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The Unconventional Amino Acid Starvation Response of the Malaria Parasite, Plasmodium falciparum

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by

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ABSTRACT OF THE DISSERTATION

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The apicomplexan parasite, *Plasmodium falciparum*, is the causative agent of the most severe form of malaria, resulting in nearly 1 million deaths each year. The parasite establishes its replicative niche within human erythrocytes, where it degrades massive amounts of host cell hemoglobin, salvaging the released amino acids for its own use. However, human hemoglobin does not contain the amino acid isoleucine, which is one of the most prevalent amino acids in the parasite's proteome. Since *P. falciparum* cannot synthesize isoleucine, it must acquire this amino acid from human serum. Optimal growth and, ultimately, the survival of *P. falciparum* depend on the availability of circulating essential nutrients such as isoleucine, which is often scarce in undernourished malaria patients.

To understand how *P. falciparum* responds to isoleucine starvation, we monitored parasite growth in isoleucine-limiting conditions. We observed that *in vitro* parasite growth is notably slower in medium containing low concentrations of isoleucine, but

completion of the life cycle, consisting of steady progression through the ring, trophozoite, and schizont stages, followed by subsequent rounds of re-invasion and gradual expansion of the culture, continues at a reduced rate. However, when subjected to isoleucine starvation, parasites progress only through the trophozoite stage. Interestingly, supplementation with isoleucine restores normal asexual growth, suggesting the involvement of sensory/response elements in the growth control mechanism of the parasite. The focus of this thesis was to characterize the dynamic metabolic properties of this remarkable starvation-induced state in *P. falciparum* and uncover the molecular basis behind this response.

In this work, it was found that isoleucine starvation effectively slows down the metabolic growth of *P. falciparum*, resulting in cell cycle inhibition, reduced protein translation, and delayed gene expression. Although appreciable parasite growth could be recovered upon isoleucine repletion even after several days of starvation, active proteolysis during extended starvation was required to maintain viability. The canonical amino acid-starvation responsive GCN2/ eIF2α signaling pair is functionally conserved in *P. falciparum*, exhibiting remarkable specificity in detecting isoleucine availability, however, its activity was not essential to preserving the parasite in a growth-competent state during starvation. These data indicate that the starvation response of *P. falciparum* is unique: although the parasite maintains an active remnant of a conventional eukaryotic amino acid-stress response pathway, its regulatory role is inconsequential. We conclude that isoleucine starvation induces a hibernating state in *P. falciparum*, an effective default pathway suitable for its parasitic lifestyle.

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CHAPTER I:

INTRODUCTION

Malaria: A global health and humanitarian crisis

Malaria remains a major threat to public health in many developing countries. This dreadful disease continues to affect roughly 200 to 500 million lives globally each year, causing the deaths of nearly one million people [1, 2], making it one of the deadliest infectious diseases known to man. Sadly, children under the age of five are the unfortunate victims who make up most of this alarming death toll [3]. This disease predominately affects those living in tropical and sub-tropical climates, which are found in areas such as Central and South America, India, Southeast Asia, and Africa, with the majority of malaria cases and fatalities occurring in sub-Saharan Africa [1] **(Figure 1)**. Commonly referred to as a disease of the poor, the economic plight of the regions most affected by malaria is further exacerbated by rising expenses for continuous preventative care, increased costs for government-managed healthcare programs, and work-absenteeism due to disease-related illness or, worse, death, amounting to over US$12 billion in revenue losses each year [4].

The causative agent of malaria is an apicomplexan, protozoan parasite from the *Plasmodium* genus, which first infects a mosquito vector, allowing for transmission to a secondary host upon blood meal acquisition via mosquito bite. Of the five *Plasmodium* species known to cause disease in humans [2], *Plasmodium falciparum* is the culprit behind the deadliest form of human malaria. Symptoms of malaria include joint and muscle pain, nausea, vomiting, and headaches, which are all common signs of the general malaise associated with many other types of infection. However, malaria infection is distinctly characterized by cyclical waves of fever and chills, the timing of which coinciding with the remarkably synchronous growth cycle of the parasite [5]. Severe

malaria infection often results in acute anemia due to the mass destruction of host red blood cells (RBCs) parasitized by *Plasmodium* [1]. Other complications such as hypoglycemia, metabolic acidosis, spleen enlargement, and kidney failure also contribute to the clinical pathology of the disease [5]. A major and often fatal complication of falciparum malaria arises once the parasite enters the brain, leading to cerebral malaria. When the disease progresses to cerebral malaria, patients experience seizures and impaired consciousness, which can escalate to coma and ultimately death [5]. Such serious complications often account for the high mortality rate of this disease in young children.

With proper and timely treatment, patient outcome following malaria infection is usually promising. However, re-infection rates remain high in malaria-endemic regions [1], thus continuing the vicious cycle of disease and poverty. Several efforts have been made to curb the endemic spread of this odious parasite, including the use of insecticide-treated bed nets for vector control and prophylactic administration of anti-malarial drugs [5]. Unfortunately, such efforts have proved inadequate given that *P. falciparum* continues to thrive. This is partly due to the many financial, logistical, and compliance barriers associated with preventative care in the developing world [5]. But also, importantly, the parasite is adapting to the once potent arsenal used to kill it, becoming increasingly resistant or tolerant to many of the currently available therapies [6]. Further compounding the problem, attempts to develop an effective vaccine have been, to date, largely unsuccessful [7]. Therefore, there remains a great need to continue studying malaria, in the hope that the future development of rational therapeutics will outpace the devious evolution of this deadly parasite.

Growth and development of *Plasmodium*

The complex life cycle of *P. falciparum* consists of both primary and secondary hosts: the mosquito vector and human, respectively **(Figure 2)**. During a blood meal, infected female *Anopheles* mosquitoes inject the sporozoite form of the parasite into the bloodstream of the human host upon biting. The sporozoites migrate from the site of the initial bite to the liver, where they invade the residing hepatocytes and differentiate into exoerythrocytic forms [8, 9]. The liver stage in falciparum malaria takes place for a period of up to 2 weeks. During this time, the parasite undergoes multiple rounds of replication, generating thousands of merozoites, which upon synchronized release, leave the liver, re-enter the bloodstream, and go on to infect erythrocytes [10].

In the RBC, *P. falciparum* undergoes three distinct intraerythrocytic stages during its characteristic 48-hour asexual development. First, upon invasion, the merozoite invaginates the RBC membrane, creating a cup-shaped form known as the ring-stage. This stage lasts for up to 24 hours and can be likened to a G0/G1 phase in cell cycle terms, given that little metabolic activity occurs here [11]. Second, the parasite transitions into the highly metabolically active trophozoite stage, lasting for 12 to 14 hours. During this stage, the parasite enters its G1 phase, acquiring nutrients required for its growth, hydrolyzing most of the host cell hemoglobin, and increasing its size [12]. In addition, initiation of DNA replication, or S-phase, takes place toward the latter end of the trophozoite stage [11, 13]. Third, the parasite continues S-phase and enters the G2- and M-phases in the schizont stage, lasting for approximately 10 hours, where multiple copies of its DNA are generated, equally partitioned, and packaged in up to 32 individual merozoites, the invasive ring-stage precursor [14]. The resulting merozoites rupture the

host RBC and quickly go on to infect new RBCs, repeating multiple rounds of asexual development, thus exponentially increasing the parasite's population while depleting the healthy RBC count of the human host. With regular coordinated invasion/ egress cycles, it is during the erythrocytic asexual proliferation of the parasite that the infected individual first begins to exhibit symptoms of malaria [15].

A portion of the merozoites exit asexual development and differentiate into male or female gametocytes, the sexual forms of the parasite [16]. RBCs containing mature asexual trophozoites and schizonts tend to sequester along the capillary endothelium due to the presentation of parasite-derived adhesive structures on the surface of the infected RBC known as knobs [17, 18]. These structures are largely absent on RBCs containing mature gametocytes and young asexual parasites, therefore, these forms are often enriched in the peripheral blood [19]. This enrichment allows the gametocyte forms to be readily taken up by a mosquito during a blood meal. It is in the mosquito where the sexual phase of the parasite initiates, ultimately giving rise to the sporozoite form which can again be transmitted to another human host, thus completing the cycle.

Nutrient acquisition in Plasmodium and the essentiality of isoleucine

Obligate intracellular organisms often lose the ability to make certain metabolites due to genome condensation, a selective process that dispenses genes coding for effector proteins or even entire enzymatic pathways when the desired end-product is abundantly available from the parasitized host [20]. According to genome analysis [21], *P. falciparum* does not encode the enzymes necessary to synthesize several biologically relevant molecules such as sugars [22], purine nucleotides [23], the B_5 vitamin

pantothenate [24], and a number of amino acids [25]. Therefore, the parasite is dependent on the host to supply it with these essential molecules to sustain its growth.

In the case of sugar utilization, extracellular glucose is imported and metabolized for energy production by both the host RBC and the parasite [26]. During intraerythrocytic development, the rapid consumption of glucose by the parasite requires that it be continuously available, particularly since *P. falciparum* does not maintain a surplus energy store [27]. In terms of nucleotide uptake, the parasite depends on a purine-salvage pathway that allows it to import and convert various extracellular purine derivatives and nucleosides to purine nucleotides used for DNA and RNA synthesis [28]. Regarding the B_5 vitamin pantothenate, which is used to derive the ubiquitous metabolic cofactor, coenzyme A (CoA), the parasite imports this molecule via new permeability pathways (NPPs) introduced into the RBC membrane during the course of an infection [24, 29, 30]. For amino acids, which are required for protein synthesis and serve as substrates for use in other metabolic pathways [31, 32], the parasite can utilize three methods of acquisition: 1) catabolism of the host cell hemoglobin [33], 2) *de novo* biosynthesis (of only a few) [25], and 3) active uptake of extracellular free amino acids via NPPs [26].

All of the molecules discussed above have been found to be essential to support the optimal growth and development of the parasite [34]; however, in terms of the amino acid requirements of *P. falciparum*, it appears that only exogenous isoleucine is necessary to sustain its continuous intraerythrocytic growth [33]. This is contrary to previous reports that determined that *P. falciparum* growth depends on the exogenous supply of at least seven amino acids, namely tyrosine, proline, cysteine, glutamate, glutamine,

methionine, and isoleucine [34]. The parasite maintains enzymes for the biosynthesis of three of these amino acids [25] and all, except the latter, can be obtained through hemoglobin degradation, as isoleucine is the only amino acid not present in adult human hemoglobin [35, 36]. Considering that the parasite is also unable to synthesize any of the branched-chain amino acids [25], it follows that isoleucine must be acquired from an extracellular source (*i.e.* human serum or supplied in the *in vitro* culture medium).

The essentiality of isoleucine to the growth of *P. falciparum* was discovered upon monitoring the progression of its intraerythrocytic developmental cycle (IDC) while maintained in various amino acid-dropout media conditions. Only in the absence of isoleucine did parasites fail to proliferate. Furthermore, parasite growth was virtually normal when only isoleucine was supplemented to amino acid-free culture medium, thus requiring the parasite to obtain all other amino acids from hemoglobin degradation or potentially, in the case of 6 amino acids, *de novo* biosynthesis [33]. It is possible that the addition of other amino acids in earlier studies may have appeared to enhance parasite growth due to the *in vitro* culturing peculiarities of the parasite strain used in the analysis. Nonetheless, the updated study discussed above clearly disputes the absolute requirement for extracellular amino acids other than isoleucine.

The host RBC is equipped with an endogenous transport system suitable for the uptake of neutral amino acids [26]. However, it has been shown that *P. falciparum* infected RBCs take up amino acids, including isoleucine, more efficiently than uninfected RBCs [37]. This increase in amino acid flux is mediated by the new permeation pathway, a parasite-derived transport system that increases the permeability of the RBC membrane to various small molecules [30]. The traversal of substrates across

the parasitophorous vacuolar membrane (PVM) appears to be via large, non-selective pores [38] and uptake across the parasite plasma membrane (PPM) is mediated by parasite-encoded integral membrane transporters, some of which have been identified and characterized as having substrate-specific properties [39]. In regard to isoleucine flux, it has been shown that the parasite most likely uses an antiport system in which intracellular leucine, presumably released from digested hemoglobin, is exchanged for extracellular isoleucine, which rapidly accumulates within the parasite [37]. Considering that *P. falciparum* is wholly dependent on an external supply of isoleucine, the transporter that mediates its uptake is naturally regarded as a critical antimalarial target.

Impact of nutrient stress in a malaria infection

In the context of a human infection, the nutritional status of malaria patients has been implicated in modulating the severity of the disease [40]. As mentioned previously, malaria-endemic regions are commonly burdened with extreme poverty, which in most cases directly correlates with poor nutrition [41]. Malnourished patients experience a variety of nutritional deficiencies (*e.g.* iron, vitamins A, B, C, and E, zinc, folate, protein), which can directly (competition between the parasite and the host for a limited supply of essential nutrients) or indirectly (diminished capacity of the host immune system to fight infection) impact parasite burden [42-46].

In the case of protein malnutrition and malaria infection, human clinical studies have yielded conflicting data regarding the effects of malnutrition on patient mortality [46-50]. However, in more controlled animal studies in which malaria-infected primates or rodents were fed protein-restricted diets, the data consistently showed that although the

animals failed to clear the infection, parasite burden remained low and prevalence of cerebral malaria was virtually absent [51-53]. Interestingly, in the rodent studies, once the low-protein diets were supplemented with the amino acids threonine, methionine, valine, and isoleucine, the animals experienced a surge in parasitemia with accompanying morbidity, while the addition of other amino acids did not have this effect [54].

Of note, rodent hemoglobin contains all of the amino acids, including isoleucine, which is present in low abundance [35], therefore unlike human malaria parasites, rodent malaria species are not totally dependent on an extracellular source of this amino acid. However, optimal *in vitro* growth of *P. falciparum* has been shown to require an isoleucine concentration above 20 μM [33]. Provided that rodent malaria species have similar growth requirements, the dramatic outgrowth of the parasite upon re-feeding suggests that *Plasmodium* may be capable of modulating its growth cycle in response to nutrient availability. With regard to human malaria, this premise is particularly intriguing, considering that in malnourished children, plasma isoleucine levels often fall well below 20 μM [55] [normal serum isoleucine concentrations in healthy individuals are typically in the 80 - 100 μM range [56]], which is correlative with protein limitation, since in mammals, this amino acid is essential and must be acquired through the diet [25].

In the earlier human clinical studies mentioned above, malaria-infected patients most likely suffered from multiple nutrient deficiencies in addition to protein malnutrition, hence the ambiguity regarding the impact of malnutrition on parasite growth and disease progression. However, the protein-restriction studies carried out with rodent and primate malarias indicate that the parasite exhibits some degree of amino acid-

sensitive growth regulation, which could represent a stress response mechanism conserved in *Plasmodium spp*.

Parasite stress response

Stress response mechanisms allow organisms to adapt to and survive in less than favorable conditions. Malaria parasites also utilize various mechanisms that allow them to overcome host defenses and establish successful infections [57-60]. Regarded as a general response to stress in the parasite, commitment to gametocytogenesis confers a degree of protection against the harsh host environment [61], since gametocytes are terminally differentiated and effectively metabolically inert at maturation [62]. However, asexual parasites are considerably more vulnerable, therefore blood-stage parasites must use alternative means to survive the volatile conditions of the host environment, which, during the course of an infection, experiences fluctuations in temperature, oxidative bursts, and nutrient shortages [63].

A drug-tolerance mechanism associated with reduced susceptibility to the antimalarial artemisinin has been recognized as a stress response utilized by the parasite to withstand drug pressure [64, 65]. This protection from killing stems from an apparent metabolic shift that gives rise to a putative dormant state, which stalls parasite development at the ring stage [65]. It seems that once drug therapy ends, the parasite reanimates and continues its asexual developmental cycle, consequently leading to recrudescent malaria infections [66]. This concept of stress-induced dormancy further suggests that *P. falciparum* has the capacity to control its growth in a signal/ response-type relay. In other organisms, such processes often affect critical cellular functions (*e.g.*

energy production, replication, transcription, translation) and require several layers of regulation [67], an area that remains ill-defined in *Plasmodium*.

Although the putative quiescence mechanism of *P. falciparum* is not well-understood, stress-induced dormancy is a phenomenon exhibited by various organisms, including a related parasite, *Toxoplasma gondii*, the causative agent of toxoplasmosis [68]. *T. gondii* parasites exist in two forms: the proliferative tachyzoite, which can infect virtually any nucleated cell in a broad range of hosts; and the latent cyst bradyzoite, which can reside in host tissues indefinitely with the potential to reactivate [68]. The ability of *T. gondii* to enter into a dormant phase allows for the establishment of persistent, chronic infections. When exposed to stress, actively growing tachyzoites convert to the dormant bradyzoite form, a process associated with reduced cell cycle activity [69], up-regulation of heat shock proteins [70], and translational repression [71]. Various environmental stresses have been shown to trigger bradyzoite differentiation, including amino acid starvation [72], which has a well-characterized role in controlling the cellular growth of eukaryotic organisms [73, 74].

Mechanisms of growth control

Virtually all eukaryotic organisms possess a signaling protein known as the Target of Rapamycin (TOR), which functions as a master regulator, promoting cellular growth and viability under growth-permissive conditions [73]. However, the TOR complex is also negatively regulated by stimuli such as nutrient starvation, which leads to a decrease in anabolic processes (e.g. protein synthesis) and an increase in catabolic processes (e.g. autophagy) in an effort to adapt to the changing environment and maintain

cellular integrity [75]. This highly conserved protein belongs to the phosphatidylinositol kinase-related kinase (PIKK) family, whose members are characterized by a serine/ threonine protein kinase domain located at the carboxy terminal (C-terminal) end of the protein [73]. TOR derives its name from the inhibitory effects of the immunosuppressant antibiotic rapamycin, which interacts with the cellular cofactor FK506-binding protein (FKBP) before binding to and, subsequently, inhibiting the growth-promoting activity of TOR [76]. Both an FKBP-like protein and a putative TOR have been identified in the apicomplexan parasite *T. gondii*, further demonstrating the extensive evolutionary conservation of this protein [77]. Interestingly, a single FKBP homolog has also been identified and characterized in *P. falciparum* [78], however genome sequence data indicates that homologs for TOR and its accompanying signaling components are absent in *Plasmodium* [79]. This discrepancy is particularly intriguing since previous reports have demonstrated that rapamycin has considerable antimalarial activity [78, 80]. Nonetheless, the antimalarial mechanism of rapamycin in *Plasmodium* appears to be independent of conventional TOR-mediated signaling.

A second pathway that is central to controlling cellular growth is the eukaryotic initiation factor 2-alpha (eIF2α)-signaling pathway **(Figure 3)**. This pathway mediates the activation of an adaptive transcriptional response to nutrient limitation, including amino acid starvation. Phosphorylation of a conserved serine residue in eIF2α by its cognate sensor kinase results in decreased overall protein synthesis and growth inhibition. At the same time, there is increased transcription of genes involved in compensatory pathways such as those that function in amino acid biosynthesis [81].

Recent reports have described the relative importance of eIF2α-mediated signaling in the life cycle of protozoan parasites. For instance, phosphorylation of eIF2α plays a role in maintaining *T. gondii* parasites in the dormant bradyzoite cyst form [71]. Furthermore, in the sexual phase of *Plasmodium*, eIF2α kinase-mediated signaling in mosquito-stage sporozoites prevents premature conversion to the liver-stage form [82]. Also in *P. falciparum*, a putative eIF2α kinase, PfPK4, was identified [83] and deemed essential for blood stage development given that the genetic locus coding for this kinase could not be disrupted [84]. In addition to the two *Plasmodium* eIF2α kinases mentioned above, kinome profiling revealed that the parasite also maintains a third eIF2α kinase ortholog, expressed during the blood stage, with similarities to a known amino acid-starvation-sensing eIF2α kinase, GCN2, which is highly conserved from yeast to humans [85, 86].

Aim and Scope of Thesis

Substantial advancements have been made in elucidating the pathogenic properties of *Plasmodium*. However many aspects concerning the complex biology of this seemingly simple parasite remain virtually unknown. The aim of this thesis was to address questions regarding how *P. falciparum* regulates its growth, particularly during amino acid limitation, with intent to determine elements involved in its mode of persistence.

Early examination of the nutrient requirements of *P. falciparum* revealed that the parasite is completely dependent on an extracellular source of the amino acid isoleucine [33, 34]. Furthermore, the *in vitro* proliferation of the parasite is inhibited when grown in

the absence of isoleucine [33]; and *in vivo* studies with rodent malarias indicate that the robustness of the parasite's growth cycle is finely tuned with the composition of the external amino acid environment [52, 54]. In addition, homology searches indicate that *Plasmodium* maintains an ortholog of GCN2, an amino acid-starvation sensitive eIF2α kinase known to regulate growth in response to nutrient availability in other organisms [81].

Previous studies have demonstrated that eIF2α kinase activity is present in *Plasmodium* [82, 83, 87]. However there is little evidence linking any of the identified kinases with a specific activating stress or trigger, a key feature that ultimately defines the specialized regulatory function of this kinase family. In regard to the putative GCN2 ortholog, this matter is directly addressed in chapter 2 of this thesis. The identity of the amino acid-starvation sensitive eIF2α kinase in *P. falciparum* was experimentally confirmed using kinase-knockout parasite lines generated for this study, followed by the assessment of the phosphorylation status of eIF2α in these parasites under both amino acid-rich and limiting conditions. Additionally, data presented in chapter 3 examined whether the isoleucine dependence of *P. falciparum* conferred exclusive specificity to the nutrient-sensing function of the identified kinase.

Further metabolic characterization of isoleucine-starved *P. falciparum* is presented in chapter 3. Interestingly, genome analysis of *Plasmodium* indicates that the parasite lacks orthologs of the effector proteins that are known to function downstream of GCN2-regulated signaling in higher eukaryotes (*i.e.* GCN4/ATF4 [74, 81, 88]). Considering that *Plasmodium* is generally deficient in regulatory transcription factors [89-91], coupled with the fact that the parasite also has lost the TOR pathway [79], the

absence of GCN2/eIF2α pathway mediators suggests that the tightly-regulated, conventional stress response mechanisms common to free-living organisms are not required by the obligate intracellular parasite to sustain its growth and viability. In short, data presented in chapter 3 of this thesis indicate that the amino acid starvation-associated growth control measures employed by *Plasmodium* have been reduced to their simplest, most elementary form, functioning irrespective of canonical translational control, with features characteristic of hypometabolism. These features were inconsistent with those described for the artemisinin-associated putative dormant state of *P. falciparum* [65, 92], thus the amino acid starvation-induced hypometabolic state represents a novel stationary phase that apparently extends the life of the parasite by delaying its growth. This thesis provides the first description of this phenomenon in blood stage *P. falciparum*.

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Figure Legends

Figure 1. Illustration of global malaria prevalence in 2010

Source: World Health Organization (WHO)

Figure 2. Life cycle of *Plasmodium falciparum*

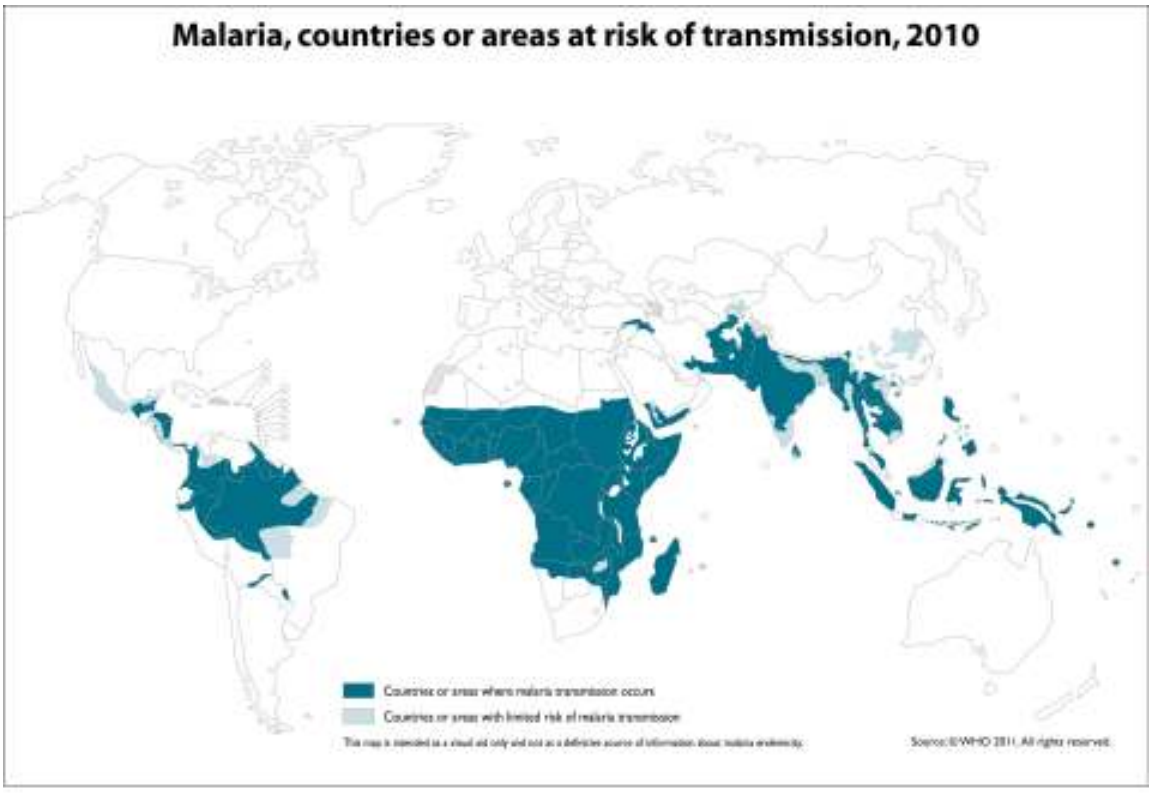
Source: Centers for Disease Control and Prevention (CDC)

http://www.dpd.cdc.gov/dpdx

Figure 3. Schematic illustration of eIF2α pathway activation via amino acid starvation.

Amino acid starvation activates the eIF2α kinase GCN2, which goes on to phosphorylate a conserved serine residue in the translation effector eIF2α. The appended phosphate moiety hinders eIF2B-mediated GTP loading of eIF2α, which is required to initiate productive protein synthesis. With eIF2α phosphorylated, translation becomes rate-limiting, thereby decreasing general protein synthesis, ultimately leading to growth inhibition. Although global translation is decreased, eIF2α phosphorylation leads to increased expression of the stress-responsive transcription factor GCN4 due to altered ribosomal scanning of GCN4 transcripts [81]. GCN4 then goes on to selectively activate the transcription of genes involved in the adaptive response.

Figure 1



The boundaries and names shown and the designations used on this map do not imply the expression of any opinion whatsoever on the part of the World Health Organization concerning the legal status of any country, territory, city or area or of its authorities, or concerning the delimitation of its frontiers or boundaries. Dotted lines on maps represent approximate border lines for which there may not yet be full agreement.

World Health Organization

Figure 2

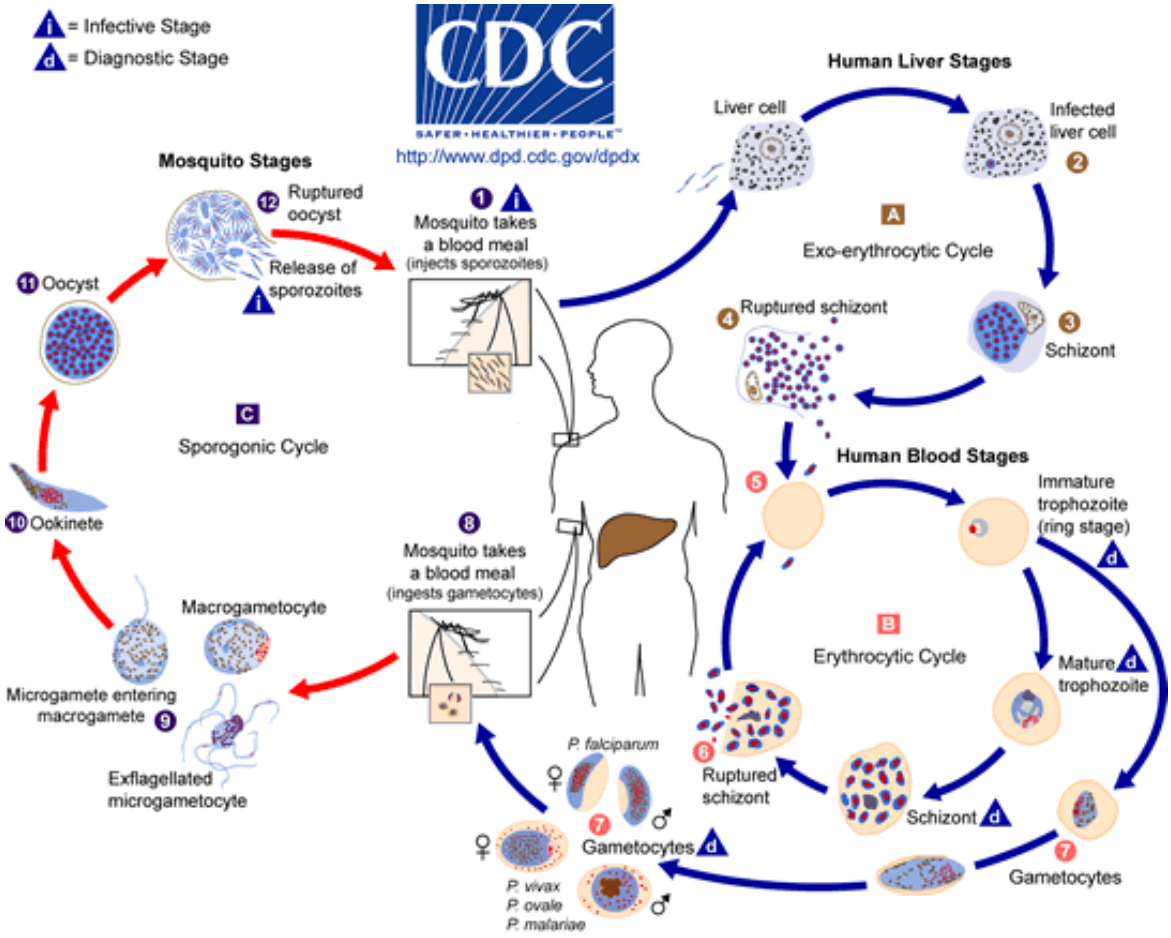
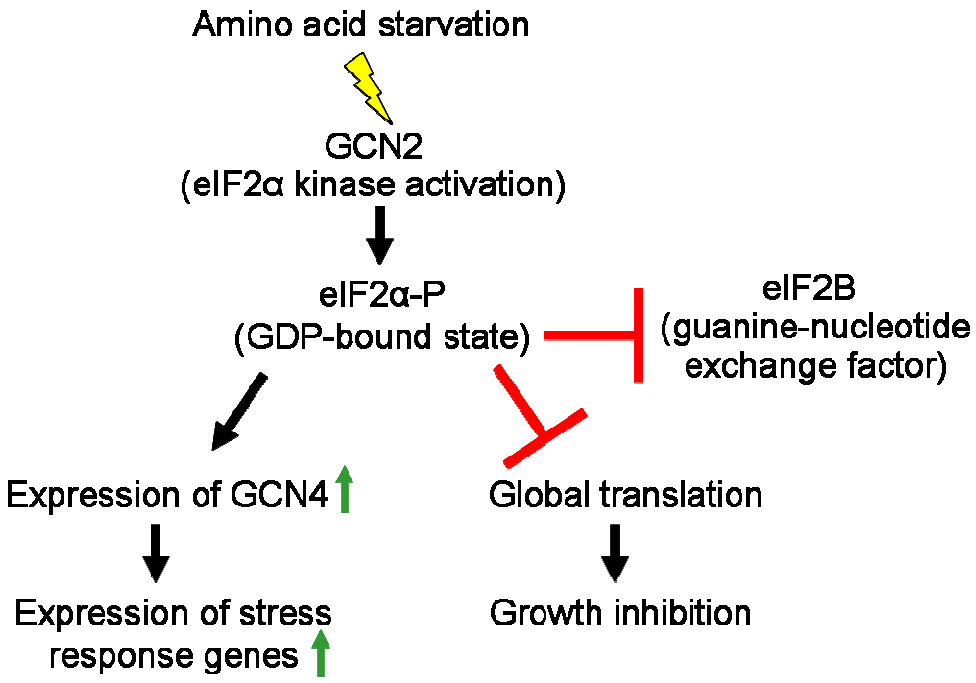

i = Infective Stage
d = Diagnostic Stage
CDC
SAFER·HEALTHIER·PEOPLE™
http://www.dpd.cdc.gov/dpdx
Human Liver Stages
Liver cell
Infected liver cell
2
A
Exo-erythrocytic Cycle
3
Schizont
4
Ruptured schizont
Human Blood Stages
5
Immature trophozoite (ring stage)
d
B
Erythrocytic Cycle
Mature trophozoite
d
6
Ruptured schizont
Schizont
d
7
Gametocytes
P. falciparum
♀
♂
7
Gametocytes
d
P. vivax
P. ovale
P. malariae
♀
♂
8
Mosquito takes a blood meal
(ingests gametocytes)
Macrogametocyte
Microgamete entering macrogamete
9
Exflagellated microgametocyte
10
Ookinete
11
Oocyst
12
Ruptured oocyst
Release of sporozoites
i
C
Sporogonic Cycle
Mosquito Stages
1
i
Mosquito takes a blood meal
(injects sporozoites)

Figure 3


Amino acid starvation
GCN2
(eIF2α kinase activation)
eIF2α-P
(GDP-bound state)
eIF2B
(guanine-nucleotide
exchange factor)
Expression of GCN4
Global translation
Expression of stress
response genes
Growth inhibition

CHAPTER II:

PFEIK1 IDENTIFIED AS THE AMINO ACID-STARVATION RESPONSIVE EIF2A KINASE IN *PLASMODIUM FALCIPARUM*

Preface

Work presented in this chapter was conducted by SEL (SB), CF, IR, JW, and LRC. SEL (SB) performed parasite starvation assays, immunoblotting analysis, and drafted portions of the manuscript relevant to these experiments and techniques. The contributions of the other authors are provided on page 50 of this chapter. Subsequent data in chapter 3 of this thesis refute the regulatory role of PfeIK1 in the amino acid starvation response of *P. falciparum* proposed here. However, the data presented in this chapter established the functional role of PfeIK1 in the sensing of amino acid starvation in *P. falciparum*.

This chapter is reprinted here essentially as published:

Fennell C*, Babbitt S*, Russo I, Wilkes J, Ranford-Cartwright L, Goldberg DE, and Doerig C. **PfeIK1, a eukaryotic initiation factor 2α kinase of the human malaria parasite Plasmodium falciparum, regulates stress-response to amino acid starvation.** Malaria J. 2009 May 12; 8:99.

*These authors contributed equally to this work

Abstract

Background

Post-transcriptional control of gene expression is suspected to play an important role in malaria parasites. In yeast and metazoans, part of the stress response is mediated through phosphorylation of eukaryotic translation initiation factor 2α (eIF2α), which results in the selective translation of mRNAs encoding stress-response proteins.

Methods

The impact of starvation on the phosphorylation state of PfeIF2α was examined. Bioinformatic methods were used to identify plasmodial eIF2α kinases. The activity of one of these, PfeIK1, was investigated using recombinant protein with non-physiological substrates and recombinant PfeIF2α. Reverse genetic techniques were used to disrupt the *pfeik1* gene.

Results

The data demonstrate that the *Plasmodium falciparum* eIF2α orthologue is phosphorylated in response to starvation, and provide bioinformatic evidence for the presence of three eIF2α kinases in *P. falciparum*, only one of which (PfPK4) had been described previously. Evidence is provided that one of the novel eIF2α kinases, PfeIK1, is able to phosphorylate the *P. falciparum* eIF2α orthologue *in vitro*. PfeIK1 is not required for asexual or sexual development of the parasite, as shown by the ability of *pfeik1*$^{-}$ parasites to develop into sporozoites. However, eIF2α phosphorylation in response to starvation is abolished in *pfeik1*$^{-}$ asexual parasites.

Conclusions

This study strongly suggests that a mechanism for versatile regulation of translation by several kinases with a similar catalytic domain but distinct regulatory domains, is conserved in *P. falciparum*.

Background

Human malaria is caused by infection with intracellular protozoan parasites of the genus *Plasmodium* that are transmitted by *Anopheles* mosquitoes. Of four species that infect humans, *Plasmodium falciparum* is responsible for the most virulent form of the disease. The transition from one stage of the life cycle to the next must be tightly regulated, to ensure proliferation and differentiation occur when and where appropriate; this is undoubtedly linked to differential gene expression. Analysis of the *P. falciparum* transcriptome during the erythrocytic asexual cycle reveals an ordered cascade of gene expression [1], and the various developmental stages display distinct transcriptomes; how this is orchestrated remains obscure. Initial investigation of the *P. falciparum* genome revealed a paucity of transcriptional regulators [2], although this picture has recently been challenged by the recent identification of the ApiAP2 transcription factor family [3]. There is nevertheless a large body of evidence suggesting that post-transcriptional control is an important means of gene regulation in *P. falciparum*. Examples include the relatively small number of identifiable transcription-associated proteins, abundance of CCCH-type zinc finger proteins commonly involved in modulating mRNA decay and translation rates [2] and translational repression during gametocytogenesis [4-6].
In mammalian cells, regulation of gene expression is a key mechanism in the mediation of stress responses, which may be achieved by influencing transcription or translation. The Stress Activated Protein kinases (SAPKs), specifically JNKs and p38 kinases, are subfamilies of mitogen activated protein kinases (MAPK) that are expressed in most eukaryotic cells, and respond to a variety of stress conditions [7]. Although the parasite kinome includes two MAPK homologues, none of these are members of the SAPK

subfamily [8-10]. In contrast, the *P. falciparum* kinome contains a phylogenetic cluster of three kinases with homology to eukaryotic Initiation Factor 2α (eIF2α kinases, which in other organisms regulate translation in response to stress [10]. Interestingly, the related apicomplexan parasite *Toxoplasma gondii* has been shown to differentiate from tachyzoites to bradyzoites on exposure to a number of cellular stresses, concomitant with an increase in phosphorylation of TgeIF2α, indicating a possible role for this mechanism in parasite differentiation [11].

Phosphorylation of eukaryotic initiation factor 2α at residue Ser51 in response to stress is a well-characterized mechanism of post-transcriptional control that regulates initiation of translation [12-17]. In mammalian cells this phosphorylation event is mediated by four distinct protein kinases, called the eIF2α kinases: general control non-derepressible-2 (GCN2), haem-regulated inhibitor kinase (HRI), RNA-dependent protein kinase (PKR), and PKR-like endoplasmic reticulum kinase (PERK). These enzymes contain a similar catalytic domain allowing them to phosphorylate the same substrate, but have different accessory domains that regulate kinase activation in response to different signals. In GCN2 the functional kinase domain is followed by a histidyl-tRNA synthetase (HisRS)-like domain [18], which is the major motif for activation in response to amino acid starvation; PERK has a transmembrane domain allowing it to reside in the endoplasmic reticulum membrane; the N-terminal domain can protrude into the lumen of the ER to sense unfolded proteins, while the catalytic domain extends into the cytoplasm where its substrate and effector mechanism lie; human PKR contains an RNA binding domain and responds to viral infection; and HRI contains haem binding sites to modulate

translation of globin chains according to the availability of haem. In this way the eIF2α kinases can integrate diverse stress signals into a common pathway [12-14, 19].

Translation initiation requires the assembly of the 80S ribosome on the mRNA, which is mediated by proteins known as eukaryotic initiation factors (eIFs). Formation of the 43S pre-initiation complex depends on binding of the ternary complex that consists of the heterotrimeric G-protein eIF2 (a, b and g subunits), methionyl-initiator tRNA (met-tRNAi) and GTP [13]. Initiation of translation and release of the initiation factors involves hydrolysis of GTP to GDP, which leaves an inactive eIF2-GDP complex. Before further rounds of translation initiation can occur eIF2 must be reactivated by exchange of GDP for GTP [13]. The presence of a phosphate group on the a subunit of eIF2 inhibits recycling of inactive eIF2-GDP to active eIF2-GTP by limiting the activity of the guanine nucleotide exchange factor, eIF2B [20]. The consequence of activity of the eIF2α kinases therefore is global translation repression, since initiation complexes cannot form. In spite of the generalized reduction in translation, some specific mRNAs are translated, whose products shapes the subsequent stress response. Reduced translation conserves energy and nutrients, allowing time for the cell to adapt appropriately to the stress conditions. This mechanism is conserved in the vast majority of eukaryotes. One notable exception is the Microsporidium *Encephalitozoon cuniculi*, whose kinome does not include eIF2α kinases (or other stress-response kinases), a probable adaptation to its parasitic lifestyle [21]. It is, therefore, of interest to investigate the extent to which malaria parasites may rely on eIF2α phosphorylation for stress-response and/or life cycle progression.

A cluster of three sequences that includes PfPK4, a protein kinase that was previously described as a putative eIF2α kinase [22], was identified in the *P. falciparum* kinome on the basis of catalytic domain similarity [10, 23]. Here, evidence is provided that the *P. falciparum* eIF2α orthologue is phosphorylated in response to amino acid starvation. Bioinformatics analysis reveals that *P. falciparum* encodes three eIF2α kinases, one of which, *Plasmodium falciparum* eukaryotic Initiation Factor Kinase-1 (PfeIK1), is shown here to indeed be able to phosphorylate *P. falciparum* eIF2α *in vitro*. Reverse genetics experiments show that inactivation of the *pfeik1* gene does not affect asexual growth, gametocytogenesis or further sexual development, since *pfeik1*$^{-}$ sporozoites can be formed in the mosquito vector; in contrast, *pfeik1*$^{-}$ parasites are unable to phosphorylate eIF2α in response to amino-acid starvation.

Methods

Bioinformatic analysis

BLASTP analysis was used to identify the closest human and *Plasmodium berghei* orthologues of the PfeIF2α kinases. Catalytic domains of the putative PfeIF2α kinases as defined by the alignment of *P. falciparum* kinases [10] were aligned with the four human eIF2α kinases and other *P. falciparum* and human sequences that were selected to represent all kinase subfamilies. The sequences were aligned using the HMMER package against a profile generated from our previous kinome analysis [10]. After removal of gaps and positions with a low quality of alignment, alternate phylogenies

generated with the neighbour joining method were visualized using NeighbourNet implemented on SplitsTree verion 4 [24].

BLASTP searches of PlasmoDB using metazoan eIF2α sequences were used to identify PF07_0117 as the *P. falciparum* homologue of eIF2α, which was then confirmed by reciprocal analysis. Alignment of these sequences was performed using ClustalW.

Molecular cloning

PfeIK1. A 1278bp fragment encoding the catalytic domain of PfeIK1 (PF14_0423) was amplified from a *P. falciparum* cDNA library using the *Phusion* polymerase (Finnzymes), using the following primers: forward, GGGGGGATCCATGGGGAAAAAAAAACATGG, reverse GGGGGTCGACCGTAAAAAGTACACTTTCGTG. The primers contained *Bam*HI and *Sal*I restriction sites, respectively (underlined). The *Taq* polymerase (Takara) was used to add adenine tails to enable cloning of the product into the pGEM-T Easy vector (Promega) for sequencing. The correct sequence was removed by digestion with *Bam*HI and *Sal*I and inserted into the expression vector pGEX-4T3 (Pharmacia). A catalytically inactive mutant was obtained by site directed mutagenesis of Lys^{458} to Met using the overlap extension PCR method [25] (forward: CTTATGCATTAATGATTATAAG, reverse: CTTATAATCATTAATGCATAAG).

PfeIF2α. Oligonucleotide primers were designed to amplify the complete coding sequence of PfeIF2α (PF17_0117) by PCR from a cDNA library of the *P. falciparum*

clone 3D7, using the *Phusion* polymerase (Finnzymes). The primers used were as follows: forward, GGGGGGATCCATGACTGAAATGCGAGTAAAAGC and reverse, GGGGGTCGACTTAATCTTCCTCCTCCTCGTC (restriction sites are underlined). *Taq* polymerase (Takara) was used to add adenine tails to enable cloning of the 990bp product into the pGEM-T Easy vector (Promega) for amplification and sequencing. The correct sequence was removed by digestion with *Bam*HI and *Sal*I and inserted into the expression vector pGEX-4T3 (Pharmacia). A mutant of PfeIF2α designed to be refractory to phosphorylation was obtained by site directed mutagenesis (Ser^{59} - Ala) using the overlap extension PCR technique [25], (primers: forward, CTTATGCATTAATGATTATAAG, reverse, CTTATAATCATTAATGCATAAG). All inserts were verified by DNA sequencing (The Sequencing Service, Dundee, UK) prior to expression of recombinant proteins or transfection of *P. falciparum*.

Recombinant protein expression

Expression of recombinant GST fusion proteins was induced in *E. coli* (strain BL21, codon plus) with 0.25mM Isopropyl Thiogalactoside (IPTG). After induction, bacteria were grown at 16°C overnight and the resulting bacterial pellets were stored at –20°C until use. All subsequent work was done on ice, centrifugation steps at 4°C. Protein extraction was performed by digestion of bacterial pellets 5 mins using lysozyme (Sigma), followed by 10 mins in lysis buffer (1xPBS, 2mM ethylenediaminetetraacetic acid (EDTA), 1mM dithiothreitol (DTT), 0.5% Triton x100, 1mM Phenyl Methyl Sulphonate (PMSF), Benzamidine Hydrochloride Hydrate (BHH), 1X complete cocktail protease inhibitors (Roche)). Bacterial lysates were sonicated at 20% amplitude

(Bioblock Scientific, Vibracell 72405), 5x 15 sec pulses/ 15 sec rest, and cleared by centrifugation 13000g, 15 mins. GST-fusion proteins were purified by incubation of cleared lysates on glutathione agarose beads (Sigma) for 2 hours, followed by four washes with lysis buffer and eluted for 20 minutes in elution buffer (Tris 40mM, pH8.7, 75mM NaCl, 15mM reduced glutathione). Protein concentration was monitored using the Bradford assay (Biorad reagent) A_{595}nm. Kinase assays were carried out immediately after purification.

Kinase assay

Kinase reactions (30µl) were carried out in a standard kinase buffer containing 20mM Tris-HCl, pH 7.5, 20mM $MgCl_2$, 2mM $MnCl_2$, phosphatase inhibitors; 10mM NaF, 10mM β-glycerophosphate, 10µM ATP and 0.1MBq [γ-^{32}P] ATP, using 2µg recombinant kinase, and 10µg non-physiological substrate (α-casein, β-casein), or recombinant GST-PfeIF2α. Reactions were allowed to proceed for 30 minutes at 30^{o}C and stopped by addition of reducing Laemmli buffer, 3 minutes, 100^{o}C. Samples were separated by SDS-PAGE and phosphorylation of kinase substrates assessed by autoradiography of the dried gels.

***Plasmodium falciparum* genetic manipulation**

A gene disruption plasmid was produced for PF14_0423 in the plasmid pCAM-BSD [26] that contains the gene conferring resistance to blasticidin. The oligonucleotide pair GGGGGGATCCGTAATGAAAGTAAAAAATAAG/ GGGGCGCCGGCGAGGTGAAATATAATGAATTGTTCC, containing *Bam*HI and

NotI sites (underlined) was used to amplify a 789bp fragment for insertion to pCAM-BSD. Ring stage parasites were electroporated with 50 – 100 □g plasmid DNA, as previously described [26]. Blasticidin (Calbiochem) was added to a final concentration of 2.5 □g/ml 48 hours after transfection to select for transformed parasites. Resistant parasites appeared after 3-4 weeks and were maintained under selection. After verification by PCR that *pfeik1*$^{-}$ parasites were present, the population was cloned by limiting dilution in 96 well plates (0.25/0.5/1.0 parasite per well). Genotypic analysis enabled selection of independent *pfeik1*$^{-}$ clones for further phenotypic analysis.

Parasite culture and mosquito infection

Plasmodium falciparum clone 3D7 was cultured as previously described [27]. In brief, asexual cultures were maintained in complete RPMI at a haematocrit of 5%, between 0.5% and 10% parasitaemia. Asexual growth cycle was analyzed by flow cytometric assessment of DNA content as previously described [28]. Gametocytogenesis was induced as described previously [29]; briefly, gametocyte cultures were set up at 0.5-0.7% parasitaemia in 6% haematocrit (using human blood not more than 7 days after the bleed), in an initial volume of 15ml in 75cm^2 flasks. Cultures were maintained for 4-5 days until 8-10% parasitaemia was reached and parasites appeared stressed, after which the volume was increased to 25ml. For each clone a mixture of day 14 and day 17 gametocyte cultures were fed to *Anopheles gambiae*, through membrane feeders as described [29]. Female mosquitoes were dissected 10 days post-infection and midguts examined by light microscopy for presence of oocysts. Sporozoite invasion of salivary glands was assessed by removal of salivary glands 16 days post-infection and

examination by light microscopy. DNA was extracted from oocyst-positive midguts using previously published methods [30]. Fisher's exact test was used to compare infection prevalence between oocyst and sporozoite stages, where appropriate.

Preparation of parasite pellets

Parasite pellets were obtained by saponin lysis: erythrocytes were centrifuged at 1300g for 2 min at room temperature, washed in an equal volume of Phosphate Buffered Saline (PBS), pH 7.5, and centrifuged at 1300g for 2 mins at 4^{o}C. Erythrocytes were lysed on ice by resuspension and repeated pipetting in 0.15% saponin in PBS. The PBS volume was then increased and parasites recovered by centrifugation at 5500g for 5 mins, at 4^{o}C. After two washes in PBS, the parasite pellets were stored at –80^{o}C.

***Plasmodium falciparum* amino acid starvation assay**

Plasmodium falciparum 3D7 parasites and clonal lines of *pfeik1*$^{-}$ and *pfeik2*$^{-}$ parasites were synchronized to the late ring stage, cultured in complete RPMI at 2% haematocrit, and grown to approximately 8 – 10% parasitaemia. The parasites were washed two times in 1x PBS, equally partitioned and washed with either complete RPMI or RPMI medium lacking amino acids, after which, the parasites were re-plated in their respective medium. The plates were incubated at 37°C with 5% CO_2 for 5 hours. After 5 hours, one culture maintained in amino acid free medium was supplemented with complete RPMI, and re-incubated at 37°C for an additional 45 minutes. Post-incubation, the parasites were isolated by tetanolysin (List Biological) treatment, washed with 1x PBS buffer containing Complete^{TM} protease inhibitor cocktail (Roche), 2mM NaF, and 2mM Na_3VO_4. Samples

were resuspended in 2x SDS-Laemmli buffer. Parasite proteins were resolved by SDS-PAGE and transferred to nitrocellulose for immunoblotting.

Antibodies and immunoblotting

Rabbit anti-phosphorylated eIF2α (Ser 51) was purchased from Cell Signaling Technology (Danvers, MA). Rat anti-BiP was acquired from the Malaria Research and Reference Reagent Resource Center (ATCC, Manassas, VA). Secondary antibodies used were conjugated with horseradish peroxidase (HRP). For immunoblotting, nitrocellulose membranes were blocked with 5% BSA in TBS-0.1% Tween 20 (TBST) for 1 hour at room temperature. Rabbit anti-phosphorylated eIF2α (Ser 51) was diluted 1:1000 in TBST. Rat anti-BiP was diluted 1:10,000 in TBST. Respective secondary antibodies were diluted 1:20,000. Bound antibodies were detected with Western Lightning^{TM} Chemiluminescence reagent (Perkin Elmer).

Southern blotting

To obtain genomic DNA, parasite pellets were resuspended in PBS and treated with 150 μg/ml proteinase K and 2% SDS at 55^{o}C for 4 hours. The DNA was extracted using phenol/chloroform/isoamyl alcohol (25:24:1), and precipitated in ethanol with 0.3M sodium acetate at -20^{o}C. Restriction digests were carried out with *Hind*III. Probes were labelled with alkaline phosphatase using the Gene Images AlkPhos Direct Labelling kit (Amersham).

Results and Discussion

Stress-dependent phosphorylation of the P. falciparum *eIF2α orthologue*

BLASTP searches of PlasmoDB using metazoan eIF2α sequences were used to identify PF07_0117 as the *P. falciparum* orthologue, which was confirmed by reciprocal analysis. The alignment of *P. falciparum* eIF2α with sequences from *Toxoplasma gondii,* human, rice and *E. cuniculi* is shown in **Figure 1A**. Overall, the *P. falciparum* sequence shares ~70% identity with *T. gondii* eIF2α and ~ 50%, ~ 40% and ~28% with the orthologues in humans, rice and *E. cuniculi*, respectively. Importantly, the serine that is targeted for phosphorylation is conserved in all species. Furthermore, eIF2α contacts the kinase through a large number of residues that interact with the surface of the kinase domain. These residues are also conserved in most species, as are residues that protect the regulatory serine from the activity of other kinases [31] **(Figure 1A)**; interestingly, several of these are not conserved in the *E. cuniculi* orthologue, which is consistent with the absence of eIF2α kinases in this organism [21].

The presence of the target serine residues, and of residues which in other species are involved in interaction with eIF2α kinases, suggests that PfeIF2α may be regulated by phosphorylation under stress conditions. To test this hypothesis, cultured intraerythrocytic parasites were starved of amino acids, and the phosphorylation status of PfeIF2α was monitored by western blot using an antibody that specifically recognizes the phosphorylated form (Ser51) of human eIF2α, reasoning that the high level of sequence conservation between the human and plasmodial sequences would allow cross-reaction of the antibody **(Figure 1B)**. Indeed, the antibody recognized the expected 37-kDa band in

parasite extract, and the intensity of the signal was considerably stronger in the lane containing extracts from parasites that had been stressed by amino-acid starvation than in extracts from unstressed parasites, despite equal quantities of the eIF2α factor (as quantitated with a non-phosphodependent antibody). Furthermore, this effect was removed by restoring the amino acids in the culture medium. This demonstrates that the *P. falciparum* equivalent residue of human eIF2α Ser51 is phosphorylated in response to starvation.

Identification of eIF2α kinases in P. falciparum

Bioinformatics approaches were then used to identify *P. falciparum* protein kinase(s) potentially responsible for this response. An analysis of the complete complement of *P. falciparum* protein kinases [10] identified a distinct phylogenetic cluster of three sequences, PF14_0423, PFA0380w and PFF1370, the latter of which (called PfPK4) had previously been characterized as an eIF2α kinase [22]. Reciprocal BLASTP analysis using the putative catalytic domains as queries confirmed the homology of these three genes with the eIF2α kinase family. A Hidden Markov Model (HMM) was used to generate an alignment of the three *P. falciparum* sequences with those of human eIF2α kinases; sequences of kinases from other families were used as outgroups. The resulting alignment was used to generate a phylogenetic tree **(Figure 2A)**, which clearly shows that the three *P. falciparum* genes cluster with the eIF2α kinases, as opposed to other kinase families, confirming their relatedness to this family.

Interestingly PfeIK1 (PF14_0423), on which the present study focuses, clusters most closely with GCN2, which is suggestive of a role in response to nutrient levels. The PF14_0423 gene model proposed in PlasmoDB [32] predicts a single intron that falls close to the 5' end of the sequence so that the kinase domain is encoded entirely within the second exon. All the residues that are required for catalytic activity [33] are present in the kinase domain, suggesting the gene encodes an active enzyme. The sequence shares the feature of insertions within the catalytic domain with other eIF2α kinases [34] **(Figures 2B and 2C)**. Three of the human eIF2α kinases have N-terminal extensions containing regulatory domains; the fourth, GCN2, has extensions on either side of the kinase domain (as reviewed in [35]). As PfeIK1 has extensions on both sides of the catalytic domain, it is most similar to GCN2 not only in the sequence of its catalytic domain, as the phylogenetic tree **(Figure 2A)** demonstrates, but also in overall structure **(Figure 1C)**. Furthermore, the C-terminal extension of PfeIK1 contains an "anti-codon binding" domain (Superfamily entry SSF52954) that may mediate binding to uncharged tRNAs, a function that is performed in GCN2 by the HisRS domain present in the C-terminal extension **(Figure 1C)** [18]. This adds weight to the possibility that PfeIK1 is involved in the response to amino acid starvation, like GCN2. The other functional domains present in the GCN2 extensions were not recognisable in PfeIK1.

Kinase activity of recombinant PfeIK1

In order to verify that the *pfeik1* gene encodes a functional kinase, the catalytic domain was expressed as a GST fusion protein in *E. coli*. A recombinant protein of the expected size (76 kDa) was obtained and purified for use in kinase assays. The protein

appeared as a doublet in most preparations, with both bands reacting with an anti-GST antibody. Kinase assays were performed with α- or β-casein as substrates, in the presence or absence of GST-PfeIK1 **(Figure 3A)**. A weak signal was detectable with β-casein on the autoradiogram even in the absence of the kinase, indicating a low level of contaminating kinase activity in the substrate itself. This signal was much stronger in the presence of GST-PfeIK1, and a signal was also observed with α-casein, which was not labelled in the absence of the kinase. Furthermore, a signal at a size matching that of the upper band in the GST-PfeIK1 doublet was also seen, indicating possible autophosphorylation, an established property of at least some mammalian eIF2α kinases, including GCN2 [34, 36-38]. GCN2 autophosphorylation occurs on two threonine residues in the activation loop [36], only one of which conserved in PfeIK1 **(Figure 2B)**. Autophosphorylation was more clearly seen in the absence of any exogenous substrate **(Figure 3B)**. The possible functional relevance of PfeIK1 autophosphorylation remains to be determined. Taken together, these data suggest that PfeIK1 possesses catalytic activity. To ensure that the signals were not due to co-purified activities from the bacterial extract, the assays were repeated using a catalytically inactive mutant (Lys458→Met) of GST-PfeIK1. These reactions yielded an identical pattern as the reaction containing no recombinant kinase **(Figure3A)**, confirming that the phosphorylation of the caseins is due to GST-PfeIFK1, and that the recombinant kinase can autophosphorylate.

In order to establish whether PfeIK1 is an eIF2α kinase as predicted, its activity was tested towards recombinant *P. falciparum* eIF2α expressed as a 64 kDa GST fusion.

Figure 3B (left lane) shows that GST-PfeIK1 can phosphorylate wild-type GST-PfeIF2α. The signal appears very weak, which may be explained by the fact that the recombinant kinase contains only the catalytic domain and may not mimic the enzyme in a fully activated, physiological status. Indeed, an activation mechanism for GCN2 has been proposed [37], in which a conformational alteration of the so-called "hinge region" of the catalytic domain is induced by uncharged tRNA binding to the HisRS domain, which would favour productive binding of ATP to the active site. Such a positive effect of the regulatory domain would not be possible with GST-PfeIK1, since it contains only the catalytic domain.

Consistent with the hypothesis that PfeIK1 may regulate translation through PfeIF2α phosphorylation, mutation of the predicted target for phosphorylation in the substrate (Ser59→Ala) prevents labelling with the recombinant enzyme **(Fig. 3B)**.

Generation of **pfeik1$^-$** *clones*

Microarray data available in PlasmoDB [1, 39] indicate that *pfeik1* is expressed in asexual parasites; it can be hypothesized that the kinase plays a role in the parasite's stress response, and may therefore (i) not be essential for the asexual cycle, and (ii) be involved in regulation of gametocytogenesis, similar to the function of a eIF2α kinase in *T. gondii* stage transition from tachyzoite to bradyzoite. *P. falciparum* clones that do not express PfeIK1 were generated to test these hypotheses. The strategy used to disrupt expression of the kinase relies on single cross-over homologous recombination, and has been used successfully to knock-out other *P. falciparum* protein kinase genes [40, 41]. Briefly, a plasmid based on the pCAM-BSD vector [26] containing a cassette conferring

resistance to blasticidin and an insert comprising the central region of the PfeIK1 catalytic domain, was transferred by electroporation into asexual parasites of the 3D7 clone. Homologous recombination is expected to generate a pseudo-diploid locus in which neither of the two truncated copies encodes a functional kinase: the 5' copy lacks an essential glutamate residue in subdomain VIII and all downstream sequence including the 3'UTR; the 3' copy lacks the both the promoter region and the essential ATP orientation motif in subdomain I **(Figure 4A)**.

Blasticidin-resistant parasite populations were obtained and shown by PCR analysis to contain parasites whose *pfeik1* locus was disrupted. Clonal lines deriving from two independent transfection experiments were established by limiting dilution, and their genotypes were analysed by PCR **(Figure 4B)**. The amplicon corresponding to the wild-type locus was not detected in clones C1 and C8 (lane 1), but was observed in wild-type parasites (lane 5). In contrast, PCR products that are diagnostic of both the 5' (lanes 3 & 7) and 3' (lanes 4 & 8) boundaries of the integrated plasmid were amplified from C8, but not 3D7 parasites (lanes 11 & 12). The C1 and C8 clones also yielded a signal with primers that are specific for the transfection plasmid, and detect retained episomes or integrated concatemers. Integration was verified by Southern blot analysis of *Hind*III-digested genomic DNA **(Figures 4C and 4D)**; the 12 kb band containing the wild-type locus is replaced in clones C1 and C8 by the expected two bands (10.4 kb and 6.8 kb) resulting from integration. The remaining 5.3 kb band is derived from linearized plasmid, or from digestion of concatemers of plasmid (which may or may not be integrated into the chromosome). These results confirm that the *pfeik1* locus was indeed disrupted in clones C1 and C8, and demonstrate PfeIK1 is not required for completion of

the asexual cycle in *in vitro* cultures. Additionally, asexual parasite cultures were synchronized and carefully monitored through several life cycles; samples were taken every 30 minutes and assessed for DNA content by flow cytometry [42]. No significant difference was observed in asexual cycle duration of the parental 3D7 clone and that of *pfeik1*$^{-}$ parasites; cycle times of 49.0 h +/- 0.5 and 49.2 h +/- 0.7, respectively, were measured **(Figure 5)**.

eIF2α is not phosphorylated in pfeik1- clones during amino acid starvation

To determine whether *pfeik1*$^{-}$ parasites were defective in responding to amino acid-limitation, we cultured these parasites in RPMI medium containing either all or no amino acids and assayed for eIF2α phosphorylation through western blot analysis **(Figure 6)**. We observed that *pfeik1*$^{-}$ parasites were unable to modulate the phosphorylation state of eIF2α in response to changing amino acid conditions, in direct contrast to wild-type parental clone 3D7. A further control was provided by performing the assay using a parasite clone lacking PfeIK2, another enzyme related to eIF2α kinases (see Fig. 2A; a full characterisation of PfeIK2 and *pfeik2*$^{-}$ parasite clones is to be published elsewhere). The *pfeik2*$^{-}$ parasites, which were generated using the same strategy as that described here for *pfeik1* and were therefore also resistant to blasticidin, readily phosphorylated eIF2α in amino acid starvation conditions, like wild-type 3D7 parasites. This demonstrates that the abolition of eIF2α phosphorylation observed in *pfeik1*$^{-}$ parasites is not due to non-specific effects resulting from the genetic manipulations performed to obtain the mutant clones. Taken together, these data identify

PfeIK1 as a crucial regulator of amino acid starvation stress response of intra-erythrocytic parasites.

pfeik1$^-$ ***clones are competent for sexual development and mosquito infection***

The *pfeik1*$^-$ parasites were able to differentiate into gametocytes (data not shown). Further, qualitative results showed that *pfeik1*$^-$ male gametocytes were competent to differentiate into gametes (*in vitro* exflagellation). To investigate whether PfeIK1 plays an essential role in subsequent life cycle stages, mosquitoes were fed with cultures of *pfeik1*$^-$ gametocytes. The numbers of oocysts associated with midguts dissected 10 days post-feeding, and the numbers of mosquitoes with sporozoite-positive salivary glands 16 days post-feeding, were then determined. This revealed that the complete sexual cycle can occur in the absence of PfeIK1, resulting in formation of oocysts and sporozoites **(Table 1)**. Infection rates and median numbers of oocysts per infected mosquito are low relative to what is routinely observed in transmission experiments with the wild-type clone 3D7. However, this is to be expected from parasites that have been kept in continuous culture for a long period of time; in the present case it had taken ~7 months in culture to obtain knockout clones suitable for mosquito infection experiments. Circumstantial evidence that low infection levels are not a direct consequence of *pfeik1* disruption is provided by the observation that our control for these experiments (sham-transfected 3D7 that had been cultured for the same duration, in parallel to the *pfeik1*$^-$ parasites), had completely lost the ability to produce gametocytes and therefore infect mosquitoes. Importantly, to verify that the parasites infecting the mosquitoes had not reverted to the wild-type genotype, midguts from infected mosquitoes were collected 10

days post-feeding, from which total DNA was extracted and used in nested PCR experiments. The wild-type locus could be amplified from mosquitoes infected with wild-type 3D7 parasites, but not from those infected with *pfeik1*$^{-}$ C8 parasites **(Figure 7**, lower panel, lanes 1, 3, 5**)**. Conversely, the amplicon diagnostic of the 3' boundary of the integrated plasmid could only be amplified from midguts of *pfeik1*$^{-}$ C8-infected mosquitoes, but not from mosquitoes infected with wild-type parasites (lanes 2, 4, 8).

On the basis of the similarities between PfeIK1 and GCN2, we hypothesized that PfeIK1 is involved in modulating the response to amino acid starvation depicted in **Figure 1B**. That this is indeed the case was demonstrated through a reverse genetics approach: parasites lacking PfeIK1 do not phosphorylate eIF2α in response to amino-acid depletion **(Figure 6)**. Future work will determine the impact of activation of PfeIK1 on both the rate of translation and the possible selection of specific messages that are translated under stress conditions. Overall, the data presented here suggest that eIF2α phosphorylation in response to amino-acid starvation is not essential to parasite survival during the erythrocytic asexual cycle (at least in an *in vitro* cultivation context), or for completion of sporogony.

Commitment to gametocytogenesis has been proposed to be linked to stress response, and eIF2α might possibly be involved in this process. At first sight, the data presented here suggest that PfeIK1 does not regulate gametocytogenesis, since *pfeik1*$^{-}$ parasite are able to undergo sexual development. However, caution must be exercised, as compensatory mechanisms can be at play in knock-out parasites. Indeed, in a similar situation concerning another protein kinase family, it was observed that disruption of the gene encoding one of the two *P. falciparum* mitogen-activated protein kinases (MAPKs),

pfmap-1, does not cause any detectable phenotype, but that *pfmap-1*$^{-}$ parasites overexpress the second parasite MAPK, Pfmap-2 [40]. A similar compensation mechanism may operate between the three PfeIKs represented in the parasite kinome **(Figure 2A)**. Even though compensatory mechanisms to permit sexual differentiation are presumably less likely to occur than those allowing the survival of asexual parasites (because of the absence of a true selection pressure), it cannot be formally excluded that PfeIK1 plays a role in gametocytogenesis in a wild-type parasite background. Investigating this possibility will require inducible and/or multiple knock-outs and the availability of mono-specific antibodies to monitor the levels of each PfeIK in parasites lacking one of them.

Conclusions

Phylogenetic analysis indicates that the *P. falciparum* kinome includes three putative eIF2α kinases. One of these, PfPK4, was previously shown to phosphorylate a peptide corresponding to the target region of human eIF2α [27]. It is demonstrated here that PfeIK1 is able to phosphorylate the conserved regulatory site on the *Plasmodium* orthologue of the translation factor *in vitro*, and that eIF2α phopshorylation in response to amino-acid starvation does not occur in *pfeik1*$^{-}$ parasites. The present study thus establishes that malaria parasites possess the molecular machinery that pertains to stress-dependent regulation of translation, and that this machinery is actually used in stress response.

Authors' contributions

CF carried out molecular cloning, kinase assays, parasite genetic manipulations and analysis, participated in bioinformatic analysis and drafted the manuscript. SB carried out parasite starvation and immunoblotting experiments. IR analysed parasite growth. JW participated in sequence alignments and generated the phylogenetic tree. LRC carried out mosquito infections and participated in their analysis. DEG participated in conception of the study. CD conceived of the study, participated in its design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

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Figure Legends

Figure 1. The *P. falciparum* eIF2α orthologue is phosphorylated in response to amino-acid starvation

A. Alignment of PfeIF2α with orthologous sequences from *T. gondii* (Tg), human (Hs), rice (Os) and *E. cuniculi* (Ec). Sequences surrounding the conserved regulatory serine, (*P. falciparum* numbering: M48 - K108) are shown. Residues that are identical in all sequences are highlighted in black, residues that are identical or similar are marked in grey. The arrow indicates the serine that is the target of eIF2α kinases. Open arrow heads (∨) indicate residues involved in contacting the kinase domain, asterisks (*) indicate conserved residues that protect the phosphorylation site from the activity of other kinases.

B. Western blot analysis of PfeIF2α phosphorylation. A 3D7 parasite culture synchronized to the late ring stage was equally partitioned into individual cultures. Growth of the parasites was continued up to 5 hours at 37°C in either complete RPMI medium (CM) or in RPMI lacking amino acids (-AA). CM was added back to one amino acid-deprived culture, and re-incubated for an additional 45 minutes. Total lysates from the parasites were prepared for SDS-PAGE, followed by immunoblotting with antibodies against phosphorylated eIF2α (anti-phospho eIF2α) and the endoplasmic reticulum (ER) marker, BiP (anti-BiP), which served as the loading control.

Figure 2. Bioinformatic analyses of *P. falciparum* eIF2α kinases

A. Phylogenetic tree showing clustering of PfeIF2α kinases with human eIF2α kinases. Sequences: PfeIK1: PF14_0114; PfeIK2: PFA0380w; PfPK4: PFF1370; PKR: GI:4506103; HRI: GI:6580979; PERK: GI:18203329; GCN2: GI:65287717; MEK1: GI:400274; IRAK1: GI:68800243; Aurora: GI:37926805; CDK2: GI:1942427; PfCK1: PF11_0377; PfNEK2: Pfe1290w; PfPK5: MAL13P1.279; PfPKA: PFI1685w; PFTKL3: PF13_0258; Pfb0815w: PfCDPK1; hCAMK1: GI:4502553; hPRKACA: GI:46909584; hCSNK1d: GI:20544145; hNEK7: GI:19424132; hSRC: GI:4885609.

B. Alignment of the catalytic domains of PfeIK1, PbeIK1 and human GCN2. Identical residues in all three kinases are in black boxes, residues that are identical in two sequences of the three sequences, or that are similar are boxed in grey. The number of residues comprising the inserts between domains IV and V are marked between //-//. Asterisks (*) mark residues conserved among kinases in general, while open arrowheads (∨) indicate residues specifically conserved among eIF2α kinases. The downwards arrow marks the threonine residues that are targets for autophosphorylation in GCN2. PlasmoDB accession numbers: PfeIK1: PF14_0423, PbeIK1: PB000582.03.0 GenBank accession number: HsGCN2: GI:65287717

C. Schematic of the domain structures of PfeIK1, PbeIK1 and GCN2. Kinase domains (KD) are in grey, hatched regions represent the inserts (I) within the kinase domains and regions with no identified function are white. Additional characterized domains of GCN2 are as follows: red; N-terminal GCN1 binding domain (GB), green; pseudo-kinase domain (ΨKD), blue; histidyl-tRNA synthetase (HisRS), yellow; ribosome binding and dimerisation domain (RB/DD).

Figure 3. Kinase activity of PfeIK1

A. GST-PfeIK1 phosphorylates the exogenous substrates α-and β-casein. Kinase assays were performed using 10μg α-casein (left 3 lanes) or β-casein (right 3 lanes), in the presence of 2μg wild-type kinase catalytic domain (WT), catalytically inactive mutant (K458M) or no kinase (-). Upper panel; autoradiogram, lower panel; Coomassie blue-stained gel.

B. GST-PfeIK1 autophosphorylates and can phosphorylate recombinant GST-PfeIF2α, but not the mutant GST-PfeIF2α-S59A. Kinase assays were performed using 2μg wild-type PfeIK1 catalytic domain (WT), or catalytically inactive mutant (K458M), or no kinase (-), in the presence of 10μg wild-type GST-PfeIF2α (left 3 lanes), targeted mutant GST-PfeIF2α-S59A (middle 3 lanes) or no substrate (right 2 lanes). The position of the substrate is highlighted by ovals.

Figure 4. Disruption of the *pfeik1* gene

A. Strategy for gene disruption. The transfection plasmid contains a PCR fragment spanning positions 1467-2255 of the entire 4.8kb *pfeik1* coding sequence (as predicted on PlasmoDB). The fragment excludes two regions essential for catalytic activity, labelled 'ATP' (a glycine rich region required for orientation of ATP) and 'E' (a glutamate residue required for structural stability of the enzyme). The positions of primers used for genotyping clones, and for nested PCR to genotype oocsyts are indicated by numbered arrows.

B. PCR analysis. Genomic DNA isolated from *pfeik1*$^{-}$ clones C1 and C8, and from 3D7 wild-type parasites, was subjected to PCR using the indicated primers (see Fig. 4A for primer locations). Lanes 1,5,9: primers 1 + 2 (diagnostic for the wild-type locus); lanes 2,6,10: primers 3 + 4 (diagnostic for the pCAM-BSD-PfeIK1 plasmid); lanes 3,7,11: primers 1 + 4 (diagnostic for 5' integration boundary); lanes 4,8,12: primers 3 + 2 (diagnostic for 3' integration boundary). M= co-migrating markers.

C. Schematic of expected sizes on Southern blot analysis of wild-type 3D7 parasites and *pfeik1*$^{-}$ parasites.

D. Southern blot analysis of the *pfeik1* locus in wild-type 3D7 and *pfeik1*$^{-}$ clones C1 and C8. Genomic DNA was digested with *Hind*III, transferred to a Hybond membrane and probed with the *pfeik1* fragment that was used as the insert in the pCAM-BSD-PfeIK1 plasmid. Positions of the bands corresponding to the wild-type locus (WT), 5' integration (5' int.), 3' integration (3' int.) and linearized plasmid (plasmid) are shown on the right. Sizes of co-migrating markers are indicated on the left.

Figure 5. Disruption of the *pfeik1* gene does not affect asexual growth rate

Representative cycles of *pfeik1*$^{-}$ parasites and the parental 3D7 strain (dashed). Cycle points were semi-automatically collected fixed and stored at 4°C every 30min over ~4 days. After permeabilization and RNAse treatment, the DNA content was analyzed by flow cytometry as previously described [42]. Mature schizonts (~16-32N), red line; S-phase (~2-8N), blue line; G1-phase (1N), black line. Percentage values as a function of time are shown; hpi: hours post- infection, referring to the mature schizont maxima as zero.

Figure 6. *pfeIK1*$^-$ parasites do not phosphorylate eIF2α in amino acid-limiting conditions

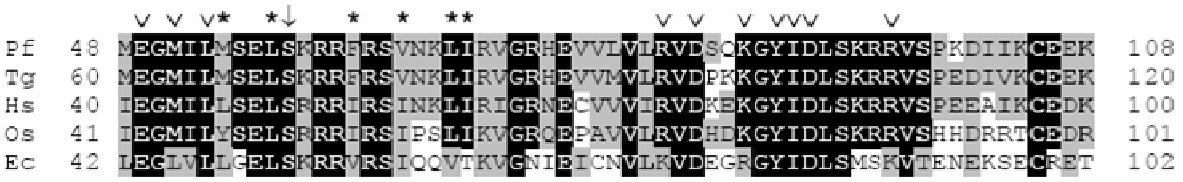
pfeik1$^-$ clones C1 and E6, as well as the 3D7 parent clone and a *pfeik2*$^-$ clone used as controls, were synchronized to the late ring stage and equally partitioned into individual cultures. Growth of the parasites was continued up to 5 hours at 37°C in either complete RPMI (CM) or in RPMI lacking amino acids (-AA). CM was added back to one amino acid-deprived culture, and re-incubated for an additional 45 minutes. Total lysates from the parasites were prepared for SDS-PAGE, followed by immunoblotting with antibodies against phosphorylated eIF2α (anti-phosho eIF2α). Antibodies against the endoplasmic reticulum marker BiP (anti-BiP) served as the loading control.

Figure 7. Analysis of the parasite genotypes in mosquito infections

Genomic DNA extracted from a wild-type 3D7-infected mosquito and from two mosquitoes infected with clone C8 was analysed by nested PCR; primer positions are indicated in Fig. 3A. The inner PCR product is shown. Lanes 1, 3 & 5 are diagnostic for the wild-type locus (primers 1 + 2, followed by 5 + 6). Lanes 2, 4 & 6 are diagnostic for the 3' boundary of plasmid integration (primers 3 + 2, followed by 7 + 6). The 3D7 infected mosquito used here serves as a control for PCR amplification of the wild-type locus from a midgut, but came from a separate experiment and hence did not provide a control for infection prevalence or intensity. Upper panel: shorter exposure; lower panel: longer exposure to reveal the wild-type band in lane 1 and its absence in the C8 samples. M= co-migrating markers.

Figure 1

A


Pf 48 MEGMILMSELSKRRGRSVNKLIRVGRHEVVLVLRVDSCKGYIDLSKRRVSPKDIIKCEEK 108
Tg 60 MEGMILMSELSKRRGRSVNKLIRVGRHEVVMVLRVDPKKGYIDLSKRRVSPEDIVKCEEK 120
Hs 40 IEGMILLSELSRRRIRSINKLIRIGRNECVVVIRVDKEKGYIDLSKRRVSPEEAIKCEDK 100
Os 41 IEGMILYSELSRRRIRSIPSLIKVGRQEPAVVLRVDHDKGYIDLSKRRVSHHDRRTCEDR 101
Ec 42 IEGLVLLGELSKRRVRSIQQVTKVGNIEICNVLKVDEGRGYIDLSMSKVTENEKSECRET 102

B

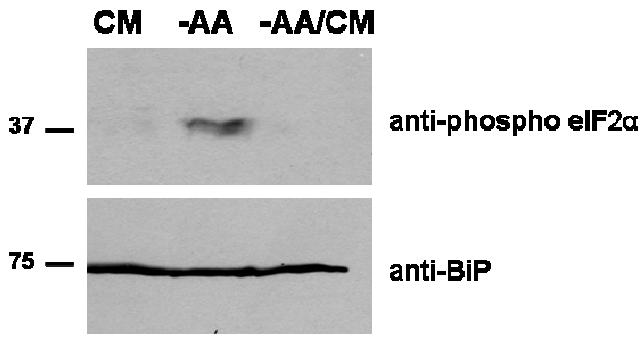
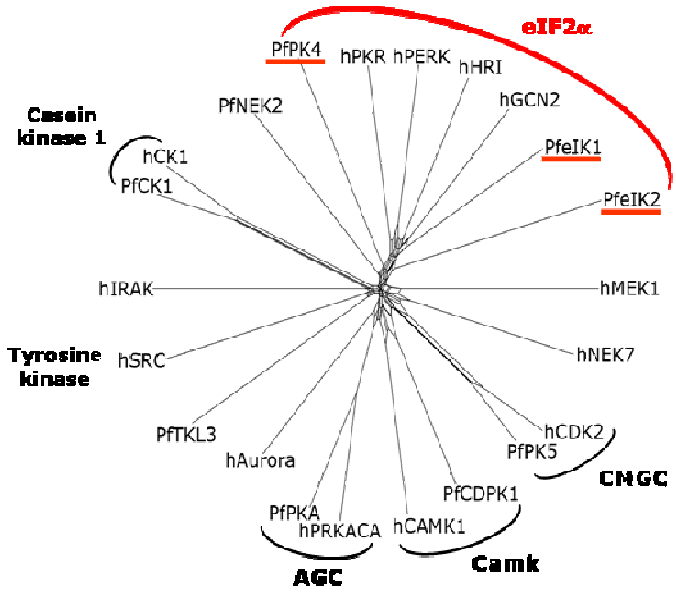

CM
-AA
-AA/CM
37
anti-phospho eIF2α
75
anti-BiP

Figure 2

A


eIF2α
PfPK4
hPKR
hPERK
hHRI
hGCN2
PfeIK1
PfeIK2
PfNEK2
Casein kinase 1
hCK1
PfCK1
hIRAK
hMEK1
Tyrosine kinase
hSRC
hNEK7
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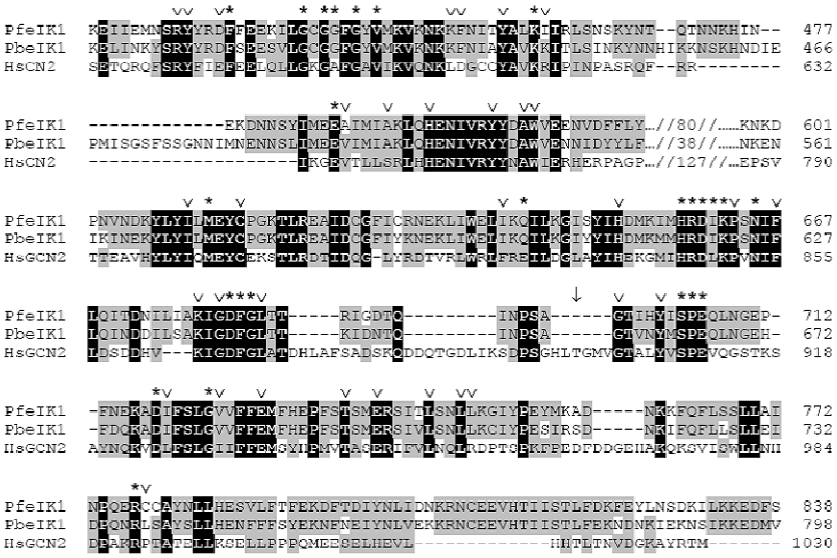
hCAMK1
AGC
Camk

B


PfeIK1
PbeIK1
HsGCN2

C

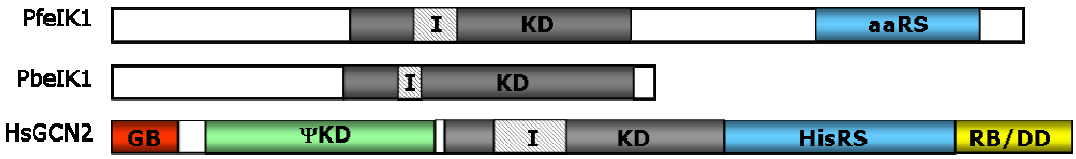

PfeIK1
I
KD
aaRS
PbeIK1
I
KD
HsGCN2
GB
ΨKD
I
KD
HisRS
RB/DD

Figure 3

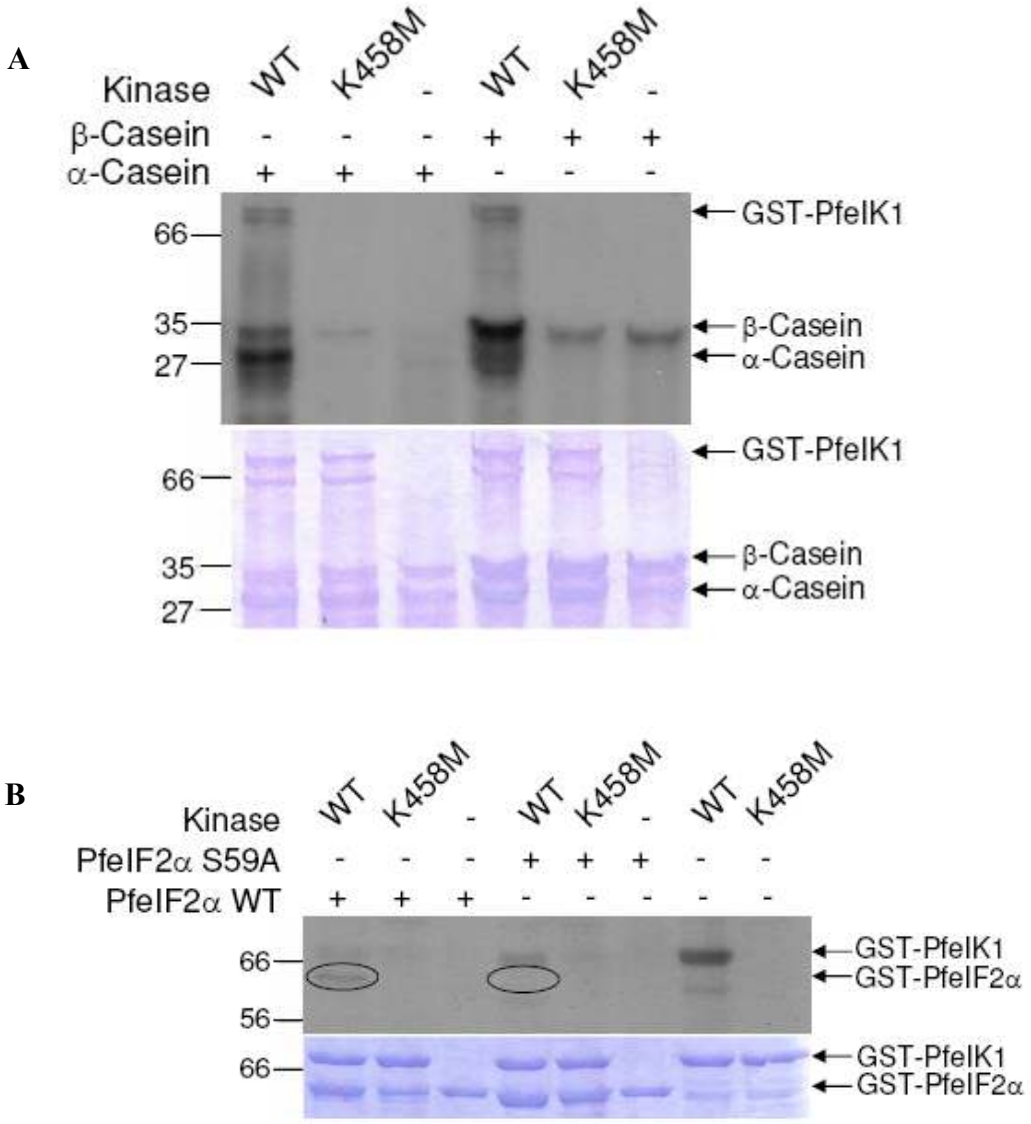

A
Kinase
WT
K458M
-
WT
K458M
-
β-Casein
α-Casein
66
35
27
GST-PfeIK1
β-Casein
α-Casein
B
PfeIF2α S59A
PfeIF2α WT
56
GST-PfeIF2α

Figure 4

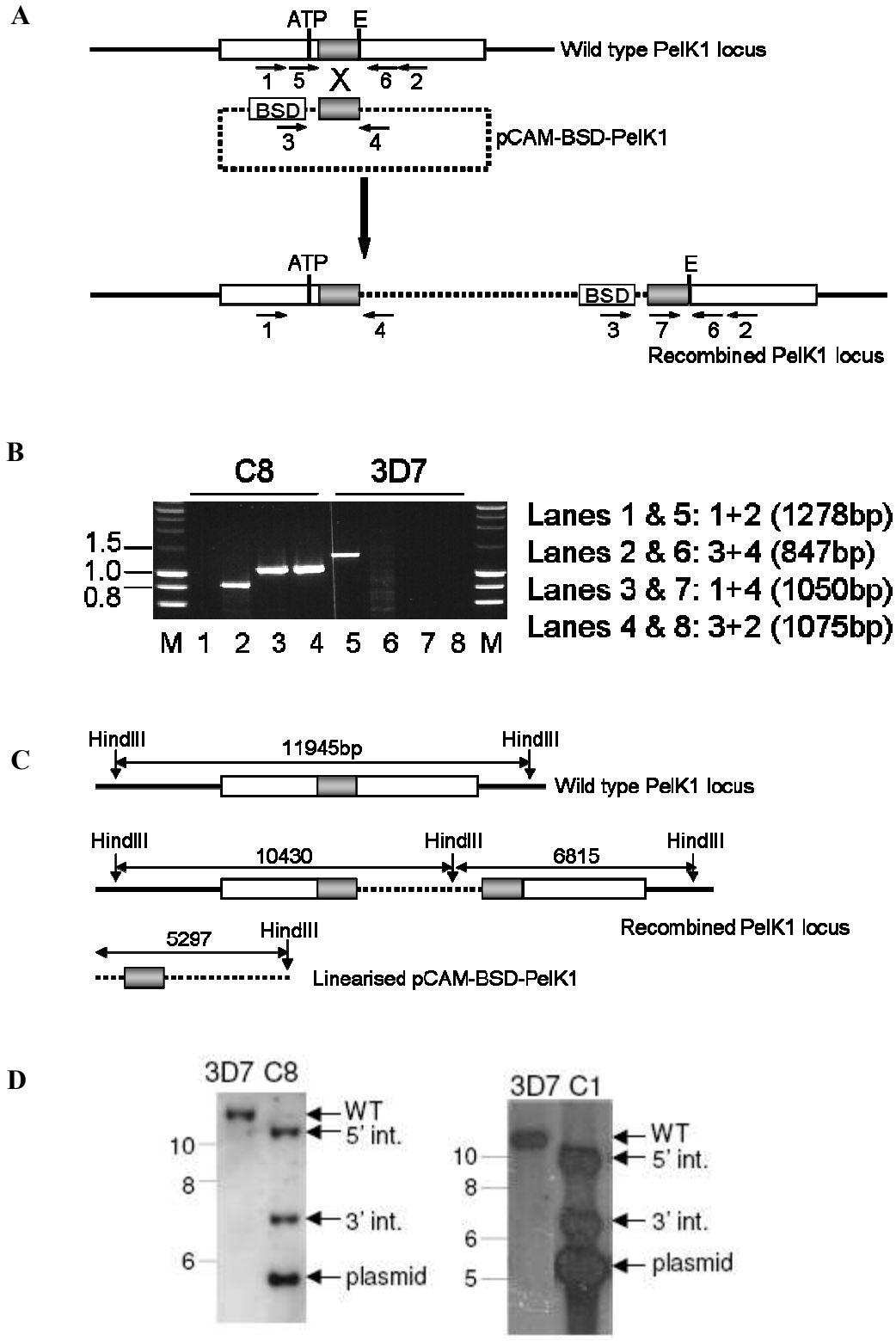

A
ATP
E
Wild type PelK1 locus
1 5 X 6 2
BSD
3 4
pCAM-BSD-PelK1
ATP
E
BSD
1 4 3 7 6 2
Recombined PelK1 locus
B
C8
3D7
1.5
1.0
0.8
M 1 2 3 4 5 6 7 8 M
Lanes 1 & 5: 1+2 (1278bp)
Lanes 2 & 6: 3+4 (847bp)
Lanes 3 & 7: 1+4 (1050bp)
Lanes 4 & 8: 3+2 (1075bp)
C
HindIII
11945bp
HindIII
Wild type PelK1 locus
HindIII
10430
HindIII
6815
HindIII
Recombined PelK1 locus
5297
HindIII
Linearised pCAM-BSD-PelK1
D
3D7 C8
WT
5' int.
3' int.
plasmid
10
8
6
3D7 C1
WT
5' int.
3' int.
plasmid
10
8
6
5

Figure 5

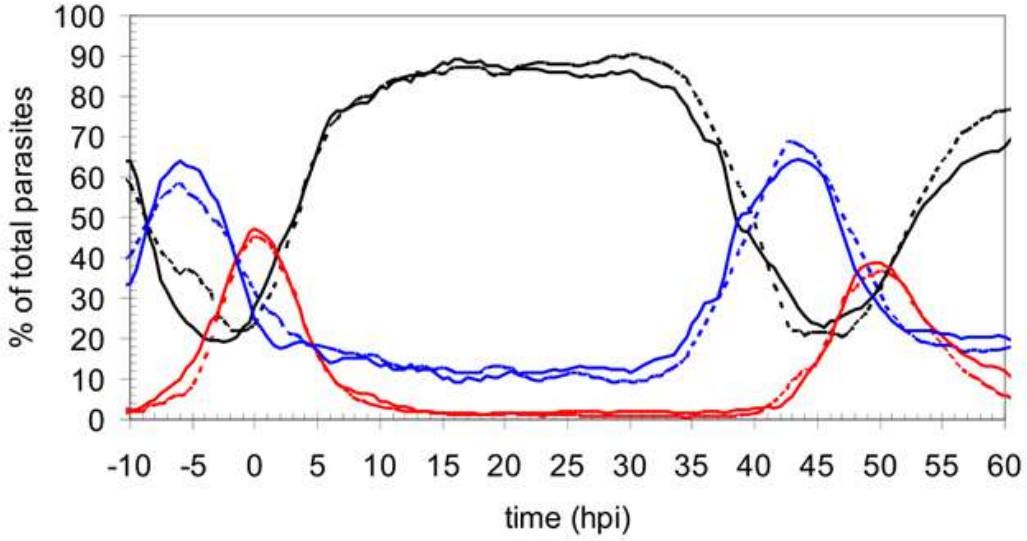

% of total parasites
100
90
80
70
60
50
40
30
20
10
0
-10
-5
0
5
10
15
20
25
30
35
40
45
50
55
60
time (hpi)

Figure 6

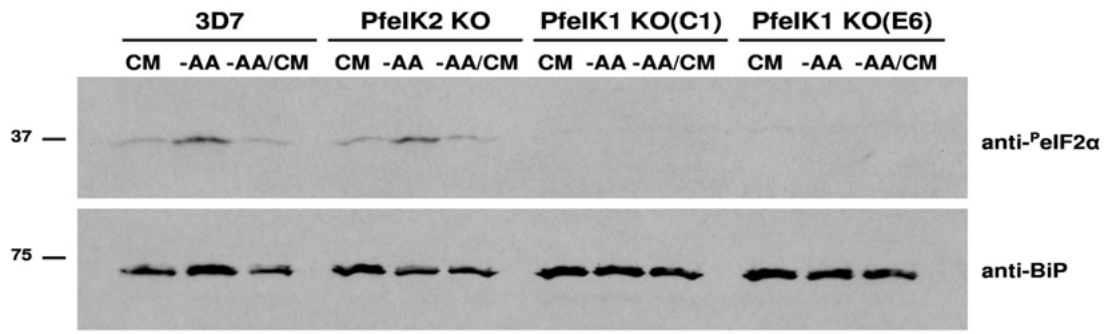

3D7
PfeIK2 KO
PfeIK1 KO(C1)
PfeIK1 KO(E6)
CM -AA -AA/CM CM -AA -AA/CM CM -AA -AA/CM CM -AA -AA/CM
37
75
anti-PeIF2α
anti-BiP

Figure 7

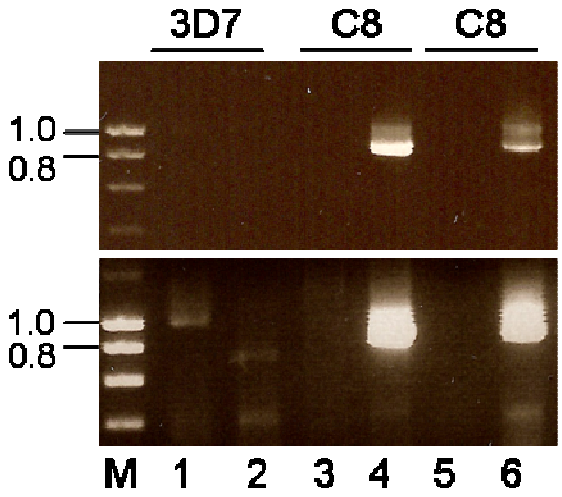


Table 1. Oocyst and sporozoite formation by* pfeik1^{-} *parasite clones.

Clone	Exp. no	% Infection (no. infected/ no. dissected)	Median oocyst no. per infected mosquito (range)	% Sporozoite positive (no. infected/ no. dissected)
C1	1	15% (2/14)	1.5 (1-2)	ND
C8	1	44% (7/16)	10 (1-34)	37% (7/19)
C8	2	20% (5/25)	2 (1-5)	ND

There was no difference in the prevalence of mosquitoes positive for the oocyst and sporozoites stages (exp. 2, p=0.74).

CHAPTER III:

PLASMODIUM FALCIPARUM RESPONDS TO AMINO ACID STARVATION BY ENTERING INTO A HIBERNATORY STATE

Preface

Work presented in this chapter was conducted by SEL (SEB), LA and EI. LA performed microarray analysis on parasite samples, and EI performed the growth recovery experiment presented in figure S1. SEL performed all other experiments and drafted the manuscript with comments incorporated from CD, ML, and DEG. This work is currently pending submission to PNAS Plus upon completion of metabolomic analysis.

Shalon E. Babbitt[1], Lindsey Altenhofen[2], Eva Istvan[1], Clare Fennell[3]*, Christian Doerig[3,4], Manuel Llinàs[2], and Daniel E. Goldberg[1]. **Plasmodium responds to amino acid starvation by entering into a hibernatory state.** *Pending submission to PNAS Plus*

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Abstract

The human malaria parasite *Plasmodium falciparum* is auxotrophic for most amino acids. Its amino acid needs are largely met through the degradation of host erythrocyte hemoglobin, however the parasite must acquire isoleucine exogenously, as this amino acid is not present in adult human hemoglobin. We report that when isoleucine is withdrawn from the culture medium of intraerythrocytic *P. falciparum*, the parasite slows its metabolism and progresses through its developmental cycle at a reduced rate. Isoleucine-starved parasites remain viable for 72 hours and resume rapid growth upon re-supplementation. Protein degradation during starvation is important for maintenance of this hibernatory state. Microarray analysis of starved parasites revealed a 60% decrease in the rate of progression through its normal transcriptional program but no other apparent stress response. *Plasmodium* parasites do not possess a TOR nutrient sensing pathway and have only a rudimentary amino acid starvation-sensing eukaryotic initiation factor 2α (eIF2α) stress response. Starved parasites respond specifically to isoleucine deprivation by GCN2-mediated phosphorylation of eIF2α, but kinase knockout clones are still able to hibernate and recover. We conclude that *P. falciparum*, in the absence of canonical eukaryotic nutrient stress response pathways, can cope with an inconsistent bloodstream amino acid supply by hibernating and waiting for more nutrient to be provided.

Introduction

Human red blood cells (RBCs) provide the intraerythrocytic malaria parasite *Plasmodium falciparum* with an abundant nutrient supply in the form of hemoglobin. However, human hemoglobin lacks the amino acid isoleucine [1]. Isoleucine is present in over 99% of the proteins encoded by *P. falciparum* [2] and since *Plasmodium* is unable to synthesize this amino acid *de novo* [3], the parasite must obtain isoleucine from human serum [4, 5]. Humans also cannot make isoleucine, and must acquire this essential amino acid through the diet [1, 3]. In endemic regions, malaria patients are often severely malnourished, which drastically limits the availability of free amino acids in the plasma [6, 7]. Normal plasma isoleucine levels are in the 100 μM range, but in malnourished children can be less than one-tenth this concentration [6]. During *in vitro* culturing, *P. falciparum* growth is optimal above 20μM isoleucine while the parasite is unable to grow in medium devoid of isoleucine [4]. This observation raises the question of how *P. falciparum* responds to low isoleucine conditions that may exist during human infection.

Eukaryotes have canonical mechanisms for responding to amino acid deprivation. The target of rapamycin (TOR) complex, which functions as a master regulator of cell growth [8], is repressed during amino acid starvation. Conversely, the eukaryotic initiation factor 2 alpha (eIF2α) kinase, GCN2, is activated by amino acid-limiting conditions [9, 10]. Activated GCN2 mediates a reduction in translation efficiency, allowing for resource conservation, metabolic re-adjustment and promotion of an adaptive transcriptional program, inducing GCN4 in yeast and ATF4 in mammals. These transcription factors control the response to amino acid deprivation by turning on pathways for amino acid biosynthesis, among others [11, 12]. *Plasmodium* does not have

a TOR complex [13] and lacks the downstream mediators of GCN2 action. An ortholog of eIF2α and three putative eIF2α kinases have been previously identified in the *P. falciparum* genome [14-16]. One of them, PfeIK2, controls latency in sporozoite development in the mosquito [16]. Another, PfeIK1, has been recently confirmed as the amino acid sensing GCN2 ortholog, active in the blood-stage of the parasite [14]. A knockout of GCN2 in the related apicomplexan parasite *Toxoplasma gondii* has an extracellular tachyzoite fitness defect [17], but the biological role of the *Plasmodium* ortholog has not been defined and is tenuous, given the lack of GCN2-responsive transcription factors and amino acid biosynthesis pathways.

To understand how *P. falciparum* responds to and survives amino acid limitation, we monitored the growth recovery, metabolic activity, and gene expression of cultured parasites exposed to isoleucine-free medium. We show that parasites slow their metabolism and cell cycle progression, which allows them to survive prolonged isoleucine starvation. Notably, its GCN2 amino acid sensing pathway is active but does not play a role in hibernation or recovery from starvation. We conclude that *P. falciparum*, upon exposure to amino acid limitation, hibernates to allow its long-term survival.

Results

P. falciparum growth is recoverable after prolonged isoleucine starvation

We monitored growth of synchronized ring-stage *P. falciparum* parasites **(Fig. 1)**. In complete medium (CM), parasites progressed normally through the cell cycle, reinvaded fresh RBCs, and continued growth. However, in the absence of isoleucine (-

Ile), parasites slowly progressed to the trophozoite stage **(Fig. 1A)**, but did not enter S-phase, as indicated by the absence of a high DNA content peak at 24 and 72 hours-post incubation in flow cytometry traces **(Fig. 1B)**. Within hours of glucose starvation, *P. falciparum* parasites appeared as shrunken, rounded bodies with pyknotic nuclei, and fail to recover. However, during isoleucine starvation, parasite morphology remained essentially normal **(Fig. 1A)**. To determine whether isoleucine-starved parasites maintain viability, we incubated synchronized ring-stage parasites in isoleucine-free RPMI medium for varying periods of time and then supplemented each starved culture with isoleucine at the concentration found in complete RPMI (382 μM). Growth of the culture was followed for an additional 72 hours and parasitemia was measured by flow cytometry. Parasites starved for isoleucine for 24, 48, and 72 hours recovered appreciable levels of control growth **(Fig. 1C)**. When parasites were starved for longer periods (up to 9 days), parasites were no longer detectable on Geimsa stained blood smears, and recovery of growth post-isoleucine supplementation dropped precipitously **(Fig. 1C)**. In cultures with lower recovery (*i.e.* those that had been starved for 4 days or more), gametocytes were undetectable and asexual forms remained prevalent, suggesting that vast reductions in growth recovery of parasites subjected to extended starvation was due to decreased viability.

To determine whether protein translation is affected in isoleucine-starved parasites, we incubated synchronized parasites in either complete or isoleucine-free labeling medium containing [^{35}S] methionine and cysteine. Starved parasites incorporated the radiolabel into protein, but at a reduced rate, indicating a slowed metabolism **(Fig. 1D)**. Recovery from starvation was similar whether parasites were

adapted to low (20 μM) or high (200 μM) concentrations of exogenous isoleucine prior to starvation **(Fig. S1)**, suggesting that parasite survival during starvation does not require a pre-existing isoleucine pool.

Impairment of proteolysis during isoleucine starvation abrogates recovery

Members of the aspartic and cysteine protease families, plasmepsins and falcipains respectively, reside in the digestive vacuole (DV) of *P. falciparum*, where they degrade massive amounts of host cell hemoglobin, a process that serves to supply the parasite with amino acids [18]. The bulk of hemoglobin degradation takes place during the trophozoite stage, when the metabolic activity of *P. falciparum* is at its highest [19]. During long-term isoleucine starvation, parasites display evidence that hemoglobin degradation remains active, in that hemozoin (the sequestered heme byproduct of catabolism) becomes visible in the DV within 24 hours **(Fig. 1A, arrows)**.

To determine whether proteolytic activity was required to maintain viability, we incubated ring-stage parasites in isoleucine-free RPMI and exposed them to E-64d, a membrane-permeable cysteine protease inhibitor, for 24 hours at different times during the starvation period. Under the conditions used, growth could be restored in a fed control (in CM) after drug removal **(Fig. 2A)**. Parasites starved for 24 hours in the presence of E-64d recovered well after washout of the drug and re-supplementation with isoleucine. Even 72 hour-starved parasites, exposed to E-64d for the last 24 hours of the incubation, recovered nearly half of control growth, in line with the decrease in recovery seen previously under similar starvation conditions without drug **(Fig. 1C)**. However,

parasites starved for 48 hours, with E-64d present during the last half of the incubation (the trophozoite stage with peak hemoglobin degradation), were not viable **(Fig. 2A)**.

We performed a similar experiment with the aspartic protease inhibitor pepstatin A (Pep A). As with E-64d, pepstatin A-treated parasites cultured in complete medium maintained viability **(Fig. 2A).** Again, 48 hour-starved parasites treated with inhibitor for the last 24 hours were not viable while parasites starved and treated for 24 hours recovered well. Although recovery was notably lower in the pepstatin A-treated 72 hour-starved cultures than in the corresponding E-64d-treated cultures, the surviving parasites consistently outgrew those from the 48 hour-starved condition. These data suggest that proteolytic activity is most critical during the first 48 hours of starvation, soon after which the parasite appears to reach its developmental limit.

The morphology of starved parasites over time indicates that they slowly transition through the trophozoite stage, displaying early characteristics of this stage at 24 hours of starvation **(Fig. 1A)**. As noted above, when parasites were starved for 24 hours in the presence of either E-64d or pepstatin A, recovery of growth was relatively unaffected. However, when starvation time was extended beyond the withdrawal of either drug, recovery was attenuated **(Fig. 2B, compare with Fig. 2A, gray bars)**. It appears that continuous protein degradation is essential for surviving the second day of starvation, and protease inhibition throughout the first or second day of isoleucine starvation is lethal to the parasite.

The cysteine protease falcipain 2 (FP2) plays a pivotal role in hemoglobin degradation of *P. falciparum* in that its genetic disruption leads to the accumulation of undigested hemoglobin and swelling of the DV [20], a phenotype similarly seen with E-

64d treatment. We incubated a clonal line of *fp2*- parasites [4] in isoleucine-free RPMI and then supplemented each starved culture with isoleucine to allow for recovery. Parasites lacking FP2 exhibited significant defects in recovery post starvation, with only minimal growth restored in 24 hour-starved mutants and virtually no recovery of the 48 and 72 hour-starved mutant parasites **(Fig. 2C)**. These data complement the protease inhibitor studies and emphasize the requirement for DV proteolysis in surviving isoleucine starvation.

Artemisinin treatment of starved parasites

Monotherapy of falciparum malaria patients with artemisinin frequently results in parasite recrudescence [21]. Parasites recovered post-therapy remain sensitive to drug. A similar phenomenon has been observed in culture and has given rise to the concept of parasite dormancy, a state which presumably confers drug tolerance [21-23]. To determine whether isoleucine-starved parasites are in a similar dormant state, we cultured parasites in isoleucine-free medium, exposed them to artemisinin and then assessed viability. There was no increased resistance to artemisinin in starved parasites. If anything, there was a slight increase in sensitivity **(Fig. S2)**, therefore isoleucine starvation status does not appear to be equivalent to artemisinin-associated dormancy.

Expression profile of starved parasites reveals a delayed growth phenotype

Global transcription in *P. falciparum* is characterized by successive waves of gene activation tightly coordinated with the parasite's developmental progression [24, 25]. To examine mRNA abundance levels in isoleucine-starved parasites, we isolated RNA from

synchronized parasites incubated in isoleucine-free RPMI, harvesting samples after 3 and 6 hours of incubation, and every 6 hours thereafter up to 48 hours total. In parallel, RNA was isolated from a control set of synchronized parasites that were maintained in complete medium and harvested along with the starved samples. The expression level of nearly 4800 genes was analyzed from both conditions at each time point by DNA microarray **(Dataset S1)**. We observed that gene expression of control parasites (CM) best correlated with isoleucine-starved parasites (-Ile) that had been grown 2.5 to 3-fold longer [*e.g.* the 18 hr –Ile sample best correlated with the 6 hr CM sample and the 30 hr –Ile sample best correlated with the 12 hr CM sample] **(Fig. 3A and Table S1)**. The starvation-associated growth retardation occurred quickly, with significant transcriptional deviation between the fed and starved samples apparent within 6 hours of incubation **(Table S1)**. Using Pearson correlation, the expression profiles generated from this dataset were compared against those from a high resolution transcriptional array study, which sampled the complete intraerythrocytic developmental cycle (IDC) of *in vitro* cultured *P. falciparum* every hour [24]. These results demonstrate the remarkably retarded progression of the isoleucine-starved parasites through the trophozoite stage **(Figs. 3B, 3C, and Dataset S2)**. The developmental rate of the starved parasites through one life cycle decreased by 60%, ultimately ending at the mid-trophozoite stage, while the CM-fed control parasites progressed normally through the IDC, transitioning from late rings to trophozoites to schizonts, continuing on to initiate another round of invasion **(Fig. 3C, and Dataset S2)**. In other eukaryotes, amino acid starvation activates a distinct shift in transcriptional activity, in which genes that support adaptation and cell viability are selectively up-regulated [8, 12]. However, in *P. falciparum*, transcription remained

inextricably coupled with parasite development during starvation, with both gene expression and parasite morphology remaining in phase but displaying slowed progression through the IDC. There were no obvious transcriptional alterations indicative of a conventional starvation-related stress response **(Fig. 4 and Dataset S3)**. For instance, genes involved in glycolysis, translation/transcription, apicoplast and mitochondrial function, appear to follow their normal course of regulation, albeit at a slowed rate.

PfeIF2α specifically responds to isoleucine levels

In *P. falciparum*, exposure to amino acid-free RPMI results in the phosphorylation of parasite eIF2α (PfeIF2α) [14]. Considering that isoleucine is the only amino acid for which the parasite can truly be starved, we examined the specificity of this response. We incubated parasites in amino acid-free RPMI, followed by supplementation with complete medium containing all 20 amino acids, or with the following single amino acids: isoleucine, methionine, or leucine, which represent amino acids that are absent, in low abundance, or abundantly present in human hemoglobin [1], respectively. As seen previously [14], phosphorylation of PfeIF2α in amino acid-free conditions was readily observed by western blot analysis using antibodies specific for the phosphorylated motif **(Fig. 5A)**. Addition of complete medium resulted in the dephosphorylation of PfeIF2α, however, single amino acid supplementation with methionine or leucine did not elicit this response. Only the addition of isoleucine to the amino acid starved cultures resulted in dephosphorylation of PfeIF2α similar to that achieved with complete medium supplementation. Furthermore, we observed that when isoleucine was supplemented to

cultures maintained in isoleucine-free RPMI, PfeIF2α dephosphorylation could be detected by 10 minutes, with complete loss of phosphorylation by 45 minutes of incubation **(Fig. 5B)**. Remarkably, phosphorylation of eIF2α could be detected within minutes after isoleucine withdrawal **(Fig. 5C)**, suggesting the absence of a significant intracellular isoleucine store**.** Collectively, these data indicate that the eIF2α-mediated starvation response of *P. falciparum* is specifically sensitive to the isoleucine environment.

Recovery from starvation is independent of PfeIK1 signaling

The amino acid sensing eIF2α kinase of *P. falciparum* was recently identified as PfeIK1, an ortholog of yeast GCN2. Unlike the parental strain, parasite mutants lacking PfeIK1 do not phosphorylate PfeIF2α in amino acid-limiting medium conditions [14]. Since long-term starved wild-type parasites maintain PfeIF2α phosphorylation **(Fig. 5D)** and are able to recover growth post re-supplementation **(Fig. 1C)**, we investigated whether *pfeik1-* mutants lose viability after prolonged starvation. We incubated a clonal line of *pfeik1-* parasites [14] in isoleucine-free RPMI for 24 hours, then supplemented the culture with isoleucine and measured outgrowth by flow cytometry. The *pfeik1-* parasites starved for 24 hours recovered growth similar to the wild type parental strain **(Fig. 6A)**, suggesting that parasite viability during starvation does not depend on PfeIK1 signaling. The *pfeik1-* parasites exhibited a reduction in the metabolic incorporation of [^{35}S] methionine and cysteine during amino acid starvation **(Fig. S3)**, indicating a decrease in protein synthesis, but phosphorylation of PfeIF2α remained undetectable in the mutant parasites **(Fig. S4)**.

To further assess whether PfeIF2α phosphorylation plays a role in regulating the starvation stress response of *P. falciparum*, we generated parasites expressing an episomal copy of a Green Fluorescent Protein (GFP)-tagged non-phosphorylatable form of PfeIF2α, possessing an alanine in place of the conserved serine residue (S59A), or a wild-type version as a control. When exposed to isoleucine-free conditions, the mutant PfeIF2α-S59A is not phosphorylated, while both the wild type episomal copy and the endogenous PfeIF2α are robustly phosphorylated **(Fig. 6B)**. In other organisms, the expression of a phosphorylation-insensitive eIF2α typically dampens the adaptive response to stress, resulting in reduced fitness [9, 26], however parasites expressing mutant PfeIF2α-S59A grew well and recovered as well as the wild-type expressing control following extended isoleucine starvation and re-supplementation **(Fig. 6C)**. These data indicate that although PfeIK1 phosphorylation of PfeIF2α is induced by isoleucine starvation in a wild type background, the regulation of parasite growth during starvation, and concomitant entry into and exit from the hibernating state is independent not only of PfeIK1 activity, but also of PfeIF2α phosphorylation.

Discussion

In this study, we have shown that starvation for the single amino acid isoleucine elicits a metabolic response in *P. falciparum* that results in slowed parasite growth. This starvation-induced stasis is reversed upon isoleucine re-supplementation, demonstrating the remarkable resilience of *Plasmodium*. We liken this phenomenon to hibernation, in which an organism is able to dramatically decrease its metabolic rate to conserve energy and resources, ultimately leading to increased survival once growth-permissive

conditions are restored. Although asexual growth is recoverable post-starvation, our data suggests that limitations exist regarding the duration of starvation tolerable by the parasite. By 72 hours a checkpoint may be reached, presumably related to S-phase, and viability starts to tail off. Initially we suspected an increase in gametocyte conversion with extended starvation, considering that reductions in asexual parasitemia often correlate with induction of gametocytogenesis [27]. However, gametocytemia was virtually absent in the recovered cultures, suggesting that amino acid stress does not necessarily skew parasite commitment towards sexual differentiation, and that starvation-induced hibernation can protect the parasite for only a limited time before viability is compromised. Optimal growth of *in vitro* cultured *P. falciparum* requires an isoleucine concentration above 20 μM, however, slow, continuous growth is still observed in cultures maintained in lower isoleucine concentrations [4]. In natural infections of malnourished children, where isoleucine levels can fall to single digit micromolar levels [6], we propose that *P. falciparum* adjusts its metabolic growth accordingly, allowing it to survive and persist.

Notably, entry into the hibernating state did not confer protection against treatment with the antimalarial artemisinin, which has been described to induce a putative quiescent state in *P. falciparum* [23]. However, artemisinin-tolerance has only been reported for ring forms [22, 23], and isoleucine-starved parasites gradually progress past this stage, therefore the biological mechanisms for drug-associated dormancy and starvation-induced hibernation may differ at the level of cell cycle kinetics. It would nevertheless be of great interest to investigate PfeIF2α phosphorylation status in artemisinin-tolerant ring stages.

Proteolysis plays a role in maintaining the parasite in a growth-competent state, as inhibition of this activity adversely affected recovery post-isoleucine re-supplementation. As starved parasites slowly progress through the trophozoite stage, peak proteolytic activity appears to coincide with gradual maturation. Moreover, this activity becomes particularly crucial during long-term starvation. Hemoglobin, which lacks isoleucine, makes up 95% of the soluble host cell protein [19], leaving the other 5% to supply a limited pool of isoleucine to sustain the parasite during extended hibernation. There does not appear to be a significant isoleucine store, since parasites pre-conditioned in subsistence levels of isoleucine (20 μM) survived starvation as well as those conditioned in high (200 μM) isoleucine, and since parasites detect isoleucine withdrawal almost immediately, evidenced by rapid phosphorylation of PfeIF2α.

Isoleucine starvation did not activate an alternative transcriptional program in the parasite, the hallmark of a conventional starvation response [11, 12]. This observation further illustrates the parasite's astonishingly limited capacity for transcriptional regulation [28-30], and is consistent with the lack of homologs for starvation response regulators such as GCN4 and ATF4. Additionally, homologs of the prokaryotic transcription factor RelA, which regulates the amino acid starvation response in bacteria [31], could not be identified in the parasite. Although infected RBCs reportedly take up amino acids, including isoleucine, an order of magnitude more efficiently than uninfected RBCs [32], expression of putative amino acid transporters (*e.g.* PFL1515c, PF11_0334, PFL0420w) was not upregulated during starvation **(Dataset S3)**. Furthermore, *Plasmodium* encodes 9 putative autophagy-related genes (ATG), representing less than 30% of the complement of ATG genes in other eukaryotes [13]; interestingly, none of

these genes appeared to be specifically induced during starvation **(Dataset S3)**. The most remarkable feature of the transcriptome from starved parasites in this report is the apparent delay in both the decay and accumulation of stage-related transcripts, constituting a 60% decrease in the rate of developmental progression. Field isolates of *P. falciparum* exhibit differential expression patterns, including one described as having starvation response characteristics [33]. This phenomenon was not observed in our conditions, considering that isoleucine-starved parasites continued to express genes normally associated with active growth, only with significantly delayed kinetics in comparison with a fed control.

In other eukaryotes, such as yeast, plants, and mammals, the amino acid starvation response has been extensively studied, with many conserved biological features [*e.g.* TOR signaling [8], upregulation of amino acid biosynthetic enzymes [11], and induction of autophagy [34]] inherently translatable to other model systems. However, components of this response that are found in other organisms appear to be missing in *Plasmodium* [3, 13]. Even the one component involved in the canonical starvation response that is conserved, the GCN2 ortholog PfeIK1, is seemingly dispensable since its absence does not compromise the viability of parasites under isoleucine-limiting conditions, despite a well-documented ability to phosphorylate PfeIF2α during starvation. In prototrophic yeast, lack of GCN2 does not affect logarithmic growth in most single amino acid dropout medium conditions due to compensatory crosstalk between other amino acid regulatory pathways that function independently of GCN2-mediated signaling [35]. However, there is no evidence to suggest that such metabolic complexity exists in *P. falciparum*, especially considering that the parasite is deficient in amino acid biosynthesis

and is absolutely dependant on exogenous isoleucine. The *Plasmodium* genome encodes another blood stage-expressed eIF2α kinase [15], but we suspect that functional redundancy is not at play during starvation since PfeIF2α remains unphosphorylated in *pfeik1*- parasites. Indeed, we have shown that amino acid starvation of wild-type *P. falciparum* results in the phosphorylation of PfeIF2α, with remarkable selective specificity for isoleucine. Although this response is generally coupled to growth arrest, this does not appear to be the case for blood stage *P. falciparum*: This raises the question of the role played by PfeIK1. Why did malaria parasites maintain this enzyme? Could it have other functions independent of PfeIF2α phosphorylation? The surprisingly fast response in PfeIF2α dephosphorylation upon isoleucine repletion implies an efficient signaling system starting with an isoleucine sensor and feeding into an effector phosphatase; it would be of great interest to elucidate this pathway.

Presumably as an adaptation to erythrocyte parasitism, the malaria organism has evolved a stripped-down starvation response pathway. In light of our findings, we propose that growth regulation in *P. falciparum* operates predominantly by reaction rates. Low isoleucine concentrations slow the rate-limiting steps of translational processivity, thereby restricting growth. This primitive response to amino acid limitation is enough to allow the parasite to survive in its host for several days, waiting for nutrient repletion.

Materials and Methods

Parasite culturing

Plasmodium falciparum strain 3D7 and derived knockout clones were cultured [36] in human O+ erythrocytes in complete RPMI 1640, containing all 20 amino acids, supplemented with 27 mM $NaHCO_3$, 22 mM glucose, 0.37 mM hypoxanthine, 10 μg/ml gentamicin, and 5 g/l Albumax (Invitrogen). Homemade complete, isoleucine-free, and amino acid-free RPMI were prepared according to the RPMI 1640 recipe provided by Invitrogen, and supplemented with 100x RPMI 1640 Vitamins (Sigma), the appropriate respective amino acids (Sigma) at the concentrations found in RPMI 1640, and the additional supplements mentioned above. A clone of *P. falciparum* strain 3D7 (IG06), that has a 38 hour cycle, was used for most analyses. This fast-growing strain allowed us to perform more extensive time courses. Both 38-hour and conventional 48-hour clones were able to recover from starvation and correlation of gene expression profiles for corresponding stages was consistent.

Generation of episomal PfeIF2α (WT and S59A)-GFP expressing parasites

Full length PfeIF2α (PF07_0117) (omitting the stop codon) was PCR amplified from 3D7 genomic DNA using primers 5'-AATTCTCGAGATGACTGAAATGCG AGTAAAAGCAGATTTG-3' (XhoI site underlined) and 5'-AATTCCTAGGATCT TCCTCCTCCTCGTCTTCACTAGTATT-3' (AvrII site underlined), digested with XhoI and AvrII, and ligated into the same sites of the pIRCTGFP vector [37], containing the promoter region for PfHsp86, a C-terminal Green Fluorescent Protein (GFP) tag, and a human dihydrofolate reductase (hDHFR) drug selection cassette. A point mutation was introduced to change Ser59 to Ala59 in PfeIF2α using the QuikChange XL mutagenesis kit (Stratagene) and the primers 5'-GGAAGGTATGATTTTAATGTCCGAACTAGCC

AAAAGAAGATTCAGAAG-3' and 5'-CTTCTGAATCTTCTTTTGCGTAGTT CGGACATTAAAATCATACCTTCC-3'. All cloning steps were confirmed by sequencing.

Ring-stage 3D7 parasites were transfected by electroporation [38] with 100μg of purified vector DNA. Parasites carrying the plasmid were selected by adding 10nM WR99210 to the culture medium. 60% of transfected parasites were green by fluorescence microscopy, a typical plasmid maintenance result for this organism.

Flow cytometry

Parasite samples were fixed in 4% paraformaldehyde/ 0.015% glutaraldehyde in phosphate buffered saline (PBS) and stored at 4°C. For analysis, the cells were permeabilized with 0.1% Triton X-100 in PBS for 10 minutes at room temperature. One-half of the sample was treated with 100 μg/ml RNase A (Qiagen) in PBS for 20 minutes at 37°C. All samples were stained with 0.5 μg/ml acridine orange (Molecular Probes) in PBS and $3x10^4 - 1x10^5$ cells were counted on a BD Biosciences FACS Canto flow cytometer. Total cell number was measured on the forward and side scattering channels (FSC and SSC). Fluorescence was detected on both the FITC-H and the PerCP-Cy5-H channels and parasitemia gates were defined by intensity of fluorescence, with highly fluorescent infected RBCs distinctly separated from low fluorescence uninfected RBCs. Alternatively, live cells were directly stained with 0.5 μg/ml acridine orange and immediately counted on the FACS Canto using the parasitemia gating parameters described above. Data were analyzed using Flowjo software (Treestar Inc.).

Growth recovery assay

P. falciparum 3D7 parasites and clonal knockout lines were sorbitol synchronized [39] to the late ring stage, cultured in complete RPMI at 2% hematocrit, and sub-cultured to approximately 1% parasitemia. The cultures were washed twice in PBS, partitioned and washed in either complete RPMI or isoleucine-free RPMI, after which, the parasites were re-plated in triplicate in their respective medium and incubated at 37°C with 5% CO_2. Control fed and isoleucine-starved parasites were grown for 72 hours and prepared for flow cytometry to assess parasitemia. Remaining isoleucine starved cultures were supplemented with isoleucine (382 μM), after starving for various periods of time, and allowed to recover for an additional 72 hours. During starvation, isoleucine-free culture medium was refreshed every other day. Parasites were prepared for flow cytometry following recovery.

In the experiments where drug was added, either 10 μM trans-epoxysuccinyl-L-leucylamino (4-guanidine)-butane (E-64d) (Sigma), 5 μM pepstatin A (Sigma), or 50 nM artemisinin (Sigma) was added to fed or starved cultures for a 24 hour period at various times during the incubation. After the 24 hour exposure, cultures were washed twice in PBS, and re-plated in either complete medium for recovery or isoleucine-free medium for extended starvation, followed by isoleucine supplementation for recovery. Following the 72 hour recovery, parasites were prepared for flow cytometry.

Microarray analysis

A large-scale sorbitol synchronized [39] *P. falciparum* 3D7 culture at 8 – 10% parasitemia was washed twice in PBS, equally partitioned and washed in either complete

or isoleucine-free RPMI, after which, the parasites were re-plated in their respective medium and incubated at 37°C with 5% CO_2. Samples were harvested initially, and at 3 or 6 hour intervals over a 48 hour period. Culture medium was changed every 12 hours, and parasites incubated in complete medium were sub-cultured just prior to schizont rupture to maintain post-reinvasion parasitemia between 8 – 10%. The infected RBC pellet was washed with PBS and resuspended in Trizol ® Reagent (Invitrogen). Chloroform was added, followed by centrifugation at 9000 rpm for 1 hour at 4°C. Isopropanol was added to the aqueous phase to precipitate the RNA. Following centrifugation, the isolated RNA pellet was washed with 70% ethanol, dried, and dissolved in diethylpyrocarbonate (DEPC)-treated water.

cDNA synthesized from isolated RNA was fluorescently labeled with Cy5 and hybridized against a Cy3-labeled reference pool as described previously [24]. The (Cy5/Cy3) ratio, representing relative expression levels, was calculated for each sample and $\log_2$ transformed for statistical analysis. Arrays were generated, clustered, and visualized with Java Treeview [40]. R-squared correlation values were calculated in Excel (Microsoft) by comparing the transformed relative expression data for each respective sample. Pearson coefficients were calculated in Excel (Microsoft) by comparing the transformed relative expression data from this dataset with that from corresponding values generated for the high resolution IDC transcriptome reported in ref 24. Data were assembled and graphed in Excel (Microsoft) and Prism 5 (Graphpad).

Starvation assay

P. falciparum 3D7 parasites and clonal lines of *pfeik1*- parasites [14] were prepared and assayed as described previously [14]. Briefly, parasites were sorbitol synchronized [39] to the late ring stage, cultured in complete RPMI at 2% hematocrit, and grown to approximately 8 – 10% parasitemia. The parasites were washed twice in PBS, equally partitioned and washed in complete, isoleucine-free, or amino acid-free RPMI, after which, the parasites were re-plated in their respective medium. Cultures were incubated at 37°C with 5% CO_2 for various increments of time. Following incubation, parasites were either harvested immediately or supplemented with complete RPMI or with single amino acids (isoleucine, methionine, leucine) (Sigma) at concentrations found in complete RPMI and re-incubated at 37°C for up to 45 minutes. After harvesting, infected RBCs were lysed with 100 hemolytic units (HU) of tetanolysin (List Biological), washed with PBS buffer containing Complete™ protease inhibitor cocktail (Roche), 2 mM NaF and 2 mM Na_3VO_4. Samples were resuspended in SDS-Laemmli buffer. Parasite proteins were resolved by SDS-PAGE and transferred to nitrocellulose for immunoblotting [41].

Antibodies and immunoblotting

Rabbit anti-phosphorylated eIF2α (Ser51) and mouse anti-eIF2α were purchased from Cell Signaling Technology. Rabbit and rat anti-BiP were acquired from the Malaria Research and Reference Reagent Resource Center (ATCC). Secondary antibodies conjugated with horseradish peroxidase (HRP) were from GE Healthcare Life Sciences. For immunoblotting, nitrocellulose membranes were blocked with 5% BSA in TBS-0.1% Tween 20 (TBST) for 1 hour at room temperature. Rabbit anti-phosphorylated eIF2α

(Ser51) was diluted 1:1000 in TBST. Mouse anti-eIF2α was diluted 1:1000 in TBST. Rabbit or rat anti-BiP was diluted 1:10,000 in TBST. Respective secondary antibodies were diluted 1:20,000. Bound antibodies were detected with Western Lightning™ Chemiluminescence reagent (Perkin Elmer, Boston, MA).

Metabolic labeling and TCA precipitation

P. falciparum 3D7 parasites and clonal lines of *pfeik1*- parasites were sorbitol synchronized [39] to the late ring stage, cultured in complete RPMI at 2% hematocrit, and grown to approximately 8 – 10% parasitemia. The parasites were washed twice in PBS, equally partitioned and washed in either complete or isoleucine-free labeling RPMI, which did not contain methionine or cysteine. The parasites were then re-plated in their respective medium in the presence or absence of 10 μg/ml cycloheximide (CHX), and incubated at 37°C with 5% CO_2 for 6 hours. During the last hour of the incubation, 0.1 mCi [^{35}S] Express protein labeling mix (Perkin Elmer, 1175 Ci/mmol) was added to each culture. After harvesting, labeled cultures were washed with PBS buffer containing Complete™ protease inhibitor cocktail (Roche) and lysed with 100 HU of tetanolysin (List Biological). A portion of the samples were resuspended in SDS-Laemmli buffer, followed by SDS-PAGE, Coomassie staining, and autoradiography. Remaining samples were TCA precipitated by adding 1/4 volume of 100% (w/v) trichloroacetic acid (TCA) to the parasite pellet, resuspended in 200 μl PBS. Samples were incubated on ice for 10 minutes and centrifuged. The precipitated protein pellet was washed with ice cold acetone, dried, resuspended in water, and pipetted onto FilterMat (Skatron Instruments,

VA). After the filters dried, they were placed in vials with Ultima Gold scintillation fluid (Perkin Elmer) and counted on a Beckman LS6000 scintillation counter.

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Figure Legends

Figure 1: *P. falciparum* growth during and recovery from isoleucine starvation.

A) Representative images of Geimsa-stained thin blood smears prepared from parasites at 0, 24, 48, and 72 hours of incubation in either CM or Ile-free RPMI medium. Arrows in images from isoleucine-starved cultures indicate hemozoin pigmentation. B) Flow cytometry assessment of DNA content. Synchronous 3D7 parasites were grown in either complete (CM) or Ile-free (-Ile) RPMI medium and samples were harvested at 0 (red), 24 (green), 48 (blue), and 72 (brown) hours. Offset overlayed histograms of FITC-H channel fluorescence for the indicated time points and medium conditions are shown. Samples were treated with RNase, allowing haploid ring and trophozoite populations (left-most peak) to be better distinguished from polyploid schizonts (right-most peaks). The gated uninfected RBC population was removed for clarity. C) Growth recovery following isoleucine re-supplementation of parasites starved for indicated times. A control set of parasites were either fed (CM) or isoleucine-starved (no Ile) for 72 hours. Synchronized 3D7 parasites were starved for up 9 days, followed by supplementation with isoleucine. Parasitemia of all cultures was measured by flow cytometry after 72 hours of recovery. Data shown represent the mean parasitemia ± SEM, n=3. (n.d.*, none detected) D) Protein synthesis in starved parasites. Parasites were fed or starved for 6 hours, and labeled with [^{35}S] met/ cys for the last hour while incubated in complete (CM) or isoleucine-free (no Ile) labeling RPMI medium in the presence or absence of the protein synthesis inhibitor cycloheximide (CHX). Parasite proteins were resolved by SDS-PAGE for autoradiography (top panel) or TCA precipitated to determine incorporated radioactivity through scintillation counting (bottom panel). The SDS-PAGE

gel was stained with Coomassie Brilliant Blue (CB) to ensure even protein loading. Data shown represent the mean disintegrations per minute (dpm) of incorporated radioactivity ± SEM, n= 6.

Figure 2: Protease activity is required to maintain viability during isoleucine starvation.

A) Synchronous 3D7 parasites were either fed (black bars) or starved for isoleucine (gray bars) for the indicated times in the presence of 10 μM E-64d (upper panel) or 5 μM pepstatin A (Pep A, lower panel) for the last 24 hours of the incubation. Following drug removal, each culture was re-plated in CM for recovery. Parasitemia of all cultures was measured by flow cytometry after 72 hours of recovery. A control set of parasites, shown on the far left of each graph, were grown in the absence of drug for 72 hours. Data shown represent the mean parasitemia ± SEM, n=3. B) Synchronous 3D7 parasites were starved for isoleucine for the indicated times in the presence of 10 μM E-64d (upper panel) or 5 μM pepstatin A (Pep A, lower panel) for the first 24 hours of the incubation. Following drug removal (and extended starvation for the 48h and 72h samples), parasites were re-plated in CM for recovery. Parasitemia of all cultures was measured by flow cytometry after 72 hours of recovery. A control set of parasites were either fed (CM) or isoleucine-starved (no Ile) in the absence of drug for 72 hours. Data shown represent the mean parasitemia ± SEM, n=3. C) Growth recovery following isoleucine re-supplementation of synchronous 3D7 (WT, black bars) and *fp2* knockout (FP2KO, white bars) parasites incubated in Ile-free RPMI for the indicated times. Control parasites were either fed (CM) or starved for isoleucine without re-feeding (no Ile) for 72 hours. Parasitemia of all

cultures was measured by flow cytometry after 72 hours of recovery. Data shown represent the mean parasitemia ± SEM, n=3. Each experiment was repeated at least three times. Representative experiments are shown.

Figure 3: Developmental progression of hibernating parasites

RNA was isolated from synchronous 3D7 parasites that were either fed (CM) or starved for isoleucine (-Ile), with samples harvested at 3 or 6 hour intervals over the course of 48 hours. A) 3-D graph showing R-squared correlation values corresponding to the comparison of the global transcriptional data generated from parasites maintained in both medium conditions at the indicated time points. Bars represent the correlation of each starved sample with fed parasites at 3 (blue), 6 (light purple), 12 (yellow), 18 (light blue), 24 (dark purple), or 30 (pink) hours of incubation. Fed time points >30 hours omitted for clarity. The height of each bar indicates the strength of the correlation, with taller bars denoting a strong relationship and shorter bars denoting a weak relationship between the compared samples. B) Pearson coefficient values were calculated by comparing the global transcriptional data generated from parasites maintained in both medium conditions at the indicated time points against corresponding data from each time point generated in the high resolution intraerythrocytic developmental cycle (IDC) analysis from ref 24. Y-axis: Pearson coefficient; X-axis: hours post invasion (h.p.i.) in the IDC data set. The apex of the peak in each graph corresponds to the approximate point in the IDC to which the fed (CM, open symbols) or starved (-Ile, filled symbols) parasites best correlate at the indicated incubation time. Plots are shown with a loess fit of the data: 0 hr, red; 12 hr, purple; 24 hr, green; 36 hr, orange. C) Summary plot of progress through

the IDC (based on Pearson coefficient (see 2B figure legend)) of parasites that were fed (CM, black circles) or starved (no Ile, gray squares, dashed line) for the indicated incubation times. The red and blue dashed lines indicate the slope (m) of the best-fit curve for the CM and no Ile points up to 24 hours of incubation, respectively.

Figure 4: Expression of metabolic, organellar, and functional pathway genes in starved parasites

Cy5-labeled cDNA from synchronous 3D7 parasites, that were either fed (CM) or starved for isoleucine (-Ile) over a 48-hour period, was hybridized against a Cy3-labeled parasite cDNA reference pool. The expression profiles of representative genes involved in the indicated biological pathways are shown. The labels at the top denote the parasite stage of each sample at the indicated time point: R, ring; T, trophozoite; S, schizont. The panels on the right consist of plots of the $\log_2$(Cy5/Cy3) expression values over time for representative genes from each of the indicated pathways. CM, black circles; no Ile, red squares. PFI1105w, phosphogylcerate kinase; PF14_0207, DNA-directed RNA polymerase III subunit; PF13_0014, 40S ribosomal protein S7; PF13_0327, cytochrome c oxidase subunit 2; PF14_0421, apicoplast acyltransferase.

Figure 5: Parasite eIF2α phosphorylation status depends on the isoleucine environment.

A) Re-supplementation of starved parasites. Synchronous parasites cultured for 6 hours in RPMI lacking all amino acids were re-supplemented with complete medium (CM) or the indicated single amino acids (at the concentration found in complete RPMI) for 45

minutes. Parasite lysates were prepared for SDS-PAGE followed by immunoblotting with antibodies against phosphorylated eIF2α (eIF2α-P), total eIF2α, and the endoplasmic reticulum marker BiP as a loading control. B) Time course of re-supplementation. Synchronous parasites were starved for 6 hours, then re-supplemented with Ile for the indicated times. Samples were processed for analysis as in A. C) Time course of starvation. Synchronous parasites were washed in –Ile medium, centrifuged briefly and re-plated in –Ile medium. Samples were taken at the indicated times and processed for analysis as in A. D) Synchronous parasites were maintained in isoleucine-free RPMI medium for 24 hours and then re-supplemented with Ile for 45 minutes. Samples were processed for analysis as in A.

Figure 6: PfeIK1 activity is not required for maintenance of viability during hibernation.

A) Viability of *pfeIk1* knockout parasites after isoleucine (Ile) starvation. Synchronous 3D7 parasites were incubated for 24 hours in –Ile medium. Ile was added back and parasites were allowed to recover in CM for 72 hours. Parental strain, black bars; *pfeik1* knockout clones, light (E6) and dark (C1) gray bars. Control parasites were either fed (CM) or starved for isoleucine without re-feeding (no Ile) for 72 hours. Parasitemia of all other cultures was measured by flow cytometry after 72 hours of recovery. Data shown represent the mean parasitemia ± SEM, n=3. B) Response of wild type (WT) PfeIF2α and PfeIF2α S59A phosphorylation mutant to starvation. Synchronous parasites expressing an episomal Green Fluorescent Protein (GFP)-tagged copy of either wild type (epi WT) or mutant (epi S59A mut) PfeIF2α, and a parental line of 3D7 were incubated in complete

(CM) or isoleucine-free RPMI (-Ile) for 5 hours. Parasite lysates were prepared for SDS-PAGE followed by immunoblotting with antibodies against phosphorylated eIF2α (eIF2α-P) and total eIF2α. C) Viability of PfeIF2α S59A phosphorylation mutant after Ile starvation. Parasites expressing an episomal GFP-tagged wild type copy of PfeIF2α (epi WT PfeIF2α), black bars; Parasites expressing an episomal GFP-tagged mutant copy of PfeIF2α (epi S59A mut eIF2α), gray bars. Growth recovery assay was performed as in A. Data shown represent the mean parasitemia ± SEM, n=3.

Supplemental Figures

Figure S1: Parasite recovery does not depend on pre-existing isoleucine stores. Synchronous 3D7 parasites, previously maintained in RPMI medium containing various concentrations of isoleucine, were starved for isoleucine for 24 hours, then re-supplemented. Parasitemia of all cultures was measured by flow cytometry after 72 hours of recovery. Data shown represent the mean parasitemia ± SEM, n=3.

Figure S2: Hibernating parasites remain susceptible to artemisinin. Synchronous 3D7 parasites were either fed (black bars) or starved for isoleucine (gray bars) for 72 hours with 50 nM artemisinin present for the last 24 hours of the incubation. Following drug removal, each culture was re-plated in CM for recovery. A control culture was incubated in the absence of drug for 72 hours in CM or isoleucine free RPMI (no Ile) for 72 hours, followed by isoleucine supplementation and recovery. Parasitemia

was measured by flow cytometry after 72 hours of recovery. Data shown represent the mean parasitemia ± SEM, n=3.

Figure S3: Protein translation is reduced in PfeIK1 mutants during isoleucine starvation.

Protein synthesis in starved parasites. Synchronous clonal *pfeik1*- parasites were fed or starved for isoleucine for 6 hours and labeled with [^{35}S] met/ cys for the last hour while incubated in complete (CM) or isoleucine-free (no Ile) labeling RPMI medium in the presence or absence of the protein synthesis inhibitor cycloheximide (CHX). Parasite proteins were TCA precipitated and amount of incorporated radioactivity was determined in a scintillation counter. Data shown represent the mean disintegrations per minute (dpm) of incorporated radioactivity ± SEM, n= 6.

Figure S4: PfeIF2α remains unphosphorylated in PfeIK1 KO parasites during prolonged starvation.

Synchronous clonal *pfeik1*- parasites were maintained in isoleucine-free RPMI medium for 18 hours, followed by re-supplementation with isoleucine for 45 minutes. Parasite lysates were prepared for SDS-PAGE followed by immunoblotting with antibodies against phosphorylated eIF2α (eIF2α-P) and BiP as a loading control.

Figure 1

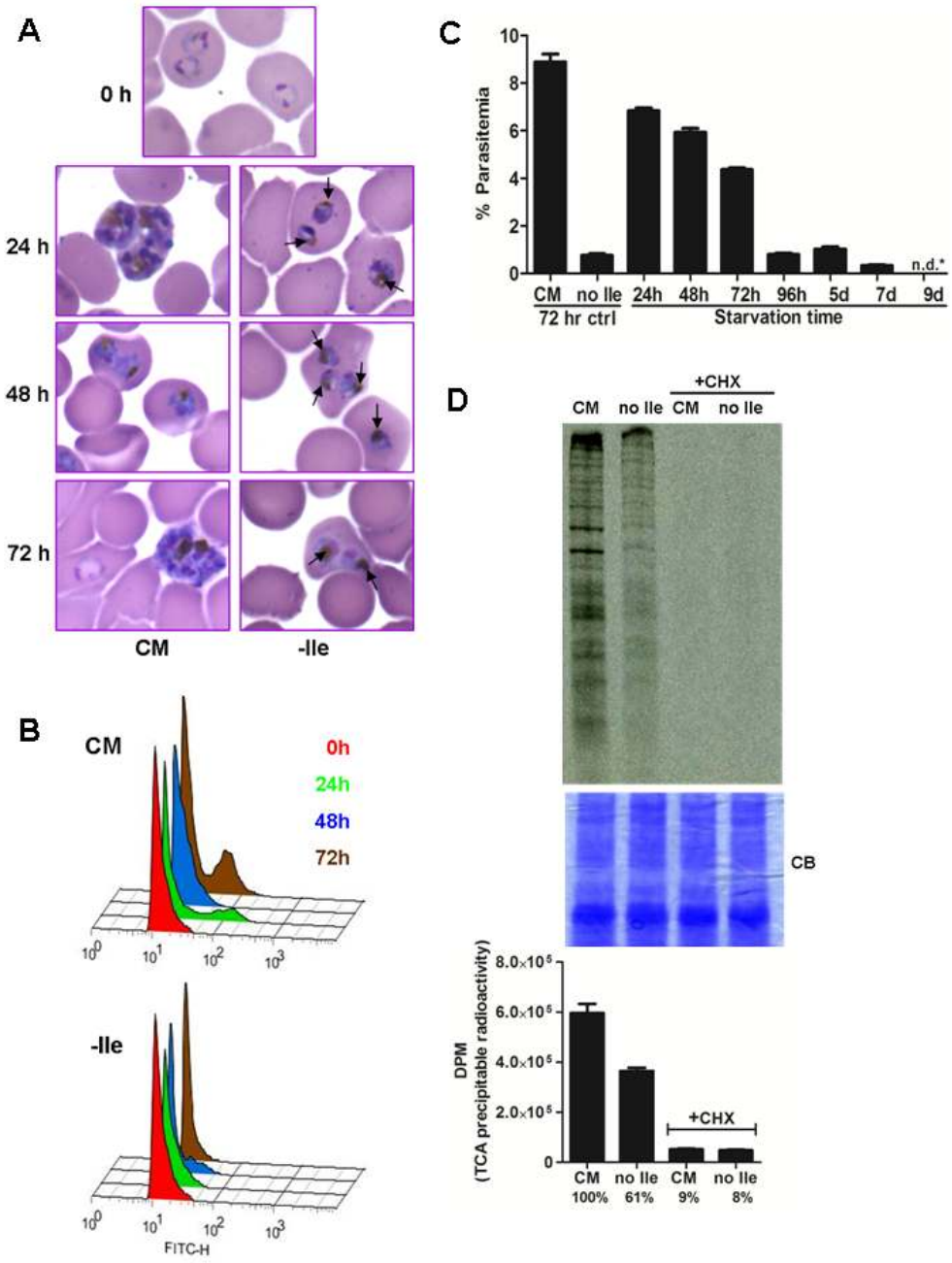
A
0 h
24 h
48 h
72 h
CM
-Ile
B
CM
0h
24h
48h
72h
-Ile
FITC-H
C
% Parasitemia
CM
no Ile
72 hr ctrl
24h
48h
72h
96h
5d
7d
9d
n.d.*
Starvation time
D
+CHX
CM
no Ile
CM
no Ile
CB
DPM
(TCA precipitable radioactivity)
+CHX
CM
100%
no Ile
61%
CM
9%
no Ile
8%

Figure 2

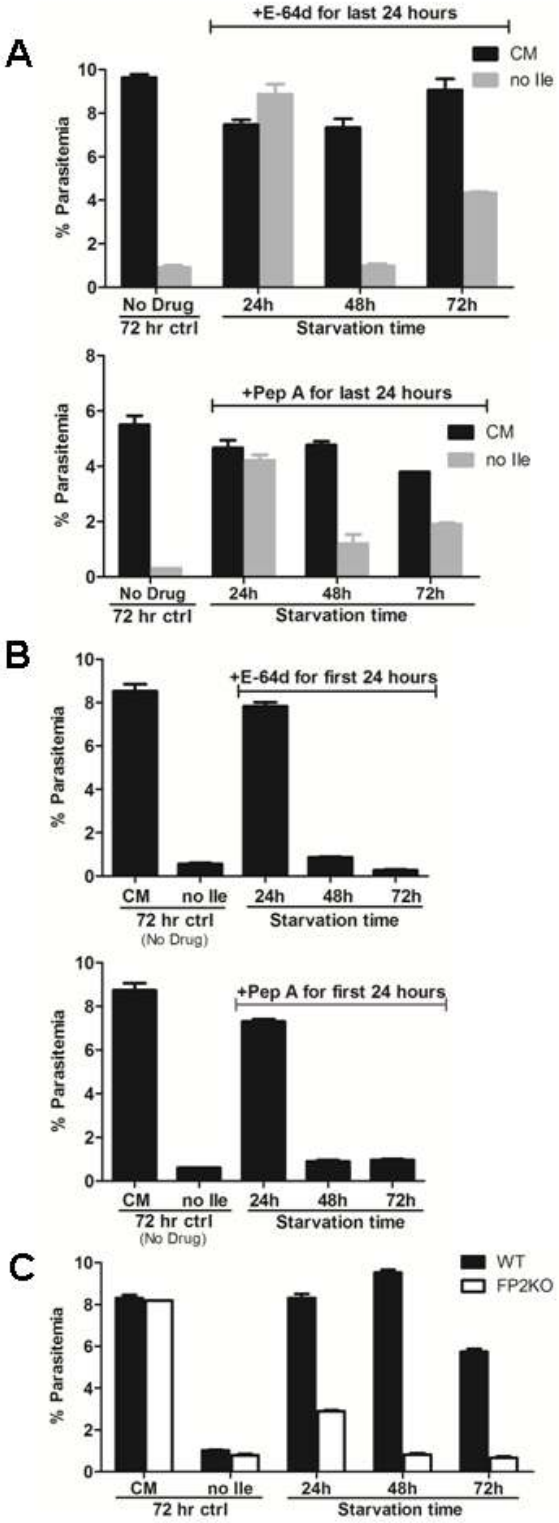
A
+E-64d for last 24 hours
CM
no Ile
% Parasitemia
No Drug
72 hr ctrl
24h
48h
72h
Starvation time
+Pep A for last 24 hours
B
+E-64d for first 24 hours
CM
no Ile
72 hr ctrl
(No Drug)
+Pep A for first 24 hours
C
WT
FP2KO

Figure 3

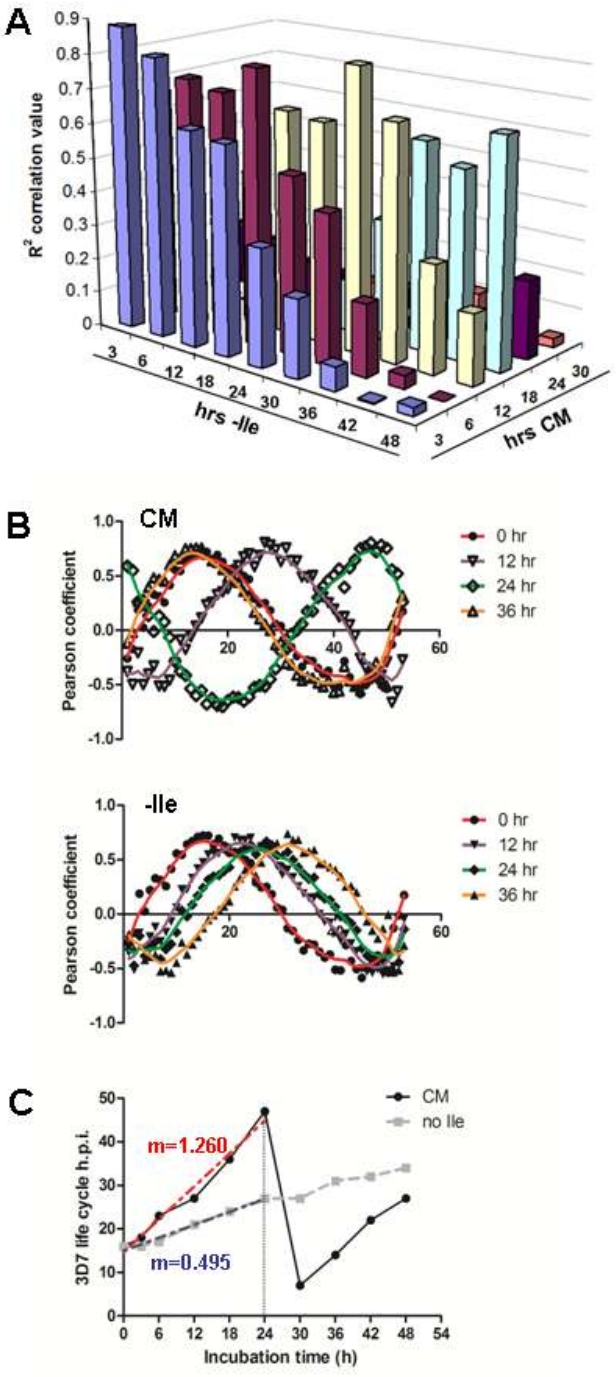
A
R² correlation value
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
3 6 12 18 24 30 36 42 48
hrs -Ile
3 6 12 18 24 30
hrs CM
B
CM
Pearson coefficient
1.0
0.5
0.0
-0.5
-1.0
20
40
60
0 hr
12 hr
24 hr
36 hr
-Ile
Pearson coefficient
1.0
0.5
0.0
-0.5
-1.0
20
60
0 hr
12 hr
24 hr
36 hr
C
CM
no Ile
m=1.260
m=0.495
3D7 life cycle h.p.i.
50
40
30
20
10
0
0 6 12 18 24 30 36 42 48 54
Incubation time (h)

Figure 4

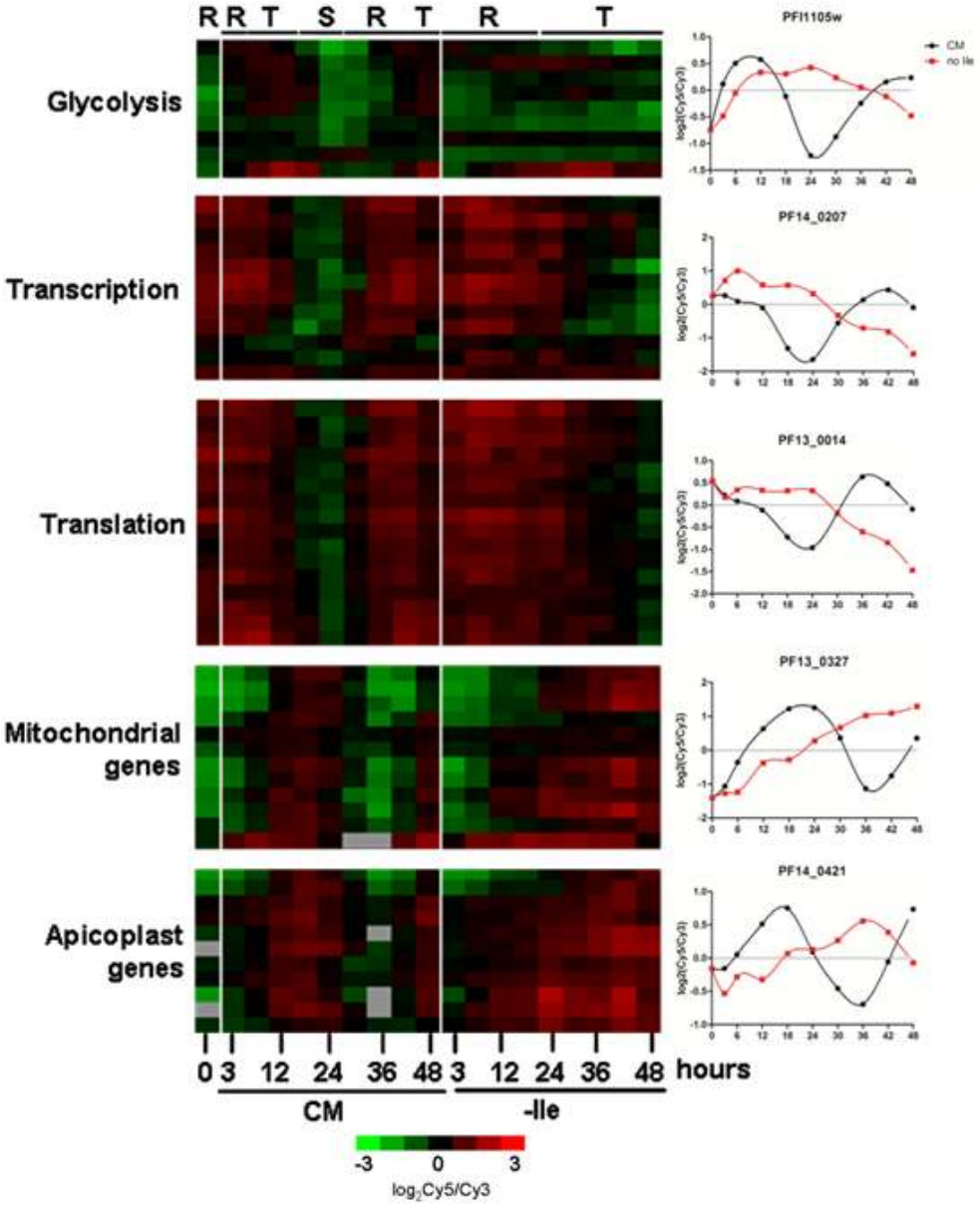
R R T S R T R T
Glycolysis
Transcription
Translation
Mitochondrial genes
Apicoplast genes
PFI1105w
PF14_0207
PF13_0014
PF13_0327
PF14_0421
CM
no Ile
0 3 12 24 36 48 3 12 24 36 48 hours
CM
-Ile
-3 0 3
log2Cy5/Cy3

Figure 5

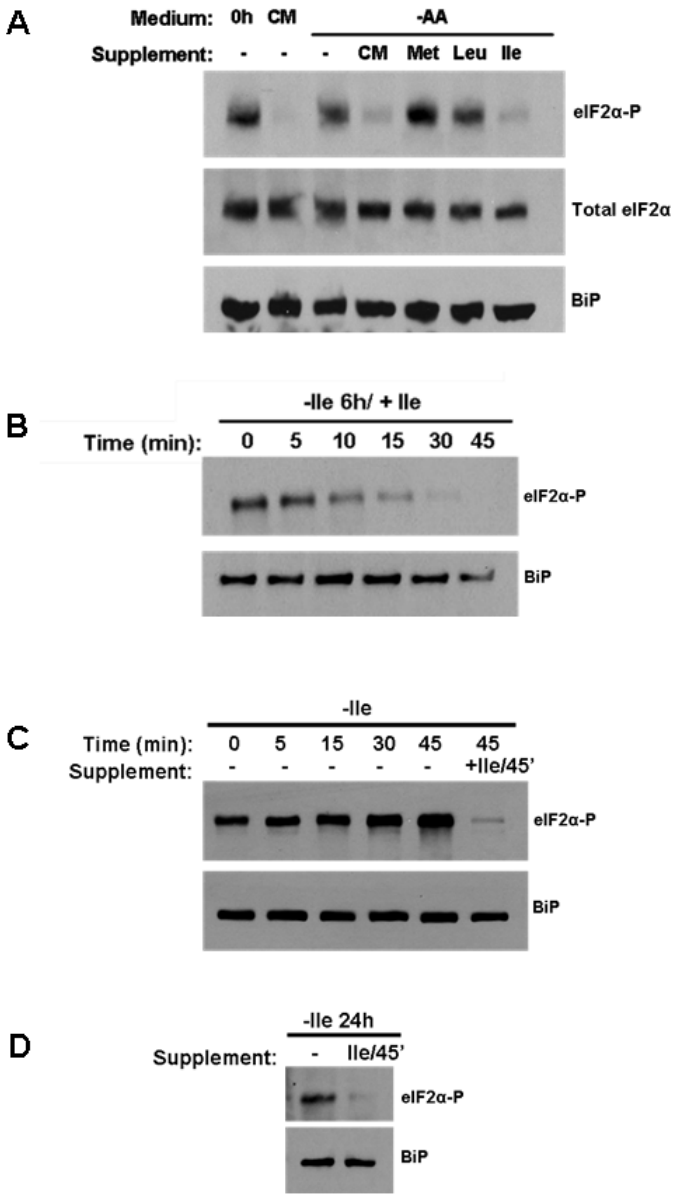
A
Medium: 0h CM -AA
Supplement: - - - CM Met Leu Ile
eIF2α-P
Total eIF2α
BiP
B
-Ile 6h/ + Ile
Time (min): 0 5 10 15 30 45
eIF2α-P
BiP
C
-Ile
Time (min): 0 5 15 30 45 45
Supplement: - - - - - +Ile/45'
eIF2α-P
BiP
D
-Ile 24h
Supplement: - Ile/45'
eIF2α-P
BiP

Figure 6

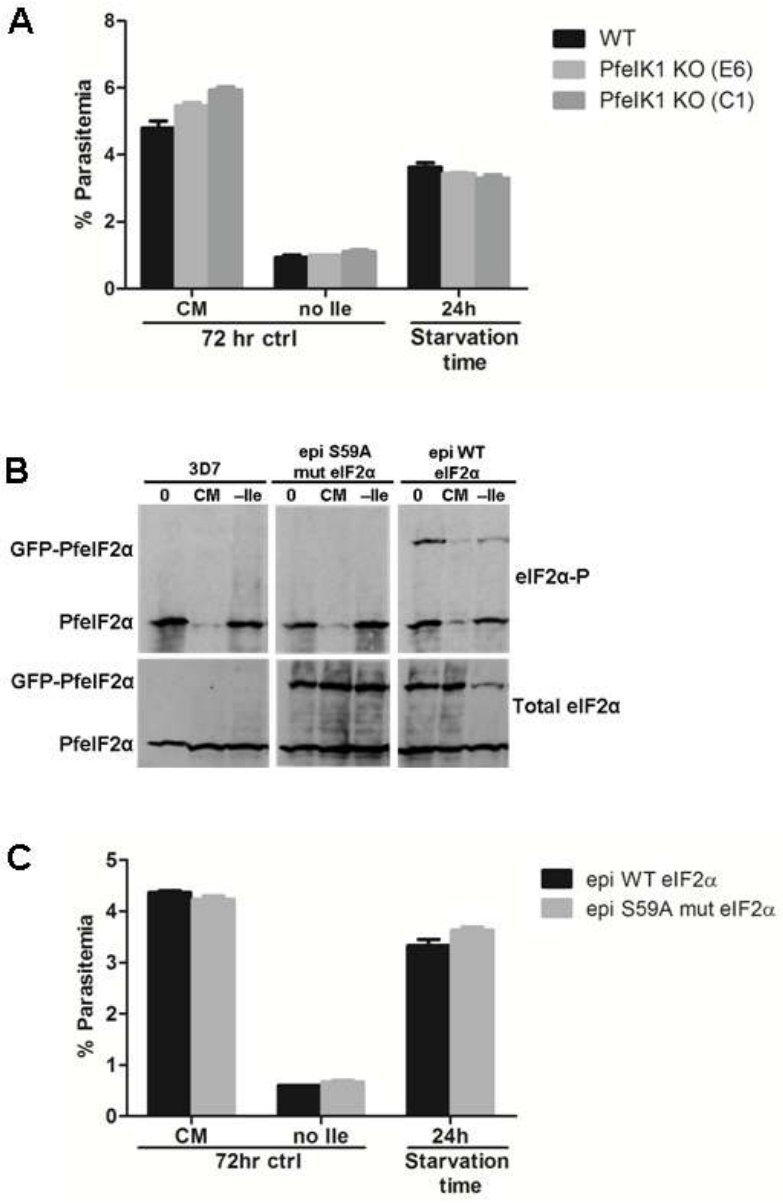
A
% Parasitemia
WT
PfeIK1 KO (E6)
PfeIK1 KO (C1)
CM
no Ile
24h
72 hr ctrl
Starvation time
B
3D7
epi S59A mut eIF2α
epi WT eIF2α
0 CM –Ile
GFP-PfeIF2α
PfeIF2α
eIF2α-P
Total eIF2α
C
% Parasitemia
epi WT eIF2α
epi S59A mut eIF2α
CM
no Ile
24h
72hr ctrl
Starvation time

Figure S1

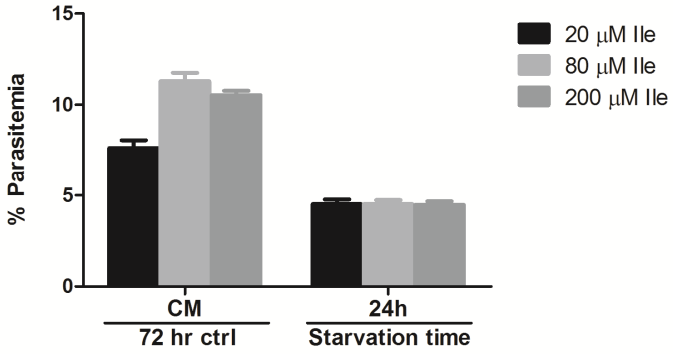
15
10
5
0
% Parasitemia
20 μM Ile
80 μM Ile
200 μM Ile
CM
72 hr ctrl
24h
Starvation time

Figure S2

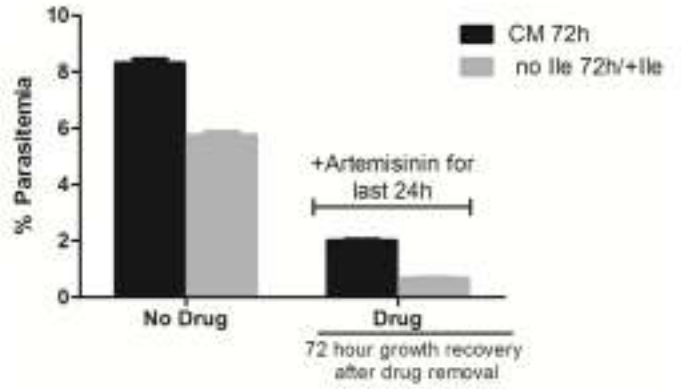
10
8
6
4
2
0
% Parasitemia
CM 72h
no Ile 72h/+Ile
+Artemisinin for
last 24h
No Drug
Drug
72 hour growth recovery
after drug removal

Figure S3

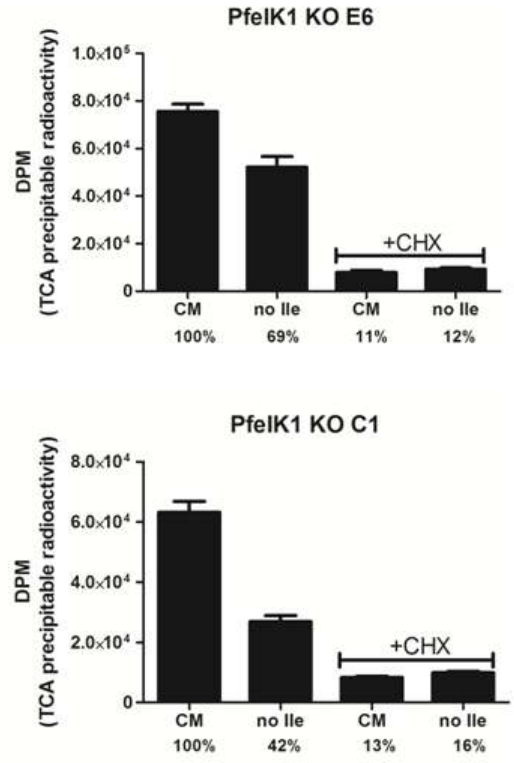
PfeIK1 KO E6
DPM
(TCA precipitable radioactivity)
1.0x10⁵
8.0x10⁴
6.0x10⁴
4.0x10⁴
2.0x10⁴
0
+CHX
CM
100%
no Ile
69%
CM
11%
no Ile
12%
PfeIK1 KO C1
DPM
(TCA precipitable radioactivity)
8.0x10⁴
6.0x10⁴
4.0x10⁴
2.0x10⁴
0
+CHX
CM
100%
no Ile
42%
CM
13%
no Ile
16%

Figure S4

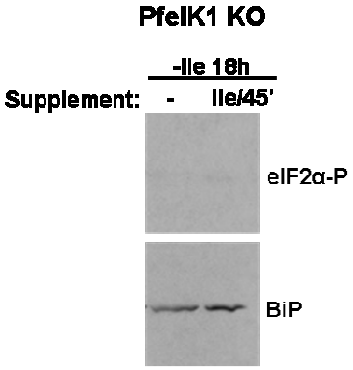

Table S1. R-squared correlation of gene expression between fed and isoleucine-starved parasites

	0h	3h+	6h+	12h+	18h+	24h+	30h+	36h+	42h+	48h+	3h-	6h-	12h-	18h-	24h-	30h-	36h-	42h-	48h-
0h																			
3h+	0.6473																		
6h+	0.4188	0.8637																	
12h+	0.0488	0.2812	0.5455																
18h+	0.0924	0.0169	0.0088	0.3638															
24h+	0.0733	0.2273	0.1961	0.0384	0.1831														
30h+	0.1541	0.0042	0.0145	0.1415	0.0753	0.2540													
36h+	0.7558	0.5164	0.2568	0.0018	0.1871	0.0336	0.3803												
42h+	0.6160	0.9464	0.8836	0.3350	0.0091	0.2058	0.0036	0.4972											
48h+	0.1709	0.4786	0.7582	0.8586	0.1647	0.0963	0.0894	0.0475	0.5584										
3h-	0.6895	0.8805	0.6718	0.1811	0.0467	0.2005	0.0207	0.6154	0.8667	0.3350									
6h-	0.5440	0.8110	0.7292	0.2873	0.0038	0.1673	0.0016	0.4388	0.8395	0.4438	0.8443								
12h-	0.3176	0.6241	0.7088	0.4522	0.0141	0.1212	0.0185	0.2012	0.6817	0.5690	0.6139	0.8490							
18h-	0.2538	0.6068	0.7942	0.6521	0.0725	0.1065	0.0514	0.1148	0.6477	0.7501	0.5142	0.6978	0.8562						
24h-	0.1232	0.3407	0.5139	0.6394	0.1496	0.0358	0.0600	0.0437	0.4021	0.6321	0.3187	0.5495	0.7856	0.8321					
30h-	0.0435	0.2270	0.4345	0.8136	0.3534	0.0152	0.1137	0.0013	0.2704	0.7038	0.1719	0.3244	0.5292	0.7274	0.8165				
36h-	0.0007	0.0680	0.2074	0.6796	0.6035	0.0065	0.1029	0.0198	0.0902	0.4938	0.0419	0.1384	0.2934	0.4563	0.6215	0.8570			
42h-	0.0091	0.0020	0.0390	0.3117	0.5475	0.0964	0.0302	0.0411	0.0062	0.1751	0.0009	0.0643	0.1879	0.2180	0.4651	0.5065	0.7254		
48h-	0.0533	0.0250	0.0001	0.2027	0.6611	0.2275	0.0258	0.1218	0.0138	0.0780	0.0182	0.0005	0.0243	0.0618	0.1902	0.3164	0.5756	0.6750	

Yellow: Best correlation between fed control samples

Green: Worst correlation between fed control samples

Turquoise: Best correlation between fed and starved samples

Pink: Worst correlation between fed and starved samples

Orange: Best correlation between starved samples

Light purple: Worst correlation between starved samples

Gray: Point where gene expression starts to deviate significantly between fed and starved sample

CHAPTER IV:

CONCLUSIONS AND FUTURE DIRECTIONS

Conclusions

This thesis work provides the first characterization of the amino acid starvation response of the human malaria parasite, *Plasmodium falciparum*. Based on previous work regarding the apparent growth inhibition of parasites starved for the essential amino acid isoleucine [1], we sought to determine the impact of isoleucine starvation on the viability of *P. falciparum*, examine the starvation-induced metabolic changes in its growth cycle, and uncover the underlying mechanisms governing the parasite's starvation-stress response. The eukaryotic nutrient-starvation response is a well-studied process, with TOR signaling and the GCN2/ eIF2α-mediated pathway playing key regulatory roles [2, 3]. These pathways have maintained remarkable evolutionary conservation from yeast to humans; however parasites from the *Plasmodium* genus are a rare exception. All elements of the TOR complex and most of its upstream and downstream effectors are absent from the parasite's genome [4]. Moreover, work presented in this thesis provides evidence that, although functionally conserved in terms of signal-mediated kinase activation, the amino acid starvation-associated eIF2α stress response results in a virtual regulatory dead-end in the parasite. Despite the lack of conventional stress-responsive growth control methods, starvation still elicits a dramatic, yet reversible, growth phenotype in the parasite, which we liken to hibernation.

Starvation-induced hibernation

In chapter 3 of this thesis, we present data detailing the metabolic response of *P. falciparum* exposed to isoleucine-limiting conditions. In short, we observed that 1) parasite growth slowly progresses to the trophozoite stage, where development stalls, 2) starved parasites experience cell cycle arrest and reduced protein translation, but can

resume normal growth upon isoleucine supplementation, 3) the rate of gene expression in starved parasites decreases significantly, corresponding with the parasite's delayed developmental progression, and 4) protein degradation, localized to the parasite's food vacuole, continues slowly and is required to maintain parasite viability during extended starvation.

The striking morphologic transition of starved parasites, along with the requirement for continuous proteolysis, distinguishes this starvation-induced state from the putative dormant state described for artemisinin-tolerant parasites [5]. The drug-induced dormant population stalls its development at the ring stage [6] and presumably, delays or limits hemoglobin digestion, given that artemisinin potency depends on efficient hemoglobinase activity [7]. Although isoleucine starvation and artemisinin treatment both appear to depress parasite growth, the biological mechanisms that induce the hypometabolic states associated with each respective stress are most likely distinct, since the resultant phenotypic features are incompatible.

Notably, starvation for other essential nutrients such as glucose, the major energy substrate for the parasite [8] did not induce the growth-competent hibernating state apparent under isoleucine-limiting conditions. Glucose starvation resulted in fairly rapid parasite death, and thus failed to yield any recoverable parasites. Given that isoleucine starvation readily elicited this remarkable growth phenotype in *P. falciparum*, we propose that this response may represent a metabolic adaptation to cope with the inconsistent extracellular isoleucine supply encountered during infection of a human host, thus allowing the parasite to survive and persist.

In data presented in chapter 3, active proteolysis in *P. falciparum* during starvation is evident by the appearance of hemozoin in the parasite's food vacuole (FV), indicating hemoglobin degradation [9]. However, as noted previously, the parasite cannot obtain isoleucine from this source [10]. Considering that *P. falciparum* continues to gradually develop, steadily increasing its mass during prolonged starvation, we propose that the parasite also degrades other RBC cytosolic proteins that contain isoleucine, thus providing a limited pool of this amino acid to support restricted biosynthetic processes that presumably sustain the parasite during starvation. Such starvation-associated proteolysis is reminiscent of the process of autophagy, a mechanism induced in eukaryotes to maintain cell viability in nutrient-poor conditions [11]. At least 9 autophagy-related genes (ATG) are conserved in the *Plasmodium* genome [4], however their role in the starvation-stress response of the parasite is disputable considering that their expression was not specifically induced in our starvation assay. Furthermore, unpublished studies in yeast deletion mutants and mammalian cells suggest that *Plasmodium* ATG orthologs may not be functionally conserved [4]. Our data indicate that proteolysis during starvation is indeed required to ensure parasite survival, and that resident FV hemoglobinases (*i.e.* plasmepsins and falcipains) serve to fulfill this need. In autophagy, cellular contents are engulfed by autophagosomes, which go on to fuse with lysosomal vesicles containing digestive enzymes [12]. Presumably, the proteases housed within the acidic FV, which is regarded as the lysosomal organelle of the parasite [13], perform dual roles: facilitating the ordered catabolism of hemoglobin to provide nutrient to and make space for the growing parasite under normal conditions [1], and contributing to the amino acid starvation response by degrading cellular proteins, thus providing a

source of scarce amino acids, such as isoleucine, to maintain the viability of the parasite in its hypometabolic state.

The metabolic retardation of critical cellular processes such as DNA replication, gene transcription, and protein synthesis in starved parasites signifies the entry into starvation-induced hibernation. Starved parasites slowly progress through the ring and trophozoite stages, which can be regarded as the G0/G1 cell cycle points of the parasite [9, 14], however our data indicate that S-phase, where DNA replication takes place, is not initiated. This finding suggests that replication checkpoints are in place to prevent parasite proliferation in conditions that cannot adequately support growth. Furthermore, the aforementioned gradual developmental progression to the trophozoite stage may prime the parasite for immediate reactivation, in anticipation that conditions may improve. Our data support this notion in that parasite recovery from starvation post-supplementation yields near control growth levels when given the same outgrowth time frame. Of note, extended starvation does impact parasite viability, revealed by the decrease in recovered parasitemia in cultures supplemented after 4 or more days of starvation. Nonetheless, a subpopulation of viable persister parasites remains in these cultures, and can recover appreciable growth when outgrowth time is extended, suggesting that starvation-induced hibernation can indeed establish a long-lived dormant state.

Hibernating parasites exhibited decreases in the rate of transcription and translation, a feature common to the starvation responses of most organisms [15, 16]. However, *Plasmodium* is unique in that starvation did not appear to induce a specific adaptive transcriptional program, a general hallmark of starvation response [17].

Presumably, this is due to the paucity of regulatory transcription factors encoded in the parasite's genome [18, 19]. Therefore, it is thought that epigenetic and post-translational modifications, which can mediate rapid biochemical and physiological changes, play the predominate role in regulating the growth and development of the parasite [20, 21].

Growth control in Plasmodium

In an effort to uncover the biological mechanism responsible for mediating the remarkable starvation phenotype of *P. falciparum*, we took a reverse approach and simply investigated a short list of "usual suspects" known to function in the eukaryotic amino acid starvation response. Previous kinome profiling revealed that the parasite genome encodes nearly 100 kinases, 65 of which can be classified as belonging to known eukaryotic kinase families [22, 23]. However, the metabolic sensor TOR, which is the most well-characterized effector of the eukaryotic starvation response, is notably absent from the parasite's kinase repertoire [4]. The absence of TOR in a eukaryotic organism is quite unusual, given its widespread conservation [24]; however this occurrence has been described in other obligate intracellular eukaryotic pathogens such as in members of the phylum microspora [25]. Such losses have been regarded as evolutionary adaptations to host parasitism, considering that the host environment generally provides a stable supply of nutrients to sustain the parasite, thus minimizing the need for nutrient-associated stress responses [26]. Interestingly, however, the *Plasmodium* genome retains a putative kinase ortholog that is specifically involved in the eukaryotic starvation response, namely the eIF2α kinase GCN2 [27]. This kinase phosphorylates the translation effector eIF2α upon sensing the depletion of amino acids, which gives rise to a global decrease in protein synthesis, thus coupling nutrient availability with translational control and cellular

growth [3]. In chapter 2 of this work, we discovered that *P. falciparum* does indeed phosphorylate parasite eIF2α (PfeIF2α) when exposed to amino acid-free medium. Using the single cross-over recombination strategy, parasite lines containing a disruption in the genetic locus of the putative GCN2 ortholog, termed PfeIK1, were generated, and found to be defective in eIF2α phosphorylation during amino acid withdrawal, thus confirming the identity of the amino acid-starvation responsive eIF2α kinase. In chapter 3, we found that the phosphorylation status of PfeIF2α is specifically linked with isoleucine availability, in further support of the data that defined isoleucine as the sole exogenous amino acid required for *in vitro* parasite growth [1].

On the surface, these collective observations suggest that PfeIK1 mediates a conventional amino acid starvation response in *P. falciparum* by phosphorylating eIF2α upon sensing isoleucine-depleted conditions, which consequently lead to the growth inhibited hibernating state. However, upon further investigation, we discovered that the genetic disruption of PfeIK1 did not impact the ability of the parasite to slow its growth during prolonged isoleucine starvation, nor did it prevent the resumption of parasite growth upon isoleucine repletion. Furthermore, PfeIF2α remained unmodified in starved mutants, thus excluding any compensatory activity from a redundant kinase. To further assess whether PfeIF2α signaling plays a role in parasite growth regulation, we introduced an episomally-expressed non-phosphorylatable copy of PfeIF2α into *P. falciparum*, PfeIF2α-S59A. This mutation typically elicits a dominant-negative effect in other organisms, particularly under stress conditions [28, 29]. However, in *P. falciparum* these mutants were phenotypically similar to control parasites in terms of growth, starvation-associated hibernation, and recovery of growth post-starvation. These findings

indicate that the starvation-induced hibernatory state of *P. falciparum* is not governed by canonical GCN2/ eIF2α-associated signaling, which is counterintuitive, especially considering that PfeIF2α appears to be rapidly modified in response to isoleucine modulation. The established dogma of eukaryotic stress response asserts that amino acid starvation leads to eIF2α phosphorylation, which downregulates global translation and ultimately inhibits cellular growth. However, this model is challenged in *P. falciparum*, as growth inhibition and reduced protein synthesis occurred during starvation regardless of eIF2α-mediated regulation.

In contrast to *P. falciparum*, a recent study demonstrated that disruption of the GCN2 ortholog in *T. gondii*, TgIF2K-D, does indeed elicit an extracellular tachyzoite fitness defect, resulting in decreased host cell reinvasion after extended incubation in medium alone [30]. Furthermore, in another study, it was shown that *T. gondii* parasites expressing a phosphorylation-insensitive eIF2α, TgIF2α-S71A, introduced via allelic replacement, were similarly impaired [31]. Determining whether such a defect exists in our mutant strains would be problematic due to the already short half-life of newly egressed *P. falciparum* merozoites, which only remain viable outside of a host cell for roughly 10 minutes [32]. Interestingly, however, both of the *T. gondii* mutant parasite lines also exhibited a decrease in protein synthesis that was independent of TgIF2α, which remained unmodified in these mutants when deprived of their host cell environments [30]. Unlike *Plasmodium*, however, TOR is conserved in *T. gondii* [33], and could potentially compensate to some degree for the loss of GCN2/eIF2α-mediated translational control during starvation [34].

The GCN2/eIF2α starvation response modulates cellular growth not only by downregulating global protein synthesis, but also by activating an adaptive transcriptional program mediated by GCN4 (ATF4 in mammals), a basic leucine zipper transcription factor [17]. GCN4 is specifically induced during starvation and, once expressed, goes on to promote the expression of proteins that function to maintain cell viability and restore homeostatic conditions, such as those involved in amino acid transport and biosynthesis, energy metabolism, and autophagy [35]. Although homology searches indicate that GCN4 is not conserved in *Toxoplasma* [36] or *Plasmodium*, ApiAP2 proteins, which comprise the only transcription factor family identified in apicomplexan parasites [37], have been implicated in the developmental regulation of these organisms. However, the transcriptional program of *Plasmodium* is perceived as being inflexible, and thus non-responsive to changing environmental conditions. For instance, previous studies have reported that treatment with certain antimalarial drugs, which dramatically impact vital pathways in the parasite, elicited few remarkable changes in the parasite's gene expression profile [38, 39]. In line with these data, the isoleucine starvation conditions in this study also failed to induce an alternative transcriptional program in *P. falciparum*. Furthermore, none of the genes coding for ApiAP2 proteins were specifically induced during starvation. Of note, the gene expression profile of starved parasites coincided stage-wise with the delayed development of the parasite, indicating a marked decrease in metabolic rate, which corresponded with the slowing of other biological processes. Despite the lack of transcriptional alterations indicative of a conventional starvation response, *Plasmodium* manages to coordinate an extraordinary metabolic shift that

suppresses its growth and preserves its cellular integrity, which presumably allows the parasite to maintain viability when amino acids, namely isoleucine, become limiting.

Unfortunately, this study could not attribute this phenomenon to a defined pathway, therefore the possibility remains that there exists an as yet uncharacterized mechanism of nutrient-sensitive growth control in protozoan parasites. Or perhaps even more simply, it is possible that processes such as transcription and translation are merely governed by enzymatic rate-limits and substrate availability, thus reducing these processes to primordial defaults that presumably do not require the complex regulation found in divergent free-living organisms.

Conservation of the GCN2 ortholog PfeIK1

Despite the apparent dispensable function of PfeIK1 to the induction of the parasite's hibernatory state during starvation, the importance of other eIF2α kinases to the life cycle of *Plasmodium* has been well-documented [40-42]. However, this raises the question of why the parasite expends energy and resources to express PfeIK1 if its conserved functional purpose is unnecessary. In this work, we showed that the isoleucine environment specifically and rapidly modulated the phosphorylation status of PfeIF2α, which indicated that PfeIK1 and an unidentified phosphatase function efficiently in a putative signal/response-type relay. However, we also provided evidence suggesting that this response is not coupled with translational control, growth regulation, or viability maintenance as in other organisms. Therefore, what is the role of PfeIK1, if not to mediate the starvation-stress response of the parasite?

The domain structure and function of GCN2 has been well-characterized and may provide an indication into the presumed divergent purpose of *Plasmodium* orthologs. It

has been shown that GCN2 is activated by the binding of uncharged tRNA, which accumulates when amino acids are depleted [43]. The tRNA-binding domain of GCN2 is C-terminal to its active site and has sequence homology with the histidine-tRNA charging enzyme, histidyl-tRNA synthetase (HisRS) [44]. This putative region in PfeIK1 is weakly conserved, but retains limited similarity to an aminoacyl-tRNA synthetase-like domain, suggesting that tRNA binding may also serve as the activating signal for PfeIK1 that leads to PfeIF2α phosphorylation. However, the overall sequence homology of *Plasmodium* GCN2 orthologs is only minimally conserved with those from yeast and human, lacking identifiable domains homologous to the N-terminal region, which contains the RWD (ring-finger and WD repeat) domain that binds to GCN1, a positive regulator of GCN2 activity [45, 46]. Interestingly, bioinformatic analysis of GCN2's N-terminal region revealed that the genome of *P. falciparum* encodes another GCN2-like kinase containing sequence similarity with this domain (PF14_0264) [30]. Notably, *T. gondii* also reportedly maintains two GCN2-like kinases, TgIF2K-C and –D [31]. The eIF2α kinase activity of TgIF2K-D has been validated [30], however TgIF2K-C has not been characterized. The N-terminus of TgIF2K-D contains the putative regulatory RWD domain and shares similarity with the second GCN2-like kinase from *P. falciparum*, however, TgIF2K-D is more closely related to PfeIK1 [30], suggesting that the second GCN2-like kinase in *Plasmodium* may serve an auxiliary function.

In terms of starvation-associated eIF2α phosphorylation, functional redundancy between PfeIK1 and the second GCN2-like kinase is unlikely, since PfeIF2α remained unphosphorylated in starved PfeIK1 mutant parasites. However, it is possible that these kinases could share redundancy in the phosphorylation of other targets, which in the

absence of PfeIK1, could still potentially be regulated by this putative proxy kinase. Presumably, the function of PfeIK1 involves some aspect of nutrient detection, given its rapid response to isoleucine depletion. The identification of additional PfeIK1 targets would further add to the novelty of *Plasmodium's* starvation response, given that GCN2 is not known to target other protein substrates for phosphorylation besides eIF2α in other model organisms. However, considering the sequence divergence of PfeIK1, and its apparent functional inconsistencies, it is conceivable that the amino acid starvation response of *Plasmodium* involves components unique to the parasite.

Future Directions

Identification of starvation response effectors

In this study, the "usual suspects" of eukaryotic starvation response were investigated; however, no viable candidates were apparent. The process of elimination was accelerated by the lack of conserved stress response effectors in the *P. falciparum* genome, which encodes a vast number of hypothetical proteins with no known function [19]. Therefore, the mechanism controlling the parasite's starvation response may be divergent from the canonical pathways found in most eukaryotes, and thus remains elusive.

In model organisms such as yeast or bacteria, the identification of gene products involved in stress response pathways has been accomplished through high-throughput mutagenesis screens [47, 48]. Although the sequencing of *P. falciparum's* genome [19] has provided a valuable source to gain insight into the complex biology of the parasite, the investigative tools available to study its functions are not as advanced as those for

other microbes. However, a transposase-mediated mutagenesis system known as *piggyBac*, that has been adapted for *P. falciparum* [49], provides a powerful genetic tool to generate large numbers of random mutants that could be tested for defects involved in the establishment and maintenance of the hibernatory state. In this approach, a plasmid containing the transposase is co-transfected with a selection plasmid containing the *piggyBac* transposable element, which randomly inserts into TTAA DNA sites [50], which are most prevalent in *P. falciparum* [19]. The drug-selected mutant pool could then be cloned by limiting dilution to obtain pure single-mutant populations. This strategy could potentially generate an extensive library of parasite mutants. Therefore, the major caveat of this method would be encountered during the labor-intensive screening process, as growth assessments under both normal and recovery conditions will have to be made. To be considered a true hit, the mutant must exhibit wild-type growth in rich-medium conditions, but fail to recover growth after extended isoleucine starvation. It is possible that a targeted gene may have a primary homeostatic function in the parasite as well as contribute to stress response, and thus exhibit defects in both conditions. Conversely, it is possible that a targeted gene may have a redundant function that provides a masked contribution in normal growth conditions, but cooperatively contributes to parasite stress response with its analogous counterparts (*e.g.* proteases), thus yielding a false positive, which would be filtered upon gene identification. Though these targets would also provide valuable information, the mentioned criteria are expected to reduce the pool of candidates to those with specific roles in the starvation response. Disrupted genes in mutants that meet these criteria can then be identified by PCR amplification of the *P. falciparum* genomic regions flanking the transposable

element [49]. Subsequently, gene complementation can serve to validate true components of the parasite's starvation-stress response upon restoration of the wild-type phenotype. Identification of these effectors could potentially shed light on the parasite's mechanism of persistence.

Biosynthesis of proteins during starvation

In this work, we show that continuous proteolysis is required to maintain parasite viability during extended starvation. Hemoglobin degradation is evident by the appearance of hemozoin in the parasite's food vacuole (FV), however, acquisition of isoleucine must come from an alternative source since this amino acid is not present in human hemoglobin [10]. Preliminary metabolomic analysis of starved *P. falciparum* indicates that during extended starvation, basal isoleucine levels increase, suggesting that the parasite degrades cytosolic proteins that contain isoleucine (data not shown), perhaps to scavenge this amino acid for the parasite's restricted biosynthetic needs during starvation. Although protein synthesis is globally reduced during starvation, the preferential translation of proteins involved in stress-related adaptation has been shown to occur in other eukaryotes [11, 17]. Considering that starvation does not elicit a characteristic transcriptional shift in *P. falciparum*, it is possible that the parasite utilizes post-transcriptional and post-translational mechanisms of regulation that mediate the parasite's adaptive response. The mRNA composition of starved parasites over time has been examined in this study; however, it is of interest to determine whether certain transcripts are translated in excess relative to those in the fed control at the corresponding life cycle stage. Such overrepresentations may indicate that the resultant gene product

plays a role in the stress response of the parasite. To this end, polysome analysis could be used to identify mRNAs that are loaded with multiple ribosomes, indicating an increased level of translation. Although polysome isolation in *P. falciparum* has been challenging, recent improvements in the solubilization protocol have made the technique an effective tool in the study of parasite translational regulation[51], which is presumed to take precedence over conventional transcriptional control in the starvation response of *P. falciparum*.

Targeted disruption of the GCN2-like kinase

PfeIK1 was identified as the amino acid-starvation responsive GCN2 ortholog of *P. falciparum* in chapter 2 of this work; however its genetic disruption did not effect the establishment or maintenance of the starvation-induced hibernatory state. Ablation of GCN2 activity in other organisms generally results in reduced fitness when exposed to amino acid-starvation conditions [52-54], therefore the lack of a phenotype in starved PfeIK1 mutants presented a conundrum. Subsequently, another GCN2-like kinase, PF14_0264, was identified in *P. falciparum*, containing putative regulatory domains not found in PfeIK1. This kinase does not appear to have redundant eIF2α kinase activity; however we propose that this second kinase may have an alternative function related to the regulation of other targets, perhaps in tandem with PfeIK1. Targeted disruption of PF14_0264 by single crossover recombination in a PfeIK1 mutant background may expose a defect in the starvation-stress response of the parasite, provided that the presumed redundancy of these kinases for targets outside of PfeIF2α is limited to these reputed orthologs. Alternatively, it is also of interest to determine whether PF14_0264

alone makes a substantial contribution to the parasite's stress response. Therefore, this gene could be targeted for disruption in a wild-type background, and resultant mutants could be specifically assessed for defects in starvation-associated hibernation.

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APPENDIX:

SUPPLEMENTARY CHARACTERIZATION

OF THE *P. FALCIPARUM* AMINO ACID

STARVATION-STRESS RESPONSE

Preface

In this section, data regarding diverse aspects of the amino acid starvation response of *Plasmodium falciparum* are presented. These data represent preliminary experiments that require further investigation to merit validation, failed to yield a measurable effect, or were discontinued due to technical reasons.

In Part I, the *P. falciparum* ortholog of the phospho-adapter 14-3-3 was recombinantly expressed and its interactions with parasite proteins were examined, with the intent to determine whether such interactions could be modulated during starvation.

In Part II, microarray analysis was performed on RNA isolated from *P. falciparum* parasites starved of isoleucine for a brief 2 hour period to determine whether parasites exhibit a starvation-specific transcriptional shift. The gene PFI1710w was significantly upregulated in starved parasites and its contribution to the parasite starvation response was further examined.

In Part III, the putative tRNA-binding domain of PfeIK1 was heterologously expressed and functional conservation was evaluated.

Finally, In Part IV, the ultrastructural organization of isoleucine-starved parasites was investigated by transmission electron microscopy (TEM), in which several morphological abnormalities were uncovered and characterized as potential indicators of an autophagic-like response to starvation.

The majority of the data included in this section is for informational purposes only, and therefore, not intended for publication.

Part I:

Construction, expression, and binding analysis of Plasmodium falciparum 14-3-3: a eukaryotic phospho-adapter protein

Abstract

14-3-3 proteins are highly conserved, dimeric phospho-adaptors that are known to bind specific phosphorylated serine and threonine residues of various target molecules involved in a diverse range of critical signaling pathways, including growth control. 14-3-3 binding reportedly leads to modulations in the activity, stability, or localization of target proteins. Homologs of 14-3-3 have been identified in most eukaryotic species, including *P. falciparum*. Interestingly, *Plasmodium* 14-3-3 reaches peak expression levels during the trophozoite stage of development, the stage in which nutrient acquisition mechanisms are most active. *Plasmodium* 14-3-3 has not been fully characterized, therefore little is known about the role(s) this versatile protein may play in the parasite's life cycle. In this study, a recombinant version of *P. falciparum* 14-3-3 (Pf14-3-3) was generated and used to examine its interactions with parasite proteins. Considering the regulatory function that 14-3-3 serves in other eukaryotes, coupled with its distinct expression pattern in *Plasmodium*, it is conceivable that Pf14-3-3 may take part in modulating the metabolic signaling pathways that are important for parasite growth.

Introduction

Protein phosphorylation plays a significant role in the signaling processes of the cell, ultimately allowing the intracellular environment to communicate with and respond to extracellular conditions, which can lead to both transcriptional and translational modulations [1, 2]. In the process of signal transduction, multiple factors are often involved, and these factors generally act in concert to regulate cellular processes. Phosphorylation-associated signaling often requires another layer of regulation in the form of regulatory adaptor proteins, which bind to phosphorylated residues contained within specific motifs, consequently, influencing the functional properties of the phospho-protein, such as activation status, localization, stability, and interaction capabilities [3]. 14-3-3 proteins, named according to their migration pattern on a DEAE-cellulose chromatography column and on starch-gel electrophoresis [4], represent a well-conserved family of small, α-helical proteins that function in this respect, preferentially binding to phosphorylated serine and threonine residues of signaling proteins [5]. The involvement of 14-3-3 phospho-adapter proteins in signal transduction pathways has been firmly established, playing roles in the cell cycle [6], apoptosis [7], growth control [8], and stress response [9], signifying the importance of this class of proteins to the cellular biology of higher eukaryotic systems.

The evolutionary conservation of 14-3-3 is even extended to protozoan parasites [10-13], including those in the *Plasmodium* genus [14]. In general, this protein family consists of multiple isoforms, the number of which varies among species [15-17], and are known to function as homo- or heterodimers [18-20]. Experimental evidence in yeast suggests that 14-3-3 proteins are essential, considering that the concomitant ablation of

its two isoforms resulted in lethality [15]. Interestingly, *Plasmodium* maintains only a single copy of 14-3-3 [14], which may be indicative of both its essentiality and its functional specificity in the parasite. In the primate malarial species, *P. knowlesi*, 14-3-3 has been shown to exhibit peak expression during the highly metabolically active trophozoite stage [14]. Therefore, we hypothesized that 14-3-3 may interact with signaling proteins that regulate parasite growth. We sought to address this hypothesis in the human malaria parasite, *P. falciparum*.

In this work, we confirmed that protein expression of the *P. falciparum* 14-3-3 ortholog (Pf14-3-3) coincided with the trophozoite developmental stage. Furthermore, Pf14-3-3 was recombinantly expressed and used to assess protein-protein interactions from a radio-labeled parasite lysate. Additionally, we used western blot analysis to specifically detect whether phosphorylated proteins from a parasite lysate interacted with recombinant Pf14-3-3. Ultimately, these data serve to establish a foundation on which to further the characterization of 14-3-3 proteins in *Plasmodium*, particularly in determining their role in the growth regulation of the parasite.

Methods

Recombinant expression and affinity purification of Pf14-3-3

The full length* coding sequence of MAL8P1.69 (Pf14-3-3) was RT-PCR amplified from isolated 3D7 parasite RNA using the SuperScript III One Step RT-PCR kit (Invitrogen) and primers 5'- AATTGGATCCATGGCAACATCTGAAGAATTAAA-3' (BamHI site underlined) and 5'- AATTCTCGAGTCATTTCTTACCTTCGGTCTGAT-3' (XhoI site underlined), digested with BamHI and XhoI, and ligated into the same sites of the pGEX6P-1 (GE Life Sciences) bacterial expression plasmid, containing an N-terminal Glutathione-S-Transferase (GST) tag. All cloning steps were confirmed by sequencing. The resulting Pf14-3-3-pGEX6P-1 DNA plasmid was transformed into BL21 Codon Plus *E. coli* (Stratagene). A single colony of Pf14-3-3-pGEX6P-1 transformed BL21 was inoculated in 5 mL LB containing 100 μg/mL ampicillin and incubated overnight at 37°C. The overnight culture was used to seed 500 mL of fresh LB containing 100 μg/mL Ampicillin, which was then grown to OD_{600} 0.6, and induced with 1 mM IPTG for 3 hours at 30°C. Cells were harvested, resuspended in cold lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5% Igepal, 10% glycerol) containing Complete protease inhibitor cocktail mix (Roche), sonicated in an ice water bath, and centrifuged at 12000 rpm for 30 minutes. 1 mL of the protein lysate was incubated with 50 μL of washed glutathione sepharose beads (Sigma) for 1 hour with tumbling at 4°C. Following the pull down, the GST-Pf14-3-3 beads were washed 3 times in the lysis buffer. 2x SDS-Laemmli buffer [21] was added to a portion of the beads, followed by SDS-PAGE and Coomassie Blue staining.

***A recently updated annotation of this gene reveals that the 3' end contains an intron, and the actual coding region now includes an additional 18 nucleotides. Thus the rPf14-3-3 generated for this study represents a C-terminally truncated protein.**

Parasite culturing, metabolic labeling, and GST-Pf14-3-3 pull down

Plasmodium falciparum strain 3D7 were cultured [22] in human O+ erythrocytes at 2% hematocrit in complete RPMI 1640 (Gibco) supplemented with 27 mM $NaHCO_3$, 22 mM glucose, 0.37 mM hypoxanthine, 10 μg/ml gentamicin, and 5 g/L Albumax (Invitrogen) and incubated at 37°C with 5% CO_2. Parasites were sorbitol-synchronized [23], grown to the trophozoite stage, washed twice in PBS, and re-plated in RPMI-labeling medium lacking methionine and cysteine (Sigma) in the presence of 0.18 mCi [^{35}S] Express Protein Labeling Mix (Perkin Elmer, 1175 Ci/mmol) for up to 6 hours. After labeling, infected RBCs (iRBCs) were lysed with 100 hemolytic units (HU)/ mL of tetanolysin (List Biologicals) in cold PBS. Parasite pellets were resuspended in cold lysis buffer (50 mM Tris-HCl pH7.5, 150 mM NaCl, 0.5% Igepal) containing Complete protease inhibitor cocktail mix (Roche) and underwent two freeze/ thaw cycles to facilitate lysis. Parasite lysates were collected following centrifugation at 16000 rpm for 10 minutes at 4°C. Parasite lysates were mixed with lysis buffer at a 1:4 ratio, added to 20 μL GST-Pf14-3-3 beads, and incubated at 4°C overnight with tumbling. The GST-Pf14-3-3 beads were washed twice with lysis buffer and resuspended in 2x SDS-laemmli. Proteins were resolved by SDS-PAGE, followed by Coomassie Blue staining, and autoradiography.

Pf14-3-3 and phospho-protein immunodetection

Parasites were synchronized to the late ring stage, incubated in RPMI 1640 medium at 37°C with 5% CO_2 and harvested at various time points up to 8 hours. Samples were lysed with 100 HU/ mL tetanolysin (List Biologicals) in PBS and centrifuged at 16000 rpm for 10 minutes at 4°C. Parasite pellets were washed 2 times in PBS containing Complete protease inhibitor cocktail mix and resuspended in 2x SDS-Laemmli buffer, followed by SDS-PAGE. Resolved proteins were transferred to nitrocellulose followed by immunoblotting [24] with polyclonal anti-14-3-3 antibodies purchased from Abcam.

Alternatively, parasite pellets were resuspended in cold lysis buffer (50 mM Tris-HCl pH7.5, 150 mM NaCl, 0.5% Igepal) containing Complete protease inhibitor cocktail mix (Roche) and underwent two freeze/ thaw cycles to facilitate lysis. Parasite lysates were collected following centrifugation at 16000 rpm for 10 minutes at 4°C and mixed with lysis buffer at a 1:4 ratio, added to 20 µL GST-Pf14-3-3 beads, and incubated at 4°C overnight with tumbling. The GST-Pf14-3-3 beads were washed twice with lysis buffer and resuspended in 2x SDS-laemmli. Proteins were resolved by SDS-PAGE and transferred to nitrocellulose, followed by immunoblotting [24] with monoclonal anti-phospho-serine/threonine/tyrosine antibodies purchased from Abcam.

Results and Discussion

The expression profile of 14-3-3 in *P. knowlesi*, a divergent primate malaria species [25], has been previously described [14]. Therefore, we initiated the characterization of the *P. falciparum* 3D7 strain14-3-3 homolog (Pf14-3-3). BLAST analysis of the human epsilon isoform of 14-3-3 against the *Plasmodium* gene database

identified MAL8P1.69 as the *P. falciparum* 14-3-3 ortholog, consisting of 771 base pairs in its genomic sequence, with 2 introns located at the 5' end. *Plasmodium* 14-3-3 maintains at least 55% sequence identity with other eukaryotic orthologs [14], thus commercially available antibodies raised against human isoforms of 14-3-3 (anti-14-3-3) were used to detect the *P. falciparum* protein. 3D7 parasites were cultured under normal conditions, sampled at various times during development, and the expression of parasite 14-3-3 was examined by western blot analysis. The polyclonal 14-3-3 antibody recognized a doublet pattern with bands migrating slightly above and below the 30kDa marker. The expected size of Pf14-3-3 is approximately 28 kDa, corresponding with the lower molecular weight band. The higher species may be representative of phosphorylated Pf14-3-3, a regulatory modification that has been described in mammalian cells [26]. Pf14-3-3 was primarily expressed during the mid to late trophozoite stage, while virtually no expression was apparent in earlier stages (**Figure 1A and B**), indicating that Pf14-3-3 exhibits stage-dependent expression, validating the previous study in *P. knowlesi* [14].

14-3-3 is known to bind to specific phospho-serine/ threonine containing proteins [27]; therefore we examined Pf14-3-3 interactions with parasite proteins. A recombinant version of Pf14-3-3 was constructed in order to conduct *in vitro* interaction studies. The full length sequence of MAL8P1.69 *(see methods) was cloned into the pGEX6P-1 GST-fusion bacterial expression vector. After induction of protein synthesis, an N-terminal Glutathione-S-Transferase (GST)-tagged version of Pf14-3-3 could be isolated from bacterial lysates through affinity purification with glutathione beads, yielding a tagged protein of expected size at approximately 53 kDa (**Figure 2A**). Synchronous 3D7

parasites were metabolically radio-labeled with [^{35}S] methionine and cysteine for different time intervals in order to examine changes in the rPf14-3-3 interaction profile over the course of development as the parasite synthesized new proteins. Immobilized GST-Pf14-3-3 was added to the labeled parasite extracts in a pull-down assay, and bound proteins were visualized by autoradiography. Although no differential pattern was observed, several proteins appeared to bind to rPf14-3-3 (**Figure 2B**, asterisk-labeled bands), while background binding to the equally loaded GST control remained relatively low. Furthermore, by western blot analysis using antibodies against phosphorylated serine, threonine, and tyrosine residues, we determined that proteins interacting with rPf14-3-3 were indeed phosphorylated **(Figure 3)**.

This pilot study provides the first indication that Pf14-3-3 may have specific binding partners that undergo phosphorylation. Assessment of the in vivo Pf14-3-3 interactions via immunoprecipitation (IP) from parasite extracts provides a logical next step, which could lead to the identification of these putative signaling proteins. Furthermore, considering the role that 14-3-3 plays in the growth control mechanisms of other organisms, it is of interest to determine whether conditions that perturb parasite growth, such as isoleucine limitation, results in differential Pf14-3-3 binding, which may be indicative of the stress-responsive phosphorylation changes that occur in the parasite, thus resulting in the creation or loss of 14-3-3 binding sites.

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Figure Legends

Figure 1: Expression of 14-3-3 in *P. falciparum*

Synchronized 3D7 parasites were grown at 37°C in complete RPMI and harvested at the indicated time points. A) Western blot of parasite lysates showing the temporal expression of Pf14-3-3. B) Representative images of Geimsa stained thin blood smears of the indicated time points showing developmental progression of the parasites.

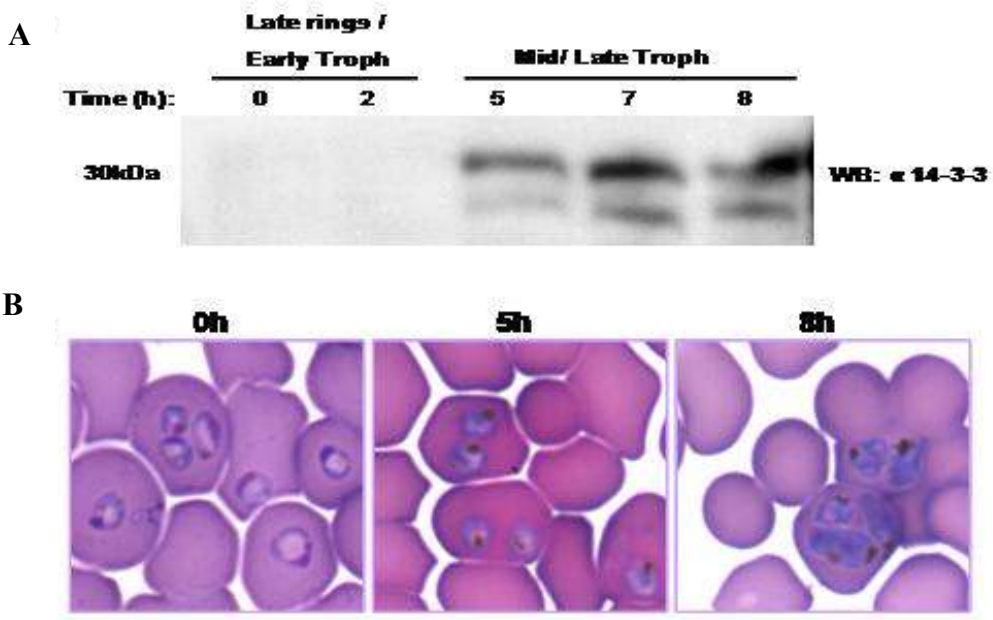
Figure 2: Recombinant Pf14-3-3 expression and interaction with *Plasmodium* proteins

A) Coomassie stained gel of GST-Pf14-3-3 lysate and enriched pull down (PD) onto glutathione beads. B) Autoradiograph of -labeled *P. falciparum* proteins that interact with immobilized GST-Pf14-3-3. The 3D7 input represents a shorter exposure. The asterisks (*) indicate signals that are above the background in the GST PD control. CB, Coomassie Blue

Figure 3: Recombinant Pf14-3-3 interaction with phosphorylated *P. falciparum* proteins

GST-Pf14-3-3 beads or control GST beads were added to a 3D7 parasite lysate in a pull-down (PD) assay. Shown is a western blot of interacting proteins detected by anti-phospho-Ser/Thr/Tyr (α^{P}S/T/Y), an antibody that recognizes phosphorylated proteins. Asterisks (*) indicate signals that are above background binding.

Figure 1


A
Late rings /
Early Troph
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Time (h):
0
2
5
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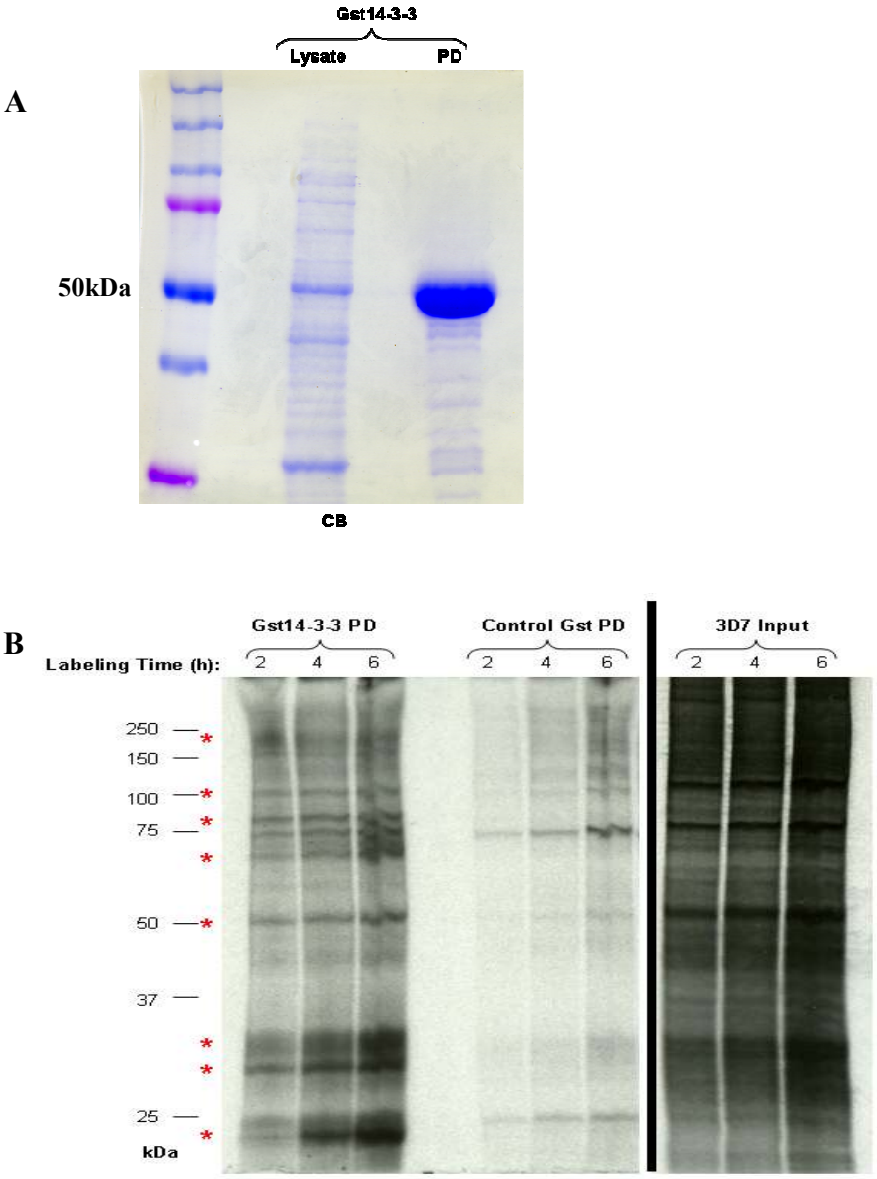
8
30kDa
WB: α 14-3-3
B
0h
5h
8h

Figure 2


A
Gst14-3-3
Lysate
PD
50kDa
CB
B
Gst14-3-3 PD
Control Gst PD
3D7 Input
Labeling Time (h):
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250
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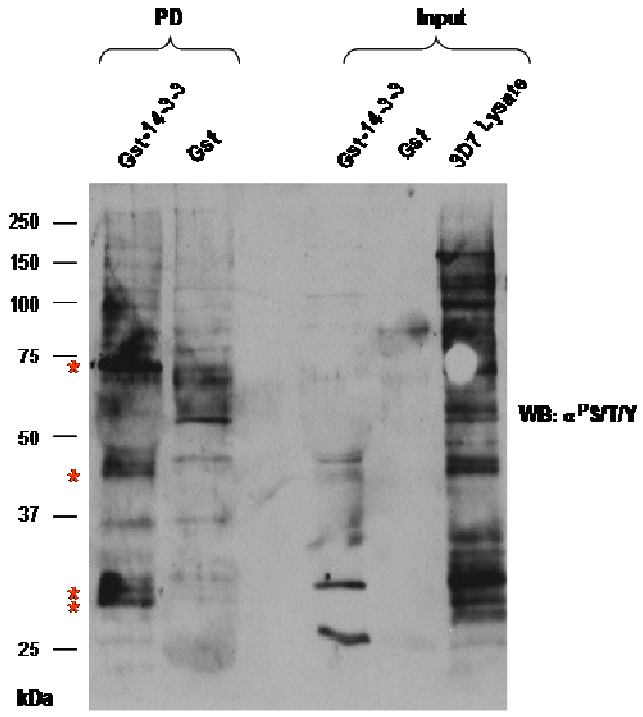
75
50
37
25
kDa

Figure 3


PD
Input
Gst-14-3-3
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WB: α-PS/T/Y

Part II:

Microarray analysis of short term isoleucine-starved P. falciparum

Abstract

Amino acid starvation is known to elicit a dramatic shift in the transcriptional program of most eukaryotes, resulting in the expression of genes involved in the adaptive response that act to maintain cellular viability during nutrient stress. Transcriptional modulation of *in vitro* cultured malaria parasites is rarely observed, even when parasites are subjected to conditions that perturb growth. However, field isolates of *Plasmodium falciparum* have been shown to display differential transcriptional profiles, including one that resembles a characteristic starvation response. In this study, gene expression analysis was performed on RNA isolated from *in vitro* cultured parasites briefly starved for isoleucine, the only exogenous amino acid required to sustain parasite growth. We report that although no substantial transcriptional shift was observed, the gene PFI1710w, which encodes a protein implicated in the induction of gametocytogenesis, experienced nearly a 14-fold increase in expression in the isoleucine-starved parasites. However, the genomic disruption of this gene did not affect parasite recovery post extended isoleucine starvation, thus we conclude that this gene does not play a major role in the starvation-stress response of *P. falciparum*.

Introduction

Gene expression in the human malaria parasite, *Plasmodium falciparum*, is tightly regulated and remarkably coordinated with its developmental cycle [1, 2]. Unlike most organisms, however, the gene expression profile of *P. falciparum* does not appear to respond to environmental stimuli, such as treatment with certain antimalarial drugs [3, 4], suggesting that gene transcription in the parasite lacks the regulatory elements that allow for adaptive modulation. However, a recent study reported that *P. falciparum*, in the context of a natural infection, exhibits distinct transcriptional profiles representing three different physiological states [5], thus challenging the widely held notion regarding the parasite's transcriptional inflexibility. These three states were characteristic of an active growth profile, a starvation response, or a general environmental stress response. The active growth state was most similar to that reported for *in vitro* cultured parasites [1], however the latter two had not been observed before, suggesting that the induction of such states may be relevant to the variable microenvironments encountered by the parasite in the human host.

In most eukaryotes, starvation for amino acids results in a rapid metabolic shift mediated by responsive alterations in the transcriptional program [6]. It has been shown that *P. falciparum* requires an extracellular supply of isoleucine to support its continuous growth, and that starvation for isoleucine results in developmental stalling [7]. Therefore, in this study, the transcriptional profile of isoleucine-starved *P. falciparum* parasites was examined, with the intent to determine whether the previously reported *in vivo* starvation profile could be reproduced in culture. We report that parasites briefly starved for isoleucine upregulate the expression of a small subset of genes, with one

particular outlier experiencing nearly a 14-fold increase in expression: a 1.8kb gene known as PFI1710w recently implicated in the stress-associated process of gametocytogenesis [8]. Although this finding suggested that isoleucine starvation may promote gametocyte conversion, subsequent studies with parasites containing a genomic disruption in this gene phenocopied wild-type behavior in isoleucine starvation and recovery conditions, therefore we conclude that this gene most likely does not contribute to the parasite's starvation stress-response.

Methods

Parasite culturing

Plasmodium falciparum strain 3D7 and derived strains were cultured [9] in human O+ erythrocytes in complete RPMI 1640, containing all 20 amino acids, supplemented with 27 mM $NaHCO_3$, 22 mM glucose, 0.37 mM hypoxanthine, 10 μg/ml gentamicin, and 5 g/L Albumax (Invitrogen). Homemade complete and isoleucine-free RPMI were prepared according to the RPMI 1640 recipe provided by Invitrogen, and supplemented with RPMI 1640 Vitamins (Sigma), the appropriate respective amino acids (Sigma) at the concentrations found in RPMI 1640, and the additional supplements mentioned above.

Parasite strains

A lab strain of *P. falciparum* 3D7 parasites was used to generate samples for microarray analysis. Additionally, a parental line of 3D7, a gametocyte-deficient line containing an 18.9kb deletion on chromosome 9 (10-2), and a 10-2 strain complemented with an episomal expression plasmid containing PFI1710w (#17) were obtained from the lab of Kim Williamson (KW) (NIH) for use in the growth recovery assay. The methods describing the generation of the deletion and complemented lines are currently unpublished and thus will be reported elsewhere.

Microarray sample preparation and analysis

A large-scale sorbitol synchronized [10] *P. falciparum* 3D7 culture at 8 – 10% parasitemia was washed twice in PBS, equally partitioned and washed in either complete or isoleucine-free RPMI, after which, the parasites were re-plated in their respective

medium and incubated at 37°C with 5% CO_2. Samples were harvested initially and after 2 hours of incubation. Infected RBCs were washed with PBS and lysed with 0.05% saponin (Sigma) in PBS. Parasite pellets were washed with PBS and resuspended in Trizol ® Reagent (Invitrogen). Following chloroform extraction, samples underwent centrifugation at 16000 rpm for 30 minutes at 4°C. Isopropanol was added to the aqueous phase to precipitate the RNA. Following centrifugation, the isolated RNA pellet was washed with 70% ethanol, dried, and dissolved in diethylpyrocarbonate (DEPC)-treated water. Samples were hybridized to Affymetrix chips and steady-state transcript levels of the fed control and the isoleucine-starved parasite samples were measured and subsequently compared for 4150 genes, generating a Pearson coefficient. To determine the life cycle stage to which the samples best correlated, the absolute expression values from the control and the isoleucine-starved samples were divided by the median-averaged asexual life-cycle expression values generated for the reference pool in the Llinàs *et al.* study. The resulting ratios were then $\log_2$-transformed and Spearman coefficients were calculated by comparing these values against the $\log_2$ ratios reported in reference 2, which detailed the expression pattern of *P. falciparum* over 53 hours of its intraerythrocytic developmental cycle (IDC). To determine the *in vivo* cluster to which the samples best correlated, Spearman coefficients were calculated by comparing the absolute expression values for the control and the isoleucine-starved samples to the values reported for the 43 individual clinical isolates in the Daily *et al.* study.

Growth recovery assay

P. falciparum 3D7 parental parasites (KW), a strain containing an 18.9kb deletion on chromosome 9 (10-2) and a 10-2 strain complemented with PFI1710w (#17) were sorbitol synchronized [10] to the late ring stage, cultured in complete RPMI at 2% hematocrit, and sub-cultured to approximately 0.5 % parasitemia. The complemented strain (#17) was cultured in the presence of 5 μM blasticidin (Sigma) to maintain positive selection on parasites carrying the plasmid containing the PFI1710w gene. The cultures were washed twice in PBS, partitioned and washed in either complete RPMI or isoleucine-free RPMI, after which, the parasites were re-plated in triplicate in their respective medium and incubated at 37°C with 5% CO_2. Control fed and isoleucine-starved parasites were grown for 96 hours and prepared for flow cytometry to assess parasitemia. Remaining isoleucine starved cultures were supplemented with isoleucine (382 μM), after starving for various periods of time, and allowed to recover for an additional 96 hours. Parasites were prepared for flow cytometry following recovery.

Flow cytometry

Samples were stained with 0.5 μg/mL acridine orange (Molecular Probes) in PBS and $3x10^4$ cells were counted on a BD Biosciences FACS Canto flow cytometer. Total cell number was measured on the forward and side scattering channels (FSC and SSC). Fluorescence was detected on both the FITC-H and the PerCP-Cy5-H channels and parasitemia gates were defined by intensity of fluorescence, with highly fluorescent infected RBCs distinctly separated from low fluorescence uninfected RBCs. Data were analyzed using Flowjo software (Treestar Inc.).

Results and Discussion

In the Daily *et al.* study, clinical isolates that displayed a transcriptional profile characteristic of a starvation response exhibited an upregulation in genes involved in oxidative phosphorylation, fatty-acid metabolism, and glycerol degradation, indicating that parasites in these samples experienced a metabolic shift, no longer relying on glycolytic metabolism to derive energy substrates, but instead obtaining such molecules from alternative sources. To determine whether isoleucine starvation activates an alternative metabolic program, we isolated RNA from synchronized *P. falciparum* 3D7 parasites that had been incubated in either isoleucine-free or complete RPMI for 2 hours. The RNA was then hybridized to Affymetrix gene chips and the expression level of 4,150 genes was measured. Upon comparing the expression values for the isoleucine-starved sample against those for the complete control, a Pearson correlation coefficient of 0.96 was calculated **(Dataset 1)**, indicating that no major transcriptional shift occurred between these samples. Furthermore, in comparison to the 43 *in vivo* samples reported in the Daily *et al.* study, both the control and the isoleucine-starved samples best correlated with the transcriptional profile representing normal glycolytic growth **(Figure 1)**, indicating that short-term isoleucine starvation does not interfere with standard carbohydrate metabolism, in contrast to the reported *in vivo* starvation profile. The parasites used to generate the data for the *in vivo* profiles were described as early rings, while the parasites from the present study correlated best with mid to late ring stage forms **(Figure 2)**, therefore it remains possible that transcriptional reorganization is dependent on life cycle staging and timing of stress induction.

Although major transcriptional changes were not apparent, about 25 genes from the isoleucine-starved parasites experienced more than a 4-fold increase in expression relative to the complete control **(Table 1)**. This subset of genes consisted of those involved in cytoadhesion, antigenic variation, and cytoskeletal organization. Also genes encoding hypothetical proteins with no known function were prevalent in this subset. However, the largest fold-change in expression was exhibited by the gene PFI1710w. This 1.8kb gene was induced nearly 14-fold, and is annotated in the *Plasmodium* genome database (PlasmoDB) as a cytoadherence-linked protein. Interestingly, however, recent reports have identified PFI1710w as an inducer of gametocytogenesis [11].

Such a substantial increase in this gene's expression suggested that isoleucine starvation may initiate gametocyte conversion, which presents a reasonable hypothesis since induction of gametocytogenesis is considered a general stress response of the parasite [12]. However, considering that sexual stage commitment occurs during schizogony of the previous cycle [12], we hypothesized that the induction of this gene during extended isoleucine starvation may also protect the viability of parasites already committed to the asexual program, perhaps in a stress response mechanism that promotes the hypometabolic features of gametocytes without the accompanying differentiation into the sexual forms. Therefore, to test this hypothesis, we obtained gametocyte-deficient parasites containing an 18.9 kb deletion in chromosome 9, which spanned the region including PFI1710w, as well as a PFI1710w-complemented strain, and starved them of isoleucine for up to 72 hours. Following starvation, isoleucine was supplemented to each culture, and parasites were allowed to recover for approximately 2 life cycles (96 hours). Interestingly, both the deletion strain, designated as 10-2, and the complemented strain,

designated as #17, behaved similarly to the wild-type (WT) parent in terms of asexual growth recovery **(Figure 3)**. In fact, control growth and growth recovery of the deletion strain, 10-2, were more comparable to that of WT, indicating that PFI1710w likely plays no major role in the starvation-stress response of *P. falciparum*. Although PFI1710w expression is associated with gametocytogenesis, we report that isoleucine starvation did not trigger a remarkable increase in gametocytemia for either the WT or complemented strain (data not shown). Furthermore, a subsequent gene expression study of isoleucine-starved parasites presented in chapter 3 of this thesis failed to show any induction of PFI1710w or any of the other genes found in the differentially expressed subset reported here. Therefore we conclude that the observed induction of PFI1710w during isoleucine starvation most likely represents an experimental artifact.

Acknowledgments

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Figure Legends

Figure 1: Transcriptional profile comparison between isoleucine-starved and control parasites with *in vivo* isolates

Heatmap illustration of Spearman correlation between the gene expression values of the physiologically distinct *in vivo* clusters reported in the Daily *et al.* study and the isoleucine-starved (ILE-) or control (CTRL) parasites. The transcriptional profiles for both the isoleucine-starved and control parasites best correlated with the 17 *in vivo* isolates (2.1S05.014-2.17S05.188) from cluster 2, representing profiles exhibiting a normal active growth pattern.

Figure 2: Transcriptional profile comparison between isoleucine-starved and control parasites across the complete intraerythrocytic developmental cycle (IDC) of *P. falciparum*

Heatmap illustration of Spearman correlation between gene expression values measured at each time point (TP) of the 53-hour IDC time series reported in the Llinàs *et al.* study and the isoleucine-starved (ILE-) or control (CTRL) parasites. The transcriptional profiles for both the isoleucine-starved and control parasites best correlated with TP13 to TP19, representing parasite development at 13 to 19 hours post-invasion, thus corresponding to mid to late ring-stage parasites.

Figure 3: Growth recovery of PFI1710w deletion and complemented 3D7 parasites post isoleucine starvation

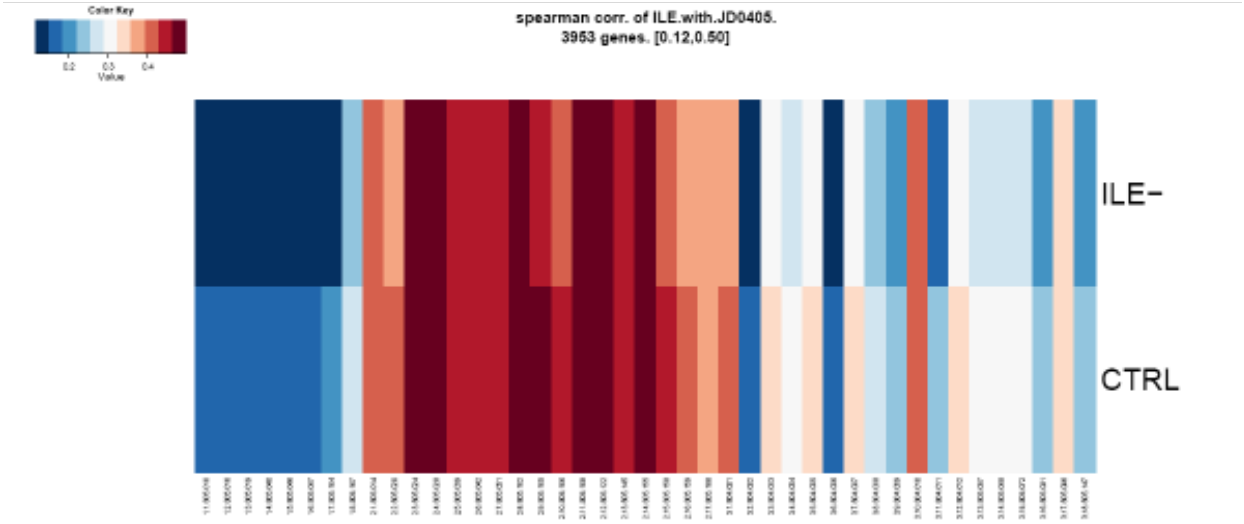
A PFI1710w deletion strain (10-2, light gray bars) and a PFI1710w complemented strain (#17, dark gray bars) along with wild-type parental parasites (WT 3D7 parent, black bars) were starved of isoleucine for the indicated times. Ile was added back and parasites were allowed to recover in CM for 96 hours. Control parasites were either fed (CM) or starved for isoleucine without re-feeding (no Ile) for 96 hours. Parasitemia of all cultures was measured by flow cytometry after 96 hours of incubation (96 h ctrl) or recovery. Data shown represent the mean parasitemia ± SEM, n=3.

Table 1. Genes upregulated in isoleucine-starved *P. falciparum*

Gene Name	PlasmoDB annotation	No Ile	Control	Fold Change Expression
PFI1710w	cytoadherence-linked protein	326.84	23.76	13.76
PFD1235w	PfEMP1 (chr4)	62.21	6.57	9.47
MAL13P1.353	hypothetical protein	106.81	12.60	8.47
PFD0110w	normocyte-binding protein 1	61.78	8.28	7.46
PF11_0180	hypothetical protein	131.40	19.41	6.77
PF11_0400	hypothetical protein	73.22	10.87	6.73
PFL0925w	Formin 2	116.37	17.54	6.63
PFB0795w	ATP synthase F1, alpha subunit	84.60	14.15	5.98
PF10_0041	U5 small nuclear ribonuclear protein	187.89	33.96	5.53
PF10_0014	pHISTa, exported protein	85.09	15.50	5.49
PFB0275w	metabolite/ drug transporter; MFS	143.79	26.51	5.42
PFL0840c	hypothetical protein	54.55	10.69	5.10
PFE1360c	methionine aminopeptidase	47.76	9.94	4.80
PFC0130c	RNA-binding protein	46.24	9.67	4.78
PFL2440w	DNA repair protein rhp16	59.82	13.20	4.53
PFF0770c	hypothetical protein with PP2C domain	73.54	16.70	4.40
PFA0020w	rifin	185.15	42.33	4.37
PF11_0507	antigen 322	44.02	10.14	4.34
PF11_0213	hypothetical protein	58.82	13.64	4.31
PF14_0393	structure-specific recognition protein	68.18	15.81	4.31
PF13_0161	hypothetical protein	91.96	21.57	4.26
PFE1160w	hypothetical protein	65.78	15.48	4.25
PFL1010c	hypothetical protein	72.86	17.59	4.14
PF14_0725	tubulin	37.35	9.03	4.14
PFL2525c	exported protein	49.65	12.15	4.09

Values reported for the isoleucine starved (No Ile) and control parasites represent mRNA expression levels in arbitrary units (AU)

Figure 1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0.4
Value
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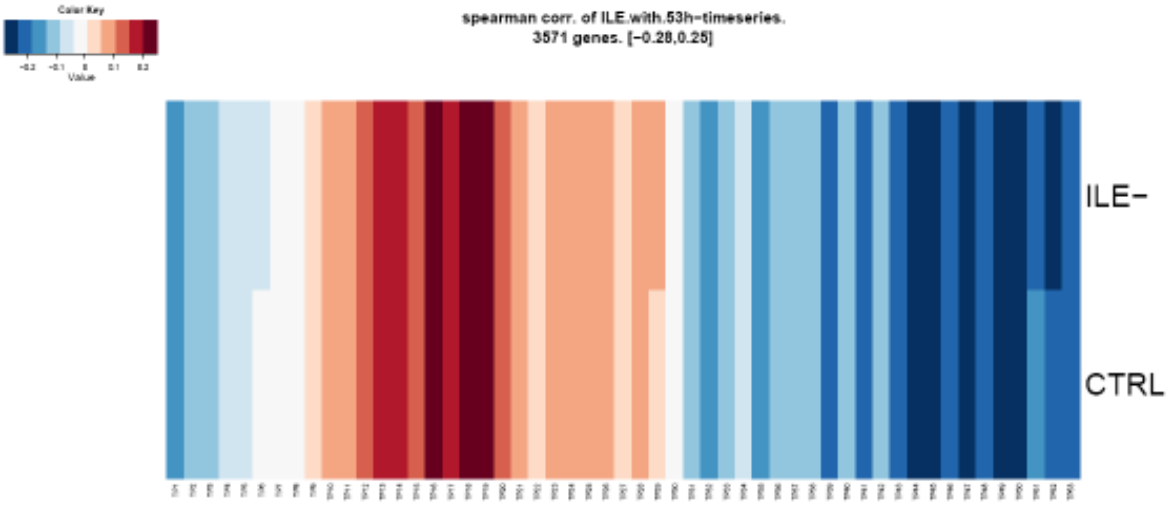
3953 genes. [0.12,0.50]
ILE−
CTRL

Figure 2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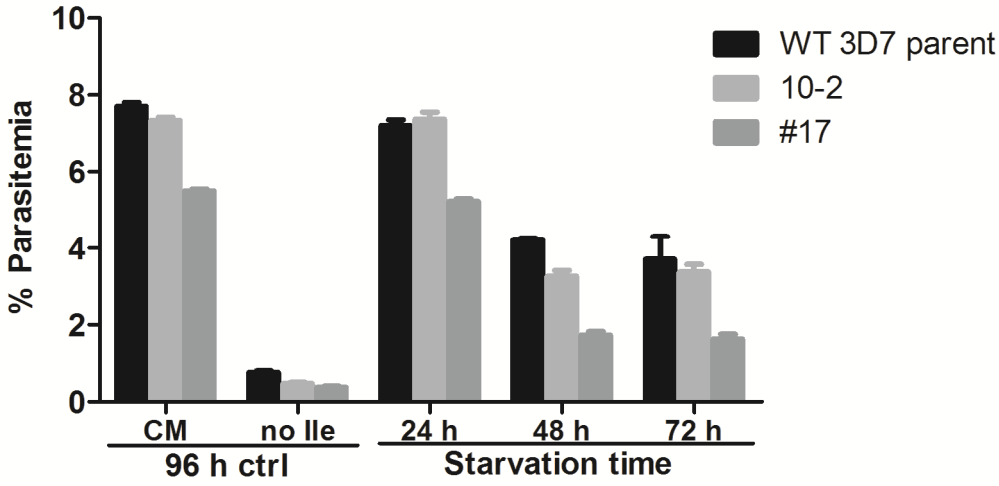
3571 genes. [-0.28,0.25]
ILE-
CTRL

Figure 3


10
8
6
4
2
0
% Parasitemia
WT 3D7 parent
10-2
#17
CM
no Ile
24 h
48 h
72 h
96 h ctrl
Starvation time

Part III:

Evaluating the tRNA-binding properties of the putative amino acyl-tRNA synthetase-like domain (PfaaRS) of PfeIK1

Abstract

Activation of the eIF2α kinase GCN2 is mediated by the binding of uncharged tRNAs, which accumulate during amino acid starvation. In yeast and most eukaryotes, the tRNA-binding domain of GCN2 orthologs is homologous to histidyl-tRNA synthetase (HisRS) and is located directly adjacent to the kinase domain. In the GCN2 ortholog of *Plasmodium falciparum*, PfeIK1, the putative tRNA-binding domain is poorly conserved with those of other eukaryotes. However, the catalytic activity of PfeIK1 is responsive to amino acid starvation; therefore it is presumed that the mechanism of kinase activation is also maintained. In this study, we attempt to examine the functional role of the putative tRNA-binding domain of PfeIK1, designated as PfaaRS.

Introduction

In yeast, starvation for amino acids leads to the activation of the eIF2α kinase GCN2. Kinase activation is stimulated by the binding of uncharged tRNA, which accumulates when amino acids are limiting [1, 2]. The tRNA-binding properties of GCN2 are mediated by a C-terminal domain that has sequence homology to histidyl-tRNA synthetase (HisRS) [3]. The GCN2 ortholog of *Plasmodium falciparum*, PfeIK1, also phosphorylates eIF2α when parasites are subjected to amino acid withdrawal (Chapter 2), presumably through a conserved activation mechanism. Although the putative tRNA-binding domain of PfeIK1 is weakly conserved with that of GCN2, this region maintains similarity with an aminoacyl tRNA synthetase (Chapter 2), and is thus predicted to bind tRNA. In this study, using a heterologous yeast expression system, we replaced the tRNA-binding domain of GCN2 with the putative binding domain of PfeIK1, termed PfaaRS, to determine whether this region could complement GCN2 activation when cells were exposed to various amino acid dropout conditions. In these yeast chimeras, we assessed phosphorylation of eIF2α as well as rescue of growth in arginine-depleted medium, which does not support the growth of yeast lacking a functional GCN2 [4]. We also recombinantly expressed PfaaRS to directly examine its tRNA binding abilities. We report that the functional complementation of PfaaRS in yeast presented numerous technical ambiguities that preclude definitive conclusions. Furthermore, although PfaaRS expression was successful, we failed to detect tRNA binding in our experimental assay; therefore, additional optimization is needed to verify the putative activation mechanism of PfeIK1.

Methods

GCN2 and PfaaRS/GCN2 hybrid cloning and yeast transformation

Full length GCN2 (YDR283C) plus 0.5kb upstream of GCN2 (representing the promoter region) was PCR amplified from plasmid YCp50 [3] using primers 5'-CCACCGCGG TGGCGGCCGAGAATAAAAAAGAATATATACTCC-3' (Eag1 site underlined) and 5'-CCCCCCCTCGAGGTCGACCTACCTCTGTAAATCGATAACAG-3' (SalI site underlined) and In-Fusion® (Clontech) cloned into the Eag1/SalI restriction sites of yeast expression plasmid pRS316 (digested with EagI/SalI), containing a URA3 selection marker. All cloning was confirmed by sequencing. The resulting plasmid, GCN2/pRS316 was then transformed into a Δgcn2 [his-, leu-, trp-, ura-, ade-] yeast strain and transformants were selected on SD-Ura plates, yielding the $GCN2^{c}$ strain. Empty pRS316 was also transformed into Δgcn2 [his-, leu-, trp- ura-, ade-] yielding a Δgcn2 URA+ strain, designated as $\Delta gcn2^{Ura}$, as a control.

To design the PfaaRS/GCN2 hybrid, the tRNA binding domain of GCN2 (bp 2917 – 4425) [5] was replaced with a codon optimized version of the putative tRNA-binding domain (PfaaRS) of PfeIK1 (PF14_0423) (bp 2707 - 4215). Briefly, a 2.109kb restriction fragment containing the codon optimized PfaaRS flanked by GCN2 sequence cloned into pUC57 was purchased from GenScript. The PfaaRS/GCN2/pUC57 plasmid was sequentially digested with PasI and PsrI and the PfaaRS/GCN2 fragment was ligated into PasI/PsrI digested GCN2/pRS316 yielding the full length PfaaRS/GCN2 chimera in pRS316. The final cloning step was confirmed by sequencing. The PfaaRS/GCN2/pRS316 plasmid was then transformed into a Δ*gcn2* [his-, leu-, ura-, ade-]

yeast strain and transformants were selected on SD-Ura plates, yielding the PfaaRS/GCN2^{c} yeast strain.

PfaaRS cloning and recombinant expression

Codon optimized PfaaRS was PCR amplified from the GCN2/PfaaRS/pUC57 plasmid using primers 5'-ACCGGTACTAGTGGATCCATGATAAACAAGAAGAAAGAA AAC-3 (BamHI site underlined) and 5'-ACGCGTTGTACACTCGAGTTA*CTTGT CATCGTCATCCTTGTAATC*GTGTATTGAAATCTT-3' (XhoI site underlined and FLAG tag sequence in italics), digested with BamHI and XhoI, and ligated into the same sites of the His-tag bacterial expression vector pET28a (Novagen). All cloning steps were confirmed by sequencing. The resulting PfaaRS^{FLAG}/pET28a plasmid was transformed into BL21 Codon Plus *E. coli* (Stratagene). A single colony of PfaaRS^{FLAG}pET28a transformed BL21 was inoculated in 5mL LB containing 100 μg/mL kanamycin and incubated overnight at 37°C. The overnight culture was used to seed 500 mL of fresh LB containing 100 μg/mL kanamycin, which was then grown to OD_{600} 0.6, and induced with 0.5 mM IPTG for 5 hours at 30°C. Cells were harvested, resuspended in cold lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5% Igepal, 10% glycerol) containing Complete protease inhibitor cocktail mix (Roche), sonicated in an ice water bath, and centrifuged at 12000 rpm for 30 minutes. 1 mL of the protein lysate was incubated with 50 μL of washed charged HisPur™ Ni-NTA resin (Thermo Scientific) for 1 hour with tumbling at 4°C. Following the pull down, the ^{His}PfaaRS^{FLAG} beads were washed 3 times in the lysis buffer and eluted with 250 μM imidazole. 2x SDS-Laemmli

buffer [6] was added to a portion of the elution, followed by SDS-PAGE and Coomassie Blue staining.

Control bacterial plasmids p274 (His-tagged wild-type *gcn2* HisRS-related domain) and p297 (His-tagged *gcn2* HisRS-related domain with mutant m2 allele) [1] were obtained from Ronald Wek's lab and recombinant protein expression was induced as above.

Starvation assay and immunoblotting

Yeast strains $\Delta gcn2^{Ura}$, $GCN2^{c}$, PfaaRS/$GCN2^{c}$, and the parental S10757: W303a URA+ (designated as WT) were grown overnight in SD-Ura medium at 30°C. Cells were harvested and washed with dH_2O, equally partitioned, inoculated into SD-Ura, SD-Leu, or SD-Ile, and incubated at 30°C for 45 minutes. Following centrifugation at 4°C, 200 µL of lysis buffer (25mM Tris-HCl pH 7.5, 50mM KCl, 10mM MgCl2, 1mM EDTA, 10% glycerol) containing 50x Complete protease inhibitor cocktail (Roche), 5 µg/ mL aprotinin, and 10 mM PMSF was added to each cell pellet along with 200 µl glass beads (size 425-600 µm) (Sigma). Samples were vortexed at 4°C, centrifuged briefly to pellet beads and cell debris, and yeast lysates were collected. Protein concentration was determined by the BCA protein assay (Pierce) and 80-100 µg of protein was prepared for electrophoresis. 2x sample buffer was added, proteins were resolved by SDS-PAGE, and transferred to nitrocellulose for immunoblotting [7] with anti-phospho-eIF2α antibodies (Cell Signaling Technology) and anti-PGK antibodies (provided by True lab).

Alternatively, to determine GCN2 expression levels only, yeast strains $\Delta gcn2^{Ura}$, $GCN2^{c}$, PfaaRS/$GCN2^{c}$, and the parental S10757: W303a URA+ (designated as WT) were grown

overnight in SD-Ura medium at 30°C, diluted to OD_{600} 0.25 in 20 mL SD-Ura medium and grown to OD_{600} 1.5 - 2. Cells were harvested, washed, lysed, and quantitated as indicated above. Following the addition of 2x sample buffer, proteins were resolved by SDS-PAGE, and transferred to nitrocellulose for immunoblotting with anti-GCN2 antibodies (provided by Hinnebusch lab).

Yeast growth assays

Yeast strains $\Delta gcn2^{Ura}$, $GCN2^{c}$, PfaaRS/$GCN2^{c}$, and the parental S10757: W303a URA+ (designated as WT) were grown overnight in SD-Ura medium at 30°C, harvested by centrifugation, and washed with dH_2O. Cells were diluted to OD_{600} 0.05 or 0.005 in dH_2O, inoculated into SD-Ura, SD-Leu, SD-Ile, or SD-Arg medium, and incubated at 30°C for up to 3 days. Assessment of yeast growth was determined by culture medium turbidity.

Alternatively, following overnight growth at 30°C and washing, the above yeast strains were diluted to OD_{600} 0.4 or 0.8 in dH_2O, spotted on SD-Ura, SD-Ura-Leu, SD-Ura-Ile, or SD-Ura-Arg plates in 2-fold serial dilutions, and incubated at 30°C for up to 4 days. Assessment of yeast growth was determined by the appearance of colonies.

tRNA binding assay

Recombinant $^{His}PfaaRS^{FLAG}$, WT GCN2 HisRS, and the GCN2 HisRS m2 mutant were resolved by SDS-PAGE and transferred to nitrocellulose membrane. The membrane was incubated in binding buffer (50 mM HEPES pH7.5, 50 mM KCl, 0.05% Triton X-100, 0.04% Ficoll, 0.04% polyvinylpyrrolidone, 0.08% BSA, 2.5 mM EDTA) containing 20

μg degraded herring sperm DNA (Sigma) per mL at 25°C overnight. Total yeast tRNA (Sigma) was dephosphorylated at the 5' end with calf alkaline phosphatase (Roche) followed by radiolabeling with 25 pmol [γ-^{32}P]ATP [6000 Ci/mmol, 142 mCi/mL] and polynucleotide kinase. The membrane was incubated with binding buffer containing the end-labeled tRNAs and 20 μg of herring sperm DNA per mL for 1 hour at 25°C. Membranes were washed in binding buffer, dried, and exposed for autoradiography.

Results and Discussion

To determine whether the putative tRNA-binding domain of PfeIK1, termed PfaaRS from here on, could functionally mimic the HisRS-like domain in yeast GCN2, we performed a domain swap and replaced GCN2-HisRS with a codon optimized version of PfaaRS. Using a heterologous yeast expression system, we transformed a Δ*gcn2* yeast strain [auxotrophic for uracil (Ura), leucine (Leu), tryptophan (Trp), histidine (His), and adenine (Ade)] with a Ura+ plasmid carrying wild-type GCN2 or the PfaaRS/GCN2 chimera, both under the control of the native GCN2 promoter. When the GCN2 complemented (GCN2^{c}) yeast were incubated in SD-Leu for 45 minutes, phosphorylation of eIF2α was readily apparent **(Figure 1A)**. Also, eIF2α was phosphorylated in the GCN2^{c} yeast when isoleucine was absent. This signal was not as robust presumably because these yeast were prototrophic for isoleucine, thus the activated general control mechanism would upregulate the biosynthesis of this amino acid, reducing the starvation signal. No eIF2α phosphorylation was detected in either SD-Leu or SD-Ile for the Δgcn2^{Ura} control **(Figure 1A)**, indicating that these mutants were unable to respond to amino acid limitation. Similarly, no signal was detected for the PfaaRS/GCN2^{c} yeast

(data not shown). It is possible that this construct simply failed to express since no protein of the expected size (~190kDa) was apparent in these yeast **(Figure 1B, Pf^c)**, in contrast to the GCN2^c strain, which exhibited a low level of GCN2 expression **(Figure 1B, G^c)**.

Interestingly, PfaaRS/GCN2^c yeast exhibited a differential growth pattern in comparison to the Δgcn2 mutant when inoculated at 0.005 OD_{600} in SD-Ile **(Table 1-1)**, suggesting that PfaaRS/GCN2 could indeed mediate a starvation response. However, this differential growth was no longer observed when these strains were inoculated at a 10-fold higher OD **(Table 1-2)**. Given this discrepancy, we examined the growth of these strains in arginine-dropout medium conditions, which reportedly does not support the growth of Δgcn2 mutants [4]. However, even in these purportedly selective conditions, growth for all strains was observed **(Figure 2 and Table 1-1 and 1-2)**, which gives rise to the possibilities of contamination or a suppressor mutation being present in the Δgcn2 strain.

We also sought to determine directly whether PfaaRS binds tRNA by expressing a recombinant version, tagged at the N-terminus with His and at the C-terminus with FLAG. A protein of expected size (63kDa) was expressed and isolated by Ni-NTA affinity from the soluble fraction **(Figure 3)**, however, this protein was not the most dominant species that eluted from the Ni-NTA resin. Several lower and higher molecular weight proteins were abundant in the eluted fraction, possibly representing degradation products or non-reduced, PfaaRS-interacting protein complexes, respectively. Nonetheless, this protein mix was used to assay tRNA-binding, along with two control protein domains previously used to establish the tRNA-binding properties of yeast GCN2

[1]. ^{His}PfaaRS^{FLAG} and the two controls were resolved by SDS-PAGE, transferred to nitrocellulose and exposed to radiolabeled tRNA in a northwestern blotting assay. However, tRNA binding was not detected for any of the protein domains (data not shown). Differential tRNA binding has already been demonstrated for the controls in a previous report using this assay [1], therefore further optimization is needed to troubleshoot this method in order to define the tRNA-binding properties of PfaaRS.

Acknowledgments

We thank Ronald Wek (University of Indiana) for wild-type and mutant *gcn2* HisRS plasmids, Alan Hinnebusch (NIH) for anti-GCN2 antibodies, Laura Westergard (Washington University) for yeast strains, plasmids, anti-PGK antibodies, and technical assistance with yeast experiments, Jennifer Dulle (Washington University) for technical assistance with yeast experiments, and Heather True-Krob (Washington University) for the use of lab space and reagents for the yeast experiments.

References

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Figure Legends

Figure 1: GCN2 complemented mutant phosphorylates eIF2α in amino acid depleted conditions

A) Δgcn2 and GCN2^{c} yeast strains were incubated in SD-Ura, SD-Leu, or SD-Ile for 45 minutes. Yeast extracts were prepared and proteins were resolved by SDS-PAGE followed by immunoblotting with antibodies against phosphorylated-eIF2α (anti-eIF2α^{P}) and PGK (anti-PGK) as a loading control. B) WT, Δgcn2 (Δ), GCN2^{c} (G^{c}), and PfaaRS/GCN2^{c} (Pf^{c}) yeast strains were grown in SD-Ura to OD_{600} 1.5 – 2, yeast extracts were prepared and proteins were resolved by SDS-PAGE followed by immunoblotting with antibodies against GCN2 (anti-GCN2). Arrow indicates expected size of GCN2.

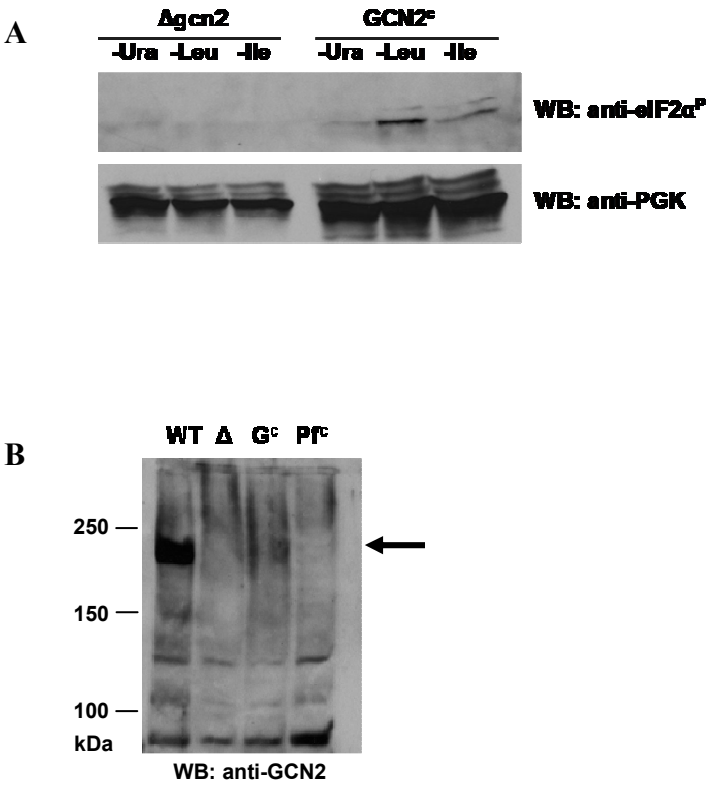
Figure 2: Growth assessment of yeast strains on selective medium

The indicated yeast strains were spotted in serial dilutions onto selective plates lacking the indicated supplements. The above images were collected 4 days post-incubation at 30°C. Growth on the selection medium indicates that the yeast strain is competent to synthesize the component(s) missing from the medium. Lack of growth indicates that the yeast strain is unable to synthesize the missing component(s).

Figure 3: Expression of ^{His}PfaaRS^{FLAG}

Recombinant ^{His}PfaaRS^{FLAG}, ^{His}WTGCN2-HisRS, and ^{His}m2mutGCN2-HisRS were expressed, Ni-NTA affinity purified, and resolved by SDS-PAGE. Proteins of the expected size (63kDa) were observed for all constructs (indicated by arrow). Other proteins were also enriched in the ^{His}PfaaRS^{FLAG} pull down (indicated by red asterisks).

Figure 1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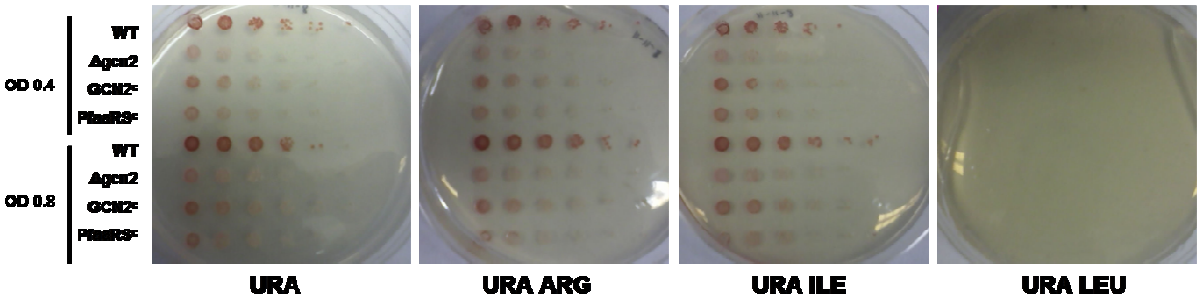
Agcn2
GCN2c
-Ura -Leu -Ile
-Ura -Leu -Ile
WB: anti-eIF2αP
WB: anti-PGK
B
WT Δ Gc Pfc
250
150
100
kDa
WB: anti-GCN2

Figure 2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WT
Agcn2
GCN2-
PhaR3-
OD 0.8
WT
Agcn2
GCN2-
PhaR3-
URA
URA ARG
URA ILE
URA LEU

Figure 3

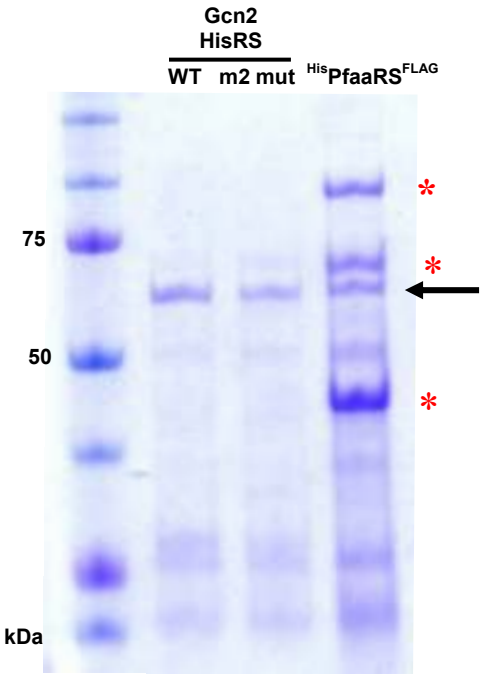

Table 1-1 Yeast growth assay (by turbidity) starting OD_{600} 0.005

Yeast Strain	-URA	-LEU	-ARG	-ILE	-ILE (3 days)
WT	+	-	+	+	+
Δgcn2^{Ura}	+	-	+	-	-
GCN2^{c}	+	-	+	-	+
PfaaRS/ GCN2^{c}	+	-	+	-	+

Growth assessed at 18 hours post-incubation unless otherwise indicated

+, confluent growth; -, no growth

Table 1-2 Yeast growth assay (by turbidity) starting OD_{600} 0.05

Yeast Strain	-URA	-LEU	-ARG	-ILE	-ILE (2 days)
WT	+	-	+	+	+
Δgcn2^{Ura}	+	-	+	-	+
GCN2^{c}	+	-	+	-	+
PfaaRS/ GCN2^{c}	+	-	+	-	+

Growth assessed at 18 hours post-incubation unless otherwise indicated

+, confluent growth; -, no growth

Part IV:

Electron microscopy of isoleucine-starved P. falciparum

Abstract

During isoleucine starvation, the human malaria parasite, *Plasmodium falciparum*, slows its growth and experiences developmental arrest at the trophozoite stage. By light microscopy, the morphology of starved parasites appears essentially normal. However the limited resolution of this technique prevents a comprehensive evaluation of cellular organization, which, in other organisms, generally undergoes dramatic effects during nutrient starvation. In this study, transmission electron microscopy (TEM) of isoleucine-starved parasites revealed prominent abnormalities in nuclear and food vacuole architecture that were most apparent after 24 hours of starvation. We hypothesize that such cellular irregularities may be representative of an autophagic-like stress response.

Introduction

The growth of *Plasmodium falciparum* depends on an exogenous supply of isoleucine [1]. Upon isoleucine withdrawal, parasite growth slows dramatically and developmental arrest occurs at the trophozoite stage, prior to initiation of DNA replication. Although parasite proliferation is inhibited in isoleucine-limiting conditions, parasites remain viable given that growth is restored upon isoleucine supplementation (Chapter 3). On Giemsa stained thin blood smears visualized by light microscopy, the morphology of isoleucine-starved *P. falciparum* is largely unremarkable and virtually indistinguishable from that of control parasites from a comparable life cycle stage. In contrast, when *P. falciparum* is starved for glucose, which is rapidly consumed by the parasite for energy [2], parasites stain dark purple with Giemsa stain, and they appear as shrunken, rounded bodies with pyknotic nuclei, features that are characteristic of cell death [3]. The maintenance of parasite viability during isoleucine starvation suggests that *P. falciparum* is better equipped to cope with amino acid fluctuations, which is suggestive of an adaptive response.

In other eukaryotic organisms, nutrient starvation induces the process of autophagy, in which cytoplasmic contents are indiscriminately degraded to salvage released lipids and amino acids that can be used as substrates for the synthesis of ATP and other molecules required to maintain cell viability [4]. The distinguishing features of this process, in the context of nutrient starvation, have been extensively characterized in terms of specialized protein markers (*i.e.* autophagy-related proteins, ATG proteins) and modulation of cellular architecture. In the latter case, electron micrographs of nutrient-deprived cells revealed the appearance of multiple vacuolar structures, termed

autophagosomes, which house cellular contents destined for degradation [5, 6], and ribbon-like membrane structures, termed phagophores, which initiate the sequestration of cytoplasmic components [7, 8]. Therefore, the presence of these structures provide a visual landmark to identify autophagic cells.

Autophagy is not well-characterized in *Plasmodium*, and to date, only a drug-induced autophagic-like cell death has been described to occur in the parasite [9]. Interestingly, however, during isoleucine starvation, food vacuole proteases remain active, and this proteolytic activity is required to maintain parasite viability (Chapter 3), suggesting that an autophagic-like process may also contribute to the starvation-stress response of *P. falciparum*. Therefore, in this study, we used transmission electron microscopy (TEM) to examine the ultrastructural architecture of isoleucine-starved parasites to ascertain whether such conditions induce changes to the parasite's cellular organization that are indiscernible by light microscopy. We report that parasites exhibit abnormal morphology with extended starvation. Furthermore, we describe the appearance of unusual structures within the food vacuole and cytoplasm of starved parasites, which may be associated with an autophagic-like stress response.

Methods

Parasite culturing

Plasmodium falciparum strain 3D7 was cultured [10] in human O+ erythrocytes in complete RPMI 1640, containing all 20 amino acids, supplemented with 27 mM $NaHCO_3$, 22 mM glucose, 0.37 mM hypoxanthine, 10 μg/ml gentamicin, and 5 g/L Albumax (Invitrogen). Homemade complete and isoleucine-free RPMI were prepared according to the RPMI 1640 recipe provided by Invitrogen, and supplemented with RPMI 1640 Vitamins (Sigma), the appropriate respective amino acids (Sigma) at the concentrations found in RPMI 1640, and the additional supplements mentioned above.

Isoleucine starvation assay

A large-scale sorbitol synchronized [11] *P. falciparum* 3D7 culture at 8 – 10% parasitemia was washed twice in PBS, equally partitioned and washed in either complete or isoleucine-free RPMI, after which, the parasites were re-plated in their respective medium and incubated at 37°C with 5% CO_2. 0.5 mL samples were harvested initially, and at 3 or 6 hour intervals over a 48 hour period. Culture medium was changed every 12 hours, and parasites incubated in complete medium were sub-cultured just prior to schizont rupture to maintain post-reinvasion parasitemia between 8 – 10%. Following harvesting, samples were prepared for transmission electron microscopy.

Transmission electron microscopy

For ultrastructural analysis, infected RBCs were fixed in 2% paraformaldehyde/2.5% glutaraldehyde (Polysciences Inc.) in PBS for 1 hour at room temperature. Samples were

washed in PBS and postfixed in 1% osmium tetroxide (Polysciences Inc.) for 1 hour. Samples were then rinsed extensively in dH_20 prior to *en bloc* staining with 1% aqueous uranyl acetate (Ted Pella Inc.) for 1 hour. Following several rinses in dH_20, samples were dehydrated in a graded series of ethanol and embedded in Eponate 12 resin (Ted Pella Inc.). Sections of 95 nm were cut with a Leica Ultracut UCT ultramicrotome (Leica Microsystems Inc.), stained with uranyl acetate and lead citrate, and viewed on a JEOL 1200 EX transmission electron microscope (JEOL USA Inc.).

Results and Discussion

To examine the ultrastructural detail of isoleucine-starved parasites, we conducted a time course in which synchronized 3D7 parasites were incubated in isoleucine-free RPMI over a period of 48 hours, with samples prepared for TEM at 3 or 6 hour intervals. In parallel, a control set of parasites were incubated in complete RPMI over the same time frame and similarly prepared. Representative images of select incubation times are shown in **Figure 1**. Parasites grown in complete RPMI exhibited normal morphology **(Figure 1A-E)**, in that cellular organelles were readily identifiable throughout the course of parasite development. Isoleucine-starved parasites appeared somewhat developmentally delayed, but relatively comparable to the control at the 12h time point (**Figure 1F)**. After 12 hours, however, the morphology of isoleucine-starved parasites became increasingly irregular with the appearance of unidentifiable cytoplasmic structures and food vacuole abnormalities **(Figure 1G-I)**. For instance, the food vacuoles of the starved parasites appeared to contain membrane bound vesicles with electron-lucent contents **(Figure 1G-I, black arrows)**, and the cytoplasmic structures appeared as

membranous, ribbon-like cisternae **(Figure 1G-H, red arrows)**. At 48 hours of starvation, nuclear morphology appeared elongated and distended **(Figure 1I, asterisks)**, however, only a single nucleus was observed per parasite. This nuclear enlargement may be due to transcriptional activity rather than replication since data presented in chapter 3 of this thesis indicates that DNA synthesis arrests during starvation.

The appearance of the membrane-bound vesicles within the food vacuole and the cytoplasmic structures in the isoleucine-starved parasites is rather unusual and was not observed in control parasites. Presumably, these structures may represent features of a starvation-associated, autophagic-like response. *P. falciparum* encodes at least 9 ATG genes [12], however, their roles in parasite biology are undefined. Therefore, to further characterize these novel structures that appear during starvation, it is of interest to determine the localization of the *P. falciparum* ATG orthologs, which in other eukaryotes, conventionally serve as markers for autophagic compartments.

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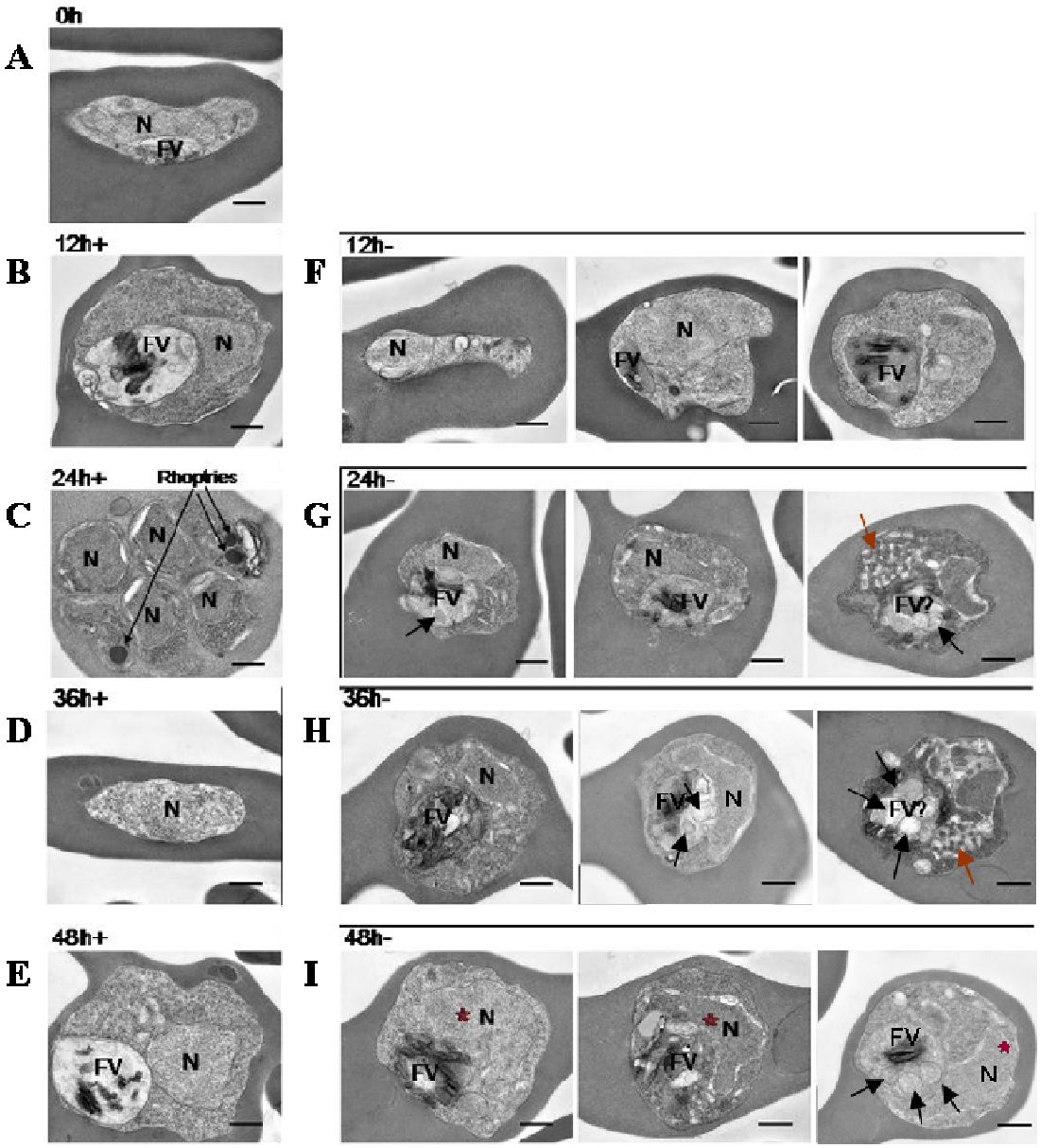
Figure Legend

Figure 1: Transmission electron micrographs of control and isoleucine-starved *P. falciparum*

A) Ring-stage parasites at the 0h initial time point; B-E) Representative images of control parasites from complete medium conditions harvested after B) 12 hours (trophozoite stage), C) 24 hours (schizont stage), D) 36 hours (rings from 2nd cycle reinvasion), and E) 48 hours (trophozoites of 2nd cycle) of incubation; F-I) Representative images of isoleucine-starved parasites harvested after F) 12 hours, G) 24 hours, H) 36 hours, and I) 48 hours of incubation. Black arrows indicate membrane-bound vesicles. Red arrows indicate cytoplasmic, ribbon-like structures. Asterisks (*) indicate abnormal nuclei. N, nucleus; FV, food vacuole; Scale bar 0.5 μm

Figure 1


A
0h
N
FV
B
12h+
FV
N
C
24h+
Rhoptries
N
D
36h+
N
E
48h+
FV
N
F
12h-
N
FV
G
24h-
FV?
H
36h-
FV?
I
48h-
FV