi from LLcMK2 grown in HG or Gal medium. Oligomycin (O), FCCP (F), rotenone and Antimycin A (R/A) were injected as indicated. Basal respiration (D) and ETC coupling efficiency (E) of isolated intracellular amastigotes. Student’s t-test was applied. Mean ± SD from one of 2 independent experiments.
Figure A3.1. *T. cruzi* infection does not alter host cell number or glycolytic rates. (A-B) Of the known facilitated glucose transporters in humans (*SLC2A1-14*), a subset were detectable in fibroblasts in earlier transcriptomic analysis (Li et al. 2016). While *SLC2A1* and *SLC2A3* are the major glucose transporters in fibroblasts and widely expressed across different tissue and cell types, expression of these genes is not significantly altered following *T. cruzi* infection. In contrast, *SLC2A12* transcript levels significantly increase in response to infection (Fig 1A), revealing responsiveness of this human glucose transporter to parasite infection. Graphs show mean ± SD of 3 independent experiments. Two-way RM ANOVA with Sidak’s multiple comparisons test was applied. * p < 0.05. (C) Quantitative PCR analysis of genomic DNA isolated from HFF monolayers infected with different multiplicity of infection (MOI) indicate no difference in host cell abundance due to infection at 48 hpi. Changes in host glucose uptake (Fig 1B) are therefore not due to changes in host cell abundance. Mean ± SD shown for 3 technical replicates from a representative experiment. (D) The fibroblast extracellular acidification rate (ECAR) is not appreciably altered by infection. Inhibition of amastigote respiration by treatment with 1 µM ELQ300 reduces the ECAR of *T. cruzi*-infected monolayers down to that of uninfected monolayers. Mean ± SD shown for 3 technical replicates from a representative experiment. Two-way ANOVA with Tukey’s multiple comparisons test was applied. ** p < 0.01, *** p < 0.001.
Figure A3.2. ELQ300 specifically inhibits *T. cruzi* amastigote mitochondrial respiration with high efficiency *in situ*. (A) Dose-dependent inhibition of isolated *T. cruzi* amastigote mitochondrial respiration (OCR) with ELQ300 has a maximal effect at 1 µM resulting in >90% inhibition. Mean ± SD shown for 3 technical replicates from a representative experiment. One-way ANOVA with Dunnett’s multiple comparisons test was applied. ** p < 0.01, *** p < 0.001. (B) ELQ300 does not inhibit host cell (HFF) mitochondrial respiration even at 10 µM. Student’s t-test was applied. Mean ± SD shown for 3 technical replicates from a representative experiment. (C-H) Use of patient dermal fibroblasts with complex III ETC deficiency to validate ELQ300 as a tool to distinguish parasite and host contributions to total OCR signal in *T. cruzi*-infected monolayers. The CIII mutant line, which has a mutation in subunit *BCS1L* of ETC complex III (Fernandez-Vizarra et al. 2007), displays reduced basal OCR (D) and spare respiratory capacity (SRC) (E) as compared to controls. One-way ANOVA with Dunnett’s multiple comparisons test was applied. *** p < 0.001, **** p < 0.0001. (F-H) ELQ300 treatment of uninfected and *T. cruzi*-infected fibroblasts at 48 hpi was used to inhibit *T. cruzi* OCR signal in CIII mutant fibroblasts (F) and Control fibroblasts (G-H). ELQ300 inhibits 90% of the increase in respiration in *T. cruzi*-infected CIII mutant cells. Mean ± SD shown for ≥3 technical replicates.
Figure A3.2 (Continued) from a representative experiment. Two-way ANOVA with Tukey’s multiple comparisons test was applied. * p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001.
Figure A3.3. Flow cytometry of mCherry and ATP5B indicate increased mitochondrial content in *T. cruzi*-infected mammalian cells. (A) Dose-response of mCherry fibroblasts to valproic acid treatment for 48 h as determined by flow cytometry. (B) Flow cytometric detection of *T. cruzi*-containing (GFP+) mito-mCherry fibroblasts in infected monolayers at 48 hpi and 66 hpi as compared to uninfected controls. (C) Increased expression of endogenous host mitochondrial protein, ATP5B, in *T. cruzi*-containing (GFP+) fibroblasts at 48 hpi as detected by flow cytometry. Each graph displays ≥10,000 events per condition and shows data from a representative experiment.
Figure A3.4. Alamethicin permeabilizes *T. cruzi* amastigotes. Isolated amastigotes were treated with alamethicin and processed for flow cytometric determination of permeabilization using propidium iodide (PI) exclusion as previously described (Shah-Simpson et al. 2016). Permeabilization with 4% PFA was used as a positive control.