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**On the role of transporters in transition
metal homeostasis in the malaria
parasite *Plasmodium falciparum***

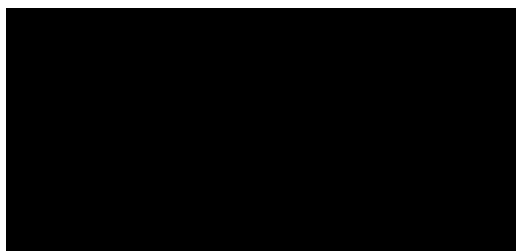
Benedict M. O. Davies

Thesis submitted for the degree of
Doctor of Philosophy (PhD)

May 2023

Declaration of Authenticity

I confirm that I am the sole author of this thesis and the work to which it refers. All referenced studies have been appropriately cited and included in the reference list, and any contributions of collaborators have been made clear in the body of the thesis. This thesis has not been previously submitted to any university or institution.



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Abstract

Despite major reductions in clinical cases and mortality over the last half century, malaria remains a major public health threat with an estimated 241 million cases and 627 000 deaths in 2021. Our arsenal of effective treatment options continues to be threatened by the emergence of drug resistance, thus highlighting a need for new drugs and drug combinations with novel modes of action. Transition metals such as iron, copper and zinc are essential to the survival of the malaria parasite *Plasmodium falciparum*, but become toxic at high concentrations. Hence, the careful regulation of transition metals is required as the parasite progresses through its complex life cycle. With that considered, the perturbation of transition metal homeostasis is an attractive approach for novel malaria control strategies. The work presented herein aimed to characterise several putative transition metal transporters from *P. falciparum*, to better understand their role in transition metal homeostasis. The sole cation diffusion facilitator expressed by *P. falciparum* (PfCDF) was shown to confer zinc tolerance to a zinc-sensitive yeast line, but not tolerance to cobalt or iron. Using similar yeast systems, it was shown that putative copper transporters PfCTR1 and PfCTR2 did not rescue the growth phenotype of a copper-sensitive yeast line, nor did the chloroquine resistance transporter, PfCRT, confer tolerance to iron in an iron-sensitive yeast line. Furthermore, reverse genetics approaches were undertaken which aimed to generate parasite lines that featured mutated putative transporter genes, to enable the study of protein essentiality, substrate specificity and sub-cellular localisation. Finally, the effects of iron on artemisinin (current frontline antimalarial drug) action were also explored. Whilst malarial proteins involved in transition metal homeostasis remain reasonably understudied, the work presented in this thesis builds on existing foundations of a promising area of investigation and leaves a clear trajectory for further exploration.

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Project Specific Attributions and Collaborators

Design and cloning of the pL6-PfDMT plasmids was done by Dr Ksenija Slavic prior to the start of my project. She also designed the pARL-PfDMT plasmids with the assistance of Dr. Moritz Treeck (Francis Crick Institute). All other data presented in this thesis was generated by the author.

1. Introduction

1.1. Malaria

Malaria is a mosquito-borne, tropical disease caused by protozoan parasites of the genus *Plasmodium* [1]. The World Health Organisation (WHO) estimated that in 2021 there were 247 million cases of malaria worldwide, leading to 619 000 deaths [2]. The disease is endemic across much of sub-Saharan Africa, Asia and Latin America with around half of world's population at risk of infection. Sub-Saharan Africa bears around 95% of the total disease burden with six countries therein accounting for over 50% of global cases and deaths.

Historically it was considered that four *Plasmodium* species caused malaria in humans. Those being *Plasmodium falciparum*, *P. vivax*, *P. ovale* and *P. malariae*. However, it has been established over the last 20, or so, years that *P. knowlesi* – a parasite of long-tailed macaques in Southeast Asia – also causes human malaria and can be considered a zoonosis [3,4]. In addition, it has been shown that *P. ovale* can be split into two distinct species – *P. ovale curtisi* and *P. o. wallikeri* [5].

Parasites of the *Plasmodium* genus have a wide host range with over 200 species described across 14 subgenera that infect other primates and mammals, as well as reptiles and birds [6–8]. Beyond the six parasite species (noted above) that are recognised as human pathogens, there have been scant reports of zoonotic transmission of *P. simium*, *P. cynomolgi* and *P. brasilianum*, however as detected cases are few it remains to be seen whether these will be deemed true human pathogens [9–11]. All the described species of *Plasmodium* are transmitted to a vertebrate host by a female mosquito of the *Anopheles*, *Culex*, or *Aedes* genera, however, the human parasites are exclusively transmitted by *Anopheles* [12].

P. falciparum has the highest public health importance of the *Plasmodium* species, and in fact any parasite of man [1]. If an infected patient is left untreated, the parasite can

cause severe disease and ultimately death. Notably, *P. falciparum* causes 99% of malaria cases in Africa and the majority of cases and deaths in all other affected regions other than the Americas where *P. vivax* is the dominant species. *P. vivax* also has a significant public health burden with a reported 4.9 million cases in 2021 [2]. Unlike *P. falciparum* however, *P. vivax* can become dormant in the liver of the infected human host [13,14]. This aspect of the biology of this parasite provides a substantial challenge to control efforts and eventual elimination attempts. This is because a *P. vivax* infection can relapse months or even years post infection and whilst dormant is undetectable by commonly used diagnostic tests such as microscopy, rapid diagnostic tests (RDT) or molecular methods such as PCR and is difficult to treat [15,16].

Plasmodium has a complex life cycle (Figure 1.1), during which it interchanges among distinct ecological niches [1]. These are the gut and salivary glands of the mosquito, the liver of the vertebrate host and the blood of the vertebrate host. Following infection by a feeding mosquito, the parasites migrate to the liver – during which the infection is clinically silent. The disease symptoms of malaria arise from the blood stage of the life cycle. This is due to the invasion and subsequent egress of the parasites from red blood cells (RBCs), which not only cause the destruction of the RBCs, but also cause the release of parasite material into the peripheral blood. Parasite toxins such as haemozoin and glycosylphosphatidylinositol (GPI) stimulate the systemic production of proinflammatory cytokines (e.g. TNF-alpha, IL-1, 6, 12) which result in fever and other flu-like symptoms, such as chills, headache, nausea and general malaise [17]. In addition, patients may also develop splenomegaly, hepatomegaly, and mild jaundice. Severe malaria complications include, cerebral malaria (coma), pregnancy-associated malaria, severe malaria anaemia, metabolic acidosis, and shock-like syndrome [18]. These severe, life-threatening, syndromes are seen in a minority (~1%) of all cases and are most common in African children [2,19]. However, in areas with lower and seasonal transmission such as Southeast Asia, the Indian subcontinent, and amongst

immunologically naïve travellers, severe malaria also occurs in adulthood [20–22].

Severe manifestations are usually caused by *P. falciparum*, however, there have been scant reports of severe malaria caused by both *P. vivax* and *P. knowlesi* [23,24].

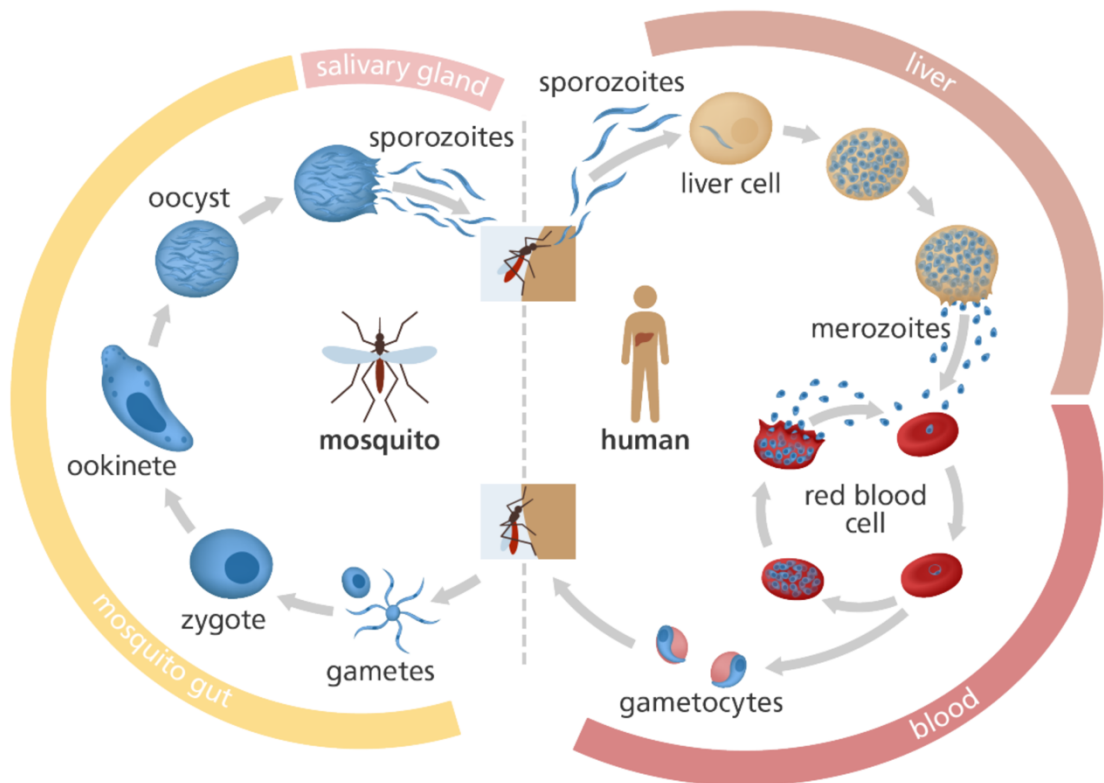


Figure 1.1. Life cycle of *Plasmodium* [25]. Infectious sporozoites are transferred into the dermis of a susceptible vertebrate host via the saliva of an infected female mosquito when she takes a blood meal. The sporozoites migrate to the liver and invade hepatocytes. Here they undergo asexual reproduction and form a liver schizont. The schizont then ruptures (after ~7 days in *P. falciparum*), releasing merozoites into the blood. The merozoites invade red blood cells (RBCs) and become an immature trophozoite, or ring form. The ring develops into a mature trophozoite and then multiplies asexually to form a schizont. As with the liver stage, the schizont ruptures releasing merozoites (~30 in the case of *P. falciparum*) back into the blood, with each subsequently able to invade an uninfected RBC. At the trophozoite stage, the parasite can develop into sexual stages of the life cycle – male and female gametocytes. These are long-lived and can persist in the blood stream for several months. When another mosquito takes a blood meal from the infected host, she ingests the gametocytes, which then develop into gametes and reproduce sexually in gut of the mosquito to form a zygote. This develops into an ookinete, which migrates to the gut lining and develops

into an oocyst, which then ruptures releasing many sporozoites. These then migrate to the salivary glands of the mosquito, where they can be transferred to another susceptible host when a blood meal is taken.

1.2. Malaria control

Efforts to control malaria rely on a multifaceted approach that includes the use of antimalarial drugs, vector control strategies, educating at risk populations about disease transmission and more recently vaccines.

1.2.1. Vector control

Vector control relies predominantly on the use of long-lasting insecticide treated bed nets (LLINs) and indoor residual spraying (IRS) but also includes larval source management and other personal protection measures [26]. LLINs prevent transmission by providing a physical barrier to the mosquito and thus offering personal protection to the user. The insecticide – usually a pyrethroid – has a repellent effect on the vector, as well being lethal. IRS involves spraying the interior walls and ceilings of houses with a long-lasting insecticide, which interrupts transmission by killing the vector as it rests on a treated surface. Together, IRS and LLINs prevented 663 million malaria cases between 2000 and 2015 [27].

It is noteworthy that resistance exists to all four of the routinely used insecticide classes: pyrethroids, carbamates, organophosphates, and organochlorines [28]. While the long-term effectiveness of both IRS and LLINs are threatened by the rapid emergence and spread of insecticide resistance among mosquito populations, these interventions remain central to malaria control and are highly cost-effective and safe [2,29]. As a child survival intervention, LLINs are as cost-effective as the measles vaccine [30]

There are various other promising tools in development including, the use of larvicides and predatory larviferous fish, entomopathogenic fungi/bacteria/viruses, environmental modification, odour baits and traps, and mosquito transgenesis [31].

The prospect of genetically altered mosquitos as a means for reducing the transmission of various vector-borne diseases, such as malaria, has gained traction over recent years and is now starting to be trialled in the field [32]. These efforts have undoubtedly been aided by the development of novel genome editing technologies such as the CRISPR-Cas9 gene editing system. The main two strategies are to produce; 1. transgenic sterile male mosquitos – which would suppress vector populations, thereby reducing disease transmission, and 2. Refractory transgenic female mosquitos – which would be inhospitable to the malaria parasite, thus blocking its onward transmission [33,34].

1.2.2. Vaccine

An ideal component of any infectious disease control programme is a highly effective vaccine. However, despite several decades of research and numerous clinical trials, there is currently no highly effective malaria vaccine. In 2022 RTS,S became the first licensed malaria vaccine and is now recommended by the WHO for young children in areas of high transmission [35]. Significantly however, RTS,S only provides limited and short-lived protection, with 55% efficacy in infants after a year following three doses, and 36% over four years after a fourth “booster” dose [36]. While the protection conferred by the vaccine is modest, at the population level one would expect RTS,S to significantly reduce malaria burden and should be used in conjunction with other available control methods, until a more efficacious vaccine becomes available [37].

Other vaccines, such as R21 – which is a modified version of RTS,S – and the whole sporozoite vaccine PfSPZ are currently being trialled in the field, however it seems very unlikely that a highly effective vaccine is on the horizon [38,39]. With this considered the

development of new drugs to complement our current repertoire will be crucial to reduce malaria burden.

1.2.3. Drugs

The use of antimalarial drugs is central to malaria control and prevention. There are several different drug classes routinely used, however, as is the case with insecticides, their longevity is threatened by the emergence of resistance – with widespread resistance already reported for many classes.

1.2.3.1. Amino alcohols and 4-aminoquinolines (e.g. quinine and chloroquine)

Quinine – an amino alcohol – has been used to treat malaria for at least 400 years and probably much longer. It was isolated from the bark of the cinchona tree in 1820 after being introduced to Europe by Jesuit missionaries returning from South America in the seventeenth century [40].

The mode of action of quinine is not yet fully understood, however it is widely accepted that it targets the haem detoxification pathway of the blood stage malaria parasite [41]. In the RBC the parasite endocytoses haemoglobin, which is broken down into its constituent parts – host-derived peptides and amino acids, which are redistributed and utilised by the parasite for growth and development, and toxic haem, which is converted into haemozoin – an inert crystal structure. Quinine likely acts by interrupting this conversion, thus killing the parasite [41].

Quinine was routinely used as a frontline antimalarial drug, however due to its numerous adverse effects and the availability of the safer and highly effective artemisinin derivatives, its use is now only recommended when an artemisinin derivative is not obtainable and for the treatment of vivax malaria [42].

Chloroquine is a synthetic analogue of quinine that was first synthesised by Bayer in the early twentieth century [43]. Following the Second World War, the use of chloroquine became widespread and was highly effective in treating clinical malaria. Like quinine, chloroquine interferes with haem detoxification in the digestive vacuole of the blood stage parasite [44].

Chloroquine resistance emerged independently in South-East Asia and South America and spread to sub-Saharan Africa from Asia. Resistance was later shown to be due to a causal association with mutations in a drug/metabolite transporter – the chloroquine resistance transporter, PfCRT [45,46]. The K76T mutation is seen in all chloroquine resistant parasite lines and, along with numerous other region-specific mutations, decreases the build-up of chloroquine in the digestive vacuole, consequently abrogating its effect [47]. Furthermore, mutations in the vacuolar ABC transporter, PfMDR1 also affect chloroquine susceptibility – notably the N86Y mutation [48,49]. It is thought that PfMDR1 affects the susceptibility of chloroquine (and other drugs) by mediating drug efflux from the vacuole [50].

Throughout the latter half of the twentieth century chloroquine resistance became widespread across the malaria endemic world, eventually rendering the drug useless for the treatment of falciparum malaria. However, malaria caused by the so-called benign parasites – *P. vivax*, *P. malariae*, and the two *P. ovale* species – can still be treated with chloroquine [42].

Other synthetic quinine derivatives such as mefloquine, amodiaquine, piperaquine and lumefantrine are also used to treat malaria, but only as part of combination therapies – usually with an artemisinin derivative (see below) [42].

Resistance to the amodiaquine, as with closely related chloroquine, is mediated by mutations in PfCRT and PfMDR1 – with the same mutations often seen in both cases [51,52]. Whilst the N86Y mutation in PfMDR1 decreases susceptibility to both

chloroquine and amodiaquine, it has been shown to increase susceptibility to mefloquine and lumefantrine [53]. It has been suggested that this is because the mutation reduces the transport of mefloquine and lumefantrine to the DV thus causing a relative increase in cytosolic drug concentration, with the presumed targets of these drugs being outside the DV [44]. Furthermore, a decrease in copy number of PfMDR1 has been shown to increase susceptibility to mefloquine, lumefantrine, quinine, as well as the artemisinin derivatives [54,55]. Increase copy number of plasmepsins II and III have been suggested as a marker for piperazine resistance, as well as various novel mutations in PfCRT found in resistance field isolates [56,57].

1.2.3.2. Endoperoxides (e.g. artemisinin & derivatives)

Artemisinin and its semi-synthetic derivatives – artesunate, artemether, and dihydroartemisinin – are the current first-line drugs for the treatment of falciparum malaria [42]. These drugs have been a mainstay of malaria treatment since their discovery and synthesis in China in the 1970s. Artemisinin derives from the *Artemisia annua*, which has been used in traditional Chinese medicine for centuries [58].

Artemisinins are extremely effective and well-tolerated and have largely replaced quinine and chloroquine as treatment options for falciparum malaria. Current WHO malaria treatment guidelines recommend that severe malaria patients are treated with intravenous artesunate followed by artemisinin-based combination therapy (ACT) [42]. For uncomplicated malaria a 3-day ACT regimen is recommended.

Whilst the artemisinin derivatives (artesunate, artemether, dihydroartemisinin) are highly potent and fast acting antimalarials, they have an extraordinarily short half-life in vivo and accordingly are administered with a longer-lived partner drug, such as mefloquine, amodiaquine, piperazine, lumefantrine, or pyronaridine, as an ACT. The mode of action of artemisinin is yet to be fully understood and has been a topic of fervent debate for many years [59]. Notably, artemisinin has been shown to inhibit the essential

sarco/endoplasmic reticulum calcium ATPase (SERCA) - PfATP6, however click chemistry approaches have implicated over 100 other putative targets in addition to PfATP6 – some of which have undergone further validation [60–63].

Artemisinin becomes activated by the iron-mediated reductive cleavage of its endoperoxide moiety, giving rise to highly reactive radicals [64]. Host-derived haem iron, rather than ferrous iron, seems to be largely responsible for artemisinin activation [61]. Inhibition of haemoglobin digestion has been shown to reduce artemisinin susceptibility, as has reduced haemoglobin uptake from the RBC [61,65]. It has also been shown that artemisinin derivatives likely increase available haem iron by inhibiting the crystallisation of haem to haemozoin [66].

Reports of widespread treatment failures to ACTs in the Mekong basin countries of South-East Asia have raised alarm bells and placed yet more importance on gaining a better understanding of the mode of action of artemisinin [67–69]. Specific mutations in the Kelch13 propeller protein have been associated with a delayed clearance phenotype, characterised by increased artemisinin tolerance in the immature ring-stage parasite, but importantly not trophozoite or schizont stages of the intraerythrocytic cycle [65,70]. Birnbaum et al. elegantly show that Kelch13, and several associated proteins, are involved in haemoglobin uptake from the red cell [65]. Their work suggests that Kelch13 mutations shown previously to be associated with the delayed clearance phenotype, could limit haemoglobin uptake in early ring-stage parasites. It is plausible that the resultant reduction in haemoglobin breakdown and therefore available haem-bound iron to activate artemisinins could account for the observed effect. If so, the delayed clearance would be due to a reduced active concentration of artemisinins during the ring-stage. This could simply be overcome by increasing artemisinin exposure to the highly susceptible trophozoite and schizont stages, which could be achieved by extending drug regimens [71]. Additionally, the use of longer lived, synthetic artemisinin derivatives such as artemisone have also been suggested [72]. It is noteworthy that

artefenomel (a synthetic artemisinin derivative) is currently being evaluated in clinical trials [73].

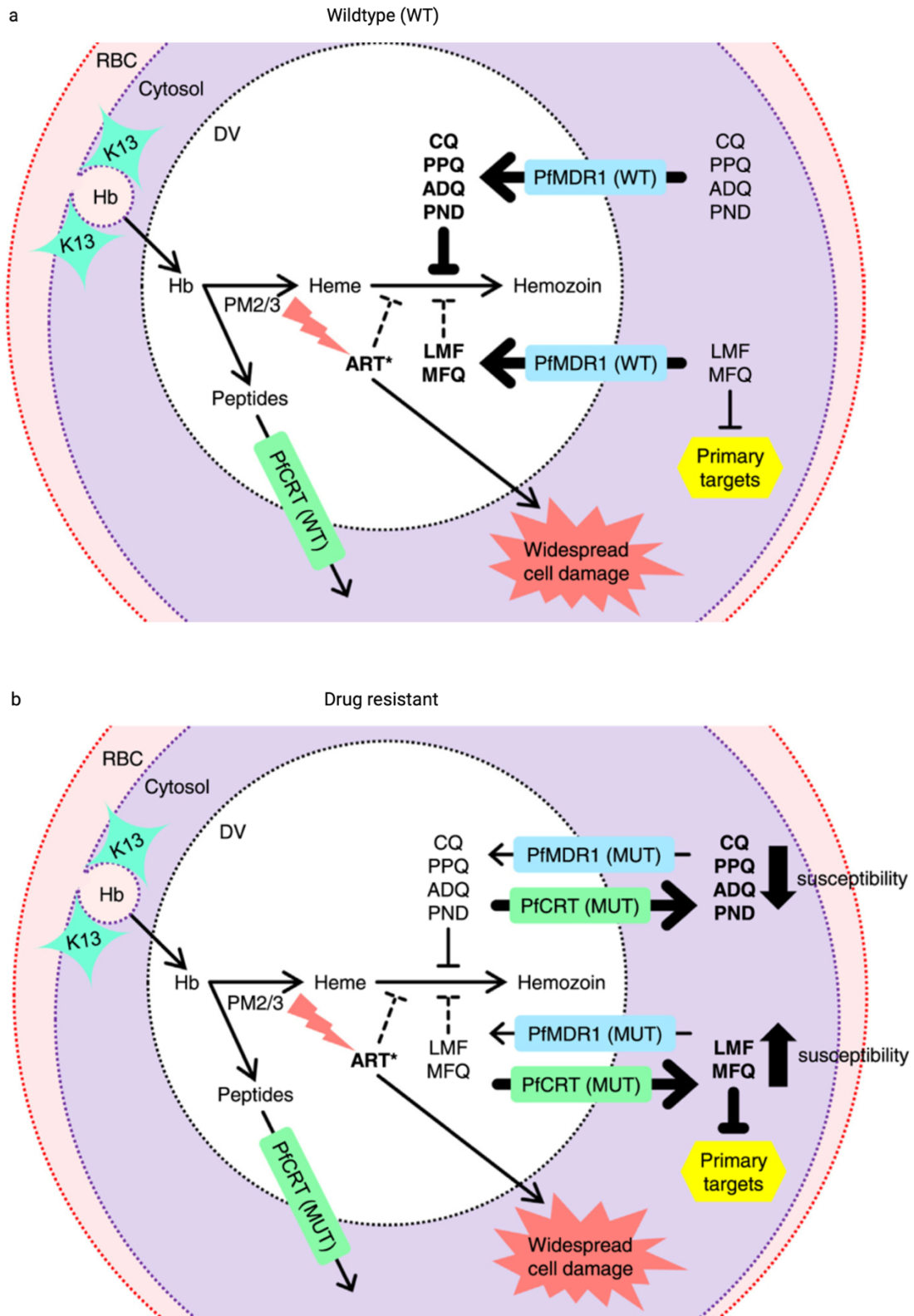


Figure 1.2. Modes of action and resistance of amino alcohol, 4-aminoquinolone and artemisinin derivative antimalarial drugs [74]. Schematics show the *P. falciparum* (purple) infected human RBC (red) and digestive vacuole (white) therein. Amino alcohols, 4-amino

quinolones and artemisinin derivatives target the detoxification of haem to haemozoin. In drug resistant parasites, mutations in either PfCRT, PfMDR1, or both, decrease drug concentrations in the digestive vacuole thereby reducing potency. Abbreviations: CQ = chloroquine; PPQ = piperaquine; ADQ = amodiaquine; LMF = lumefantrine; MFQ = mefloquine; PND = pyronaridine; ART = artemisinin; PM = plasmepsin; K13 = kelch13; Hb= haemoglobin.

1.2.3.3. 8-Aminoquinolines (Primaquine and Tafenoquine)

Primaquine is an 8-aminoquinoline antimalarial and can be used following a course of ACT due to its gametocytocidal properties to clear surviving gametocytes and thus reduce the probability of onward transmission. Furthermore, primaquine is highly effective against the liver stages of all human malaria parasites, including the hypnozoites of *P. vivax* and the *P. ovale* species [75].

Tafenoquine – a related compound – is now also being used. It has a longer half-life than primaquine and can therefore be administered as a single dose – which naturally is highly advantageous [42]. Both tafenoquine and primaquine can be used as a broad-spectrum chemoprophylactic due to its potency against the liver stages of all human malaria parasites.

Primaquine and tafenoquine are contraindicated in patients with glucose-6-phosphate dehydrogenase (G6PD) deficiency as it can cause haemolytic anaemia, which is potential fatal [75]. It is noteworthy that G6PD deficiency confers some protection against malaria and is particularly common in Africa (approximately 14% of males) and therefore a patient's G6PD status should be known prior to treatment [75–77]. They are also not recommended for pregnant women or infants [42,75].

1.2.3.4. Antifolates and sulphonamides (e.g. Sulfadoxine-Pyrimethamine)

The combination of sulfadoxine and pyrimethamine (SP) largely replaced chloroquine as the first-line malaria treatment in the mid twenty-first century, however drug resistant parasites became widespread rapidly leading to treatment failures [78]. By 2007 much of sub-Saharan Africa had moved away from SP and were implementing the use of ACTs as the first-line malaria treatment.

Sulfadoxine and pyrimethamine target folate synthesis in the blood stage malaria parasite and consequently resistance occurs due to mutations in their respective targets dihydropteroate synthase (DHPS) and dihydrofolate reductase (DHFR) [79]. Several point mutations have been shown to be associated with resistance – these include, A437G and K540E in DHPS, and N51I, C59R, S108N, and I164L in DHFR.

Despite the extensive resistance to the combination therapy, SP is still used as an intermittent preventative treatment during pregnancy (IPTp) throughout much of Africa [80]. The WHO recommends treatment in the second and third trimesters of pregnancy as a measure to reduce the risk of low infant birthweight and neonatal mortality due to malaria in pregnancy [42]. Furthermore, SP is also used, in combination with amodiaquine, as a seasonal malaria chemoprophylactic in children [81].

1.2.3.5. Atovaquone-proguanil

Atovaquone-proguanil is used as a first-line treatment for uncomplicated malaria as well as a chemoprophylactic for immunologically naïve travellers and military personnel [82]. Atovaquone is a hydroxynaphthoquinone used in combination with proguanil with which it synergises. Briefly, atovaquone disrupts the cytochrome *bc*₁ complex of the mitochondrial electron transport chain of the parasite and proguanil is converted to cycloguanil, which targets dihydrofolate reductase (DHFR) – like pyrimethamine [83,84]. Resistance results from mutations in the target of atovaquone, PfCYTb, and is specifically due to Y268S/C/N [82,85–87]. It is noteworthy that work undertaken in the

murine malaria model *Plasmodium berghei* suggests that such mutations result in a fitness cost that prevents onward transmission, thus potentially limiting the spread of the resistance allele through a population [88].

1.2.3.6. New drug classes and new combinations

Whilst countless small molecules have been identified as inhibitory to one or more of the life cycle stages of malaria parasites, relatively few have made it into clinical trials and even fewer make it into the clinic.

A potential success story is the spiroindolone compound, cipargamin – which was identified by a high-throughput compound screen – and was recently shown to rapidly clear *P. falciparum* in adults in a phase II trial in sub-Saharan Africa [89]. Cipargamin targets PfATP4 – a P-type ATPase – which induces an increase in cytosolic sodium, pH and cell swelling in blood stage *P. falciparum* [90]. Treatment failures were shown to be associated with the G358S mutation in the PfATP4-encoding gene, which suggests that drug pressure will readily select for resistance. Hence, to protect the longevity of cipargamin, it should be developed with an appropriate partner drug, rather than being deployed as a monotherapy, as was the case in the trial described [89].

Ganaplacide is an imidazolopiperazine with antimalarial activity against both the asexual blood and liver stages and has performed well in clinical trials against both falciparum and vivax malaria [91]. The drug is now being developed as a combination therapy with lumefantrine by Novartis and MMV. Novartis recently reported positive results for a completed phase 2 trial with the combination, however the data are yet to be published [92]. An ex vivo study with other human malaria parasites demonstrated that *P. knowlesi*, *P. vivax*, *P. malariae* and *P. ovale* are all more susceptible to ganaplacide than *P. falciparum* [93]. Accordingly, due to its efficacy against multiple life cycle stages and its broad-spectrum activity, ganaplacide is also being explored as a potential chemoprophylactic [94]. Whilst the mode of action of ganaplacide is unknown, it has

been shown that resistance is associated with mutations in PfCARL (*Plasmodium falciparum* cyclic amine resistance locus) [95].

Other promising compounds in clinical trials include ZY19489 and M5717 – both of which are also being trialled in combination with existing partner drugs [96–98]. There are also dozens other compounds at pre-clinical stages of development [98].

New combinations of existing drugs are also being trialled, with a notable example being artesunate with fosmidomycin and clindamycin [99]. All three compounds are potent antimalarials and the latter two are also broad-spectrum antibacterials. The combination therefore aims to treat life-threatening and severe malaria as well as concurrent bacteraemia. Other novel triple artemisinin combination therapies have also been proposed and are being trialled [100]. The rationale for this approach is by using a third drug, with a different mode of action, the longevity of artemisinins (and partner drugs) can be preserved.

Combination therapies that feature drugs with different modes of action should delay the onset of resistance. Furthermore, the rate of cure can also be improved when using drugs in combination, thus shortening the required treatment regimen and potentially increasing patient compliance – which plays a role in guarding against the emergence of resistance. Combining artemisinin derivatives with a partner drug reduces the required treatment regimen to three days (for artemisinin susceptible parasites) compared to the ten days required when used as a monotherapy.

While there is clearly an array of exciting new lead compounds, as well as new combinations of existing drugs, there is still an absolute need for further discovery and development. Resistance has been reported for all currently used drugs, as well as some of the compounds mentioned above before they have even made it out of trials. While the artemisinins are still highly effective, an overreliance on such a vital drug class would be exceedingly unwise. With time the results of such an overreliance could be

calamitous and unprecedented, with regards to the effect full-blown artemisinin resistance would undoubtedly have on global malaria burden, mortality, and morbidity.

While high-throughput phenotypic screening approaches have generated numerous lead compounds, rational drug design – which relies on prior functional characterisation of a druggable target – from the outset relies on an understanding of a particular biochemical pathway and therefore mode of action. As already discussed, understanding a drug's mode of action can be vitally important when considering drug combinations and avoidance of resistance.

Outside of malaria, rational drug design has produced many routinely used drugs including notably; the selective serotonin reuptake inhibitors – which revolutionised the management and treatment of clinical depression; imatinib – which is used to treat leukaemias; and the antiretrovirals raltegravir and enfuvirtide [101–104]. The rational targeting of biochemical pathways that are essential to the malaria parasite is therefore a sound approach to enable the development of much-needed therapeutics, but naturally requires fundamental research on said pathways.

1.3. Transporters

Membrane transport proteins, or transporters, facilitate the acquisition of essential ions and nutrients into cells, as well as facilitating the expulsion of waste and are vital to maintaining ion balances [105]. Accordingly, transporters represent a very attractive class of drug targets and have been very successfully exploited [106].

The *P. falciparum* genome encodes an estimated 144 transporters, with many being essential to the parasite at clinically relevant life cycle stages, however they remain very much understudied when compared to other organisms and therefore represent a protein class with immense potential regarding drug discovery [107–109].

As already mentioned, the ATPases; PfATP4 and PfATP6 are targets for the spiroindolones and the artemisinin derivatives respectively. Beyond those two pumps there are at least 80 essential *Plasmodium* transporters (based on them being refractory to genetic disruption in vitro and/or in vivo) that are involved in critical cellular processes and are therefore worthy of exploration as putative targets [109,110].

Transporters are integral membrane proteins and can be broadly split into two main groups – channels and carriers (Figure 1.3). Channels allow the rapid flow of ions across membranes. They are effectively aqueous tunnels that are selectively permeable to ions based on specific charge and size. Transport through a channel is controlled by a “gate” which is where changes in membrane potential, the binding of a ligand or lipid, light, temperature, or membrane tension, determine whether the channel is open or closed. [105]. Carriers facilitate the transport of solutes across a plasma membrane through binding the solute, which induces a conformational change in its structure, thus enabling the solute to traverse the membrane. Rates of transport via carriers are typically much slower than seen for channels – this is because ions can flow through channels rather than having to bind to them for transport [105]. Carriers can be further sub-classified into three groups – these are primary active, secondary active and facilitative carriers. Both primary and secondary active carriers require energy for solute transport, with this coming from the adenosine triphosphate (ATP) in the case of primary transport. Secondary active, or coupled transport, relies on the energy derived from an electrochemical gradient of one solute to drive the transport of other solutes against their own electrochemical gradients. In contrast, facilitative carriers enable transport of a solute down its concentration or, if charged, electrochemical gradient [105].

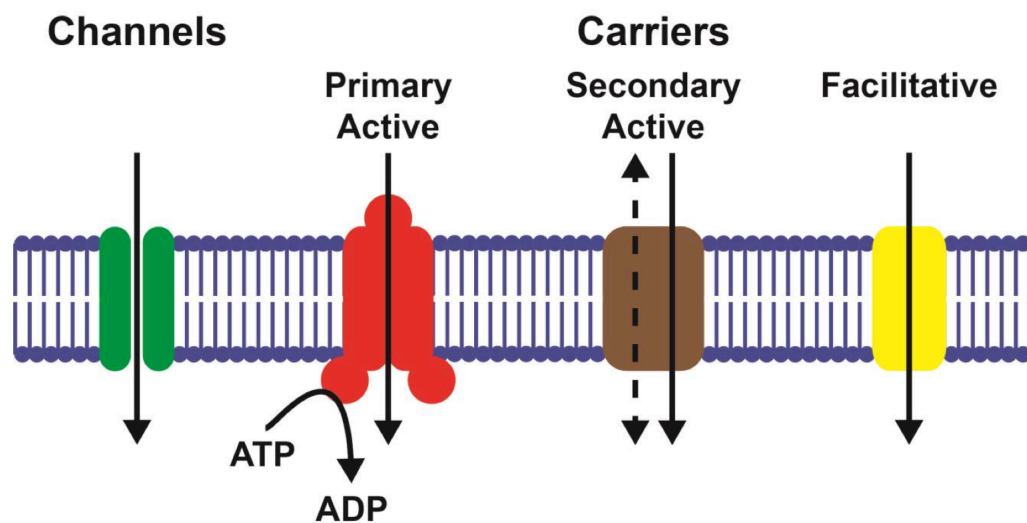


Figure 1.3. Representation of different types of transporters in a lipid bilayer [110].

Transporters can be broadly split into channels and carriers. Channels facilitating the flow of a solute is down its concentration gradient or, if charged, electrochemical gradient. Carriers facilitate ATP-driven (Primary active) or coupled (Secondary active) transport against the electrochemical gradient of the solute, or down its electrochemical gradient (Facilitative).

1.4. Transition metals

As mentioned in the previous section, transporters facilitate the acquisition of a vast array of essential nutrients and ions to cells. Transition metals, such as iron, copper, and zinc, are essential trace elements that feature in enzymatic reactions that are performed right across the life cycle of malaria parasites and indeed in all other organisms [111].

Of the nine putative transition metal transporters predicted to be encoded by *P. falciparum* all but one lacks any functional annotation [109,112]. As the presumed facilitators of transition metal acquisition and detoxification in *Plasmodium*, these transporters – especially those which have been shown to be essential – are appealing drug targets.

Accordingly, this thesis characterises a subset of under characterised transporters and explores their potential roles in the homeostasis of various metals. The remaining sections of this chapter review what is currently understood about transition metal homeostasis and the transporters involved.

1.4.1. Iron

Iron is essential to all life as it plays roles in countless biological processes [113]. These include DNA repair, glycolysis, and electron transfer. Furthermore, iron-containing proteins are present across all life, with notable examples being, haemoglobin, myoglobin, ferritin, cytochrome and rubredoxin.

To complete their multistage life cycles and thrive, malaria parasites must be able to access, store, and detoxify iron. During the malaria blood stage, the parasite, in theory, has access to an almost limitless supply of haem-bound iron from the haemoglobin of the parasitised RBC. Inside the RBC, *P. falciparum* utilises the peptide component of haemoglobin, whilst crystallising toxic haem into haemozoin. It is unclear whether the parasite can access haem-bound iron. In mammalian cells, haem is catabolised into bilirubin and carbon monoxide by haem oxygenase 1 [114]. It was long thought that malaria parasites did not encode an orthologous protein, however a haem oxygenase-like (HO-like) protein (PF3D7_1011900) was identified, and some data were published that suggested that it was capable of degrading haem into biliverdin (a bilirubin precursor) when expressed in a heterologous system [115]. Later work however could not detect biliverdin or bilirubin production by blood stage *P. falciparum*, nor could they demonstrate that the HO-like protein catalysed haem degradation [116]. This led the authors to conclude that *P. falciparum* likely lacks a canonical haem degradation pathway and instead relies on other pathways for haem detoxification and iron acquisition.

In most organisms, iron is acquired by a cell through the transport of either soluble ferrous (Fe^{2+}), or protein bound iron, or by the receptor-mediated endocytosis of a ferroprotein. These systems have been well-characterised in the budding yeast, *Saccharomyces cerevisiae*, wherein extracellular ferric iron (Fe^{3+}) is reduced by the plasma membrane metalloreductases, Fre1 and Fre2, and Fe^{2+} is imported by the carriers Fet3 and Fet4 [117]. Likewise, human enterocytes acquire dietary iron from the intestinal lumen via transport facilitated by the divalent metal transporter – DMT1, ferroportin is then responsible for the export of iron from the enterocyte into the bloodstream [118,119].

Plasmodium must acquire extracellular iron for growth during its blood stage, as is evidenced by its sensitivity to extracellular iron chelators [120]. Early work indicated that during the blood stage, *P. falciparum* acquires iron from serum transferrin [121]. Furthermore, it was suggested that to facilitate this, the malaria parasite expresses and exports its own transferrin receptor to the plasma membrane of the infected RBC, as host endogenous transferrin receptors are lost early in the maturation of RBCs [122]. Importantly however, subsequent conflicting data suggested that there is not a significant uptake of transferrin bound iron, by the *Plasmodium*-infected RBC, but rather unbound iron uptake is detectable, thus suggesting the involvement of a parasite, or host-derived cation transporter [123,124]. In addition, the ability to detect the parasite-derived, host cell surface localised transferrin receptor has also been called into question [125]. With everything considered and the dearth of published data in this area since the early 1990s, it seems overwhelmingly unlikely that transferrin uptake by the parasitised RBC represents the main strategy of iron acquisition by the blood stage malaria parasite.

Of the aforementioned putative transition metal transporters, it has been suggested that PfDMT (PF3D7_0523800) could facilitate the import of ferrous iron into the infected RBCs. PfDMT is an orthologue of the human iron carrier DMT1, which was mentioned previously. PfDMT is essential to the *P. falciparum* blood stages and appears to localise

to the parasite plasma membrane [126]. Functional characterisation of PfDMT is yet to be undertaken.

Considerably less work has been done to uncover iron acquisition mechanisms in the malaria liver stage and almost nothing in the mosquito stages. The *Plasmodium* liver stage does require iron to establish an infection, as exemplified by the effect of hepcidin-mediated liver hypoferremia on *P. berghei* [127]. Portugal et al. (2011) have shown that an active blood stage infection with *P. berghei* prevents superinfection with a mechanism controlled by the host iron modulator hepcidin. Hepcidin inhibits the host iron exporter ferroportin. During infection hepcidin expression is upregulated, thus causing iron to be sequestered in enterocytes and macrophages and importantly hepatocytes to be depleted of iron – with the latter representing a suboptimal growth environment for invading malaria parasites. It was demonstrated that when blood parasitaemia exceeded a minimal threshold (1%), sporozoites that were subsequently infected were arrested at the liver stage. This threshold correlates with host hepcidin production, which is stimulated by blood stage *P. falciparum* infection in a parasite density-dependent manner. Furthermore, it was demonstrated that administering physiologically relevant doses of hepcidin to mice significantly reduced *P. berghei* liver infection in the absence of a blood stage infection. Comparable results were seen when *P. berghei* was used to infect hepcidin-overexpressing transgenic mice.

Intriguingly, the authors suggest that their superinfection model can go some way in explaining the apparent low levels of infection complexity seen in African infants in areas of high transmission [127,128]. Low levels of infection complexity are generally accompanied by a higher parasite burden, which then gradually decrease with age and exposure. Accordingly, the high parasite burden seen in younger patients could reduce superinfection and thus infection complexity in a process controlled by hepcidin. Furthermore, one might speculate that the described hepcidin-mediated hepatic hypoferraemia is less relevant to the non-falciparum malaria parasites of humans, as

these other parasites – which typically do not reach anywhere near the same levels of blood parasitaemia as *P. falciparum* – may not reach a minimum threshold of parasite density required for the modulation of superinfection. Although it is noteworthy that increased levels of hepcidin have been reported in patients with vivax malaria, in addition to those infected with *P. falciparum* [129].

A ZIP-domain protein, ZIPCO (PF3D7_1022300), is highly expressed in the liver stage of *Plasmodium berghei* [130]. ZIP-domain protein orthologues transport various divalent cations, including iron, in other organisms [131]. ZIPCO was shown to localise to the plasma membrane of the parasite following hepatocyte invasion. A ZIPCO knock-out *P. berghei* line was shown to have a growth phenotype akin to the wild type in the blood and mosquito life cycle stages, but growth in the parasitised hepatocyte was significantly impaired. Supplementation with iron provided some rescue however, suggesting that the liver stage parasite must acquire iron by at least one other yet-to-be-revealed mechanism. Malaria parasites do also express another transporter from the ZIP family (PF3D7_0609100), however there are no published data on its function. Likewise, PfDMT may also play a role in iron acquisition by the liver stage, but this remains to be studied.

Despite being essential, iron can also be toxic to the malaria parasite, through facilitating the production of reactive oxygen species via Fenton-type chemistry. Such species are destructive to nucleic acids, lipids and proteins. It is therefore essential that surplus iron is detoxified through appropriate efflux and storage strategies.

Most, but not all, eukaryotes have evolved to regulate intracellular iron concentration with storage proteins, such as ferritin [132]. Malaria parasites have no such storage protein. Another notable exception is the yeast *Saccharomyces cerevisiae*, which utilises a digestive vacuole to sequester iron. The export of cytosolic iron – into the vacuole – is facilitated by the vacuolar iron transporter (VIT) [133].

Plasmodium expresses a VIT orthologue (PF3D7_1223700) which, although not essential to survival of *P. berghei*, was shown to be important for iron detoxification in both the blood and liver life cycle stages [134]. Cytosolic labile iron was significantly elevated in a PbVIT knock-out parasite line compared to the wild type, thus indicating that PbVIT – like its yeast orthologue – almost certainly transports iron into a storage organelle. Furthermore, heterologous expression of the *P. falciparum* orthologue PfVIT showed transport of Fe^{2+} , which was selective over other divalent cations. It was further shown, using a bacterial expression system, that PfVIT functions as an Fe^{2+} - proton exchanger [135].

The chloroquine resistance transporter, PfCRT (PF3D7_0709000) has also been implicated in iron transport. Proton-driven transport of both ferrous and ferric iron facilitated by PfCRT was shown in the *Xenopus laevis* oocyte heterologous expression system [136]. In addition to the antimalarial drug chloroquine, PfCRT has also been shown to transport short peptides from the lumen of the digestive vacuole of blood stage *P. falciparum* to the cytosol. As previously mentioned, the digestive vacuole is the site of haemoglobin breakdown. Shafik et al. show that the peptides transported by PfCRT are haemoglobin derived and thus PfCRT presumably expedites their redistribution throughout the parasite [137]. Despite ruling out the possibility that PfCRT transports numerous other potential substrates, the authors did not rule out iron transport. It remains entirely possible that, in addition to PfVIT, PfCRT also plays a role in iron detoxification by enabling it to be sequestered in the digestive vacuole, and thus, one might speculate that the apparent inessentiality of PfVIT could be attributed to mechanistic redundancy. Furthermore, the unessential vacuolar zinc transporter PfCDF (PF3D7_0715900) may also play a role in iron detoxification in *Plasmodium*, as its orthologues do in other organisms [138].

The disruption of iron homeostasis has long been discussed as an attractive potential method of malaria control [139]. Various iron chelators, such as deferoxamine and

deferiprone, have been shown to eliminate blood and liver stage malaria parasites in vitro and in vivo and have been explored as potential chemotherapeutics [120,140–143]. However, issues with toxicity and pharmacokinetics have largely prevented their use. Notably, FBS0701 – a chelator being trialled in humans for the treatment of transfusional iron overload – has been shown to inhibit the asexual and sexual blood stages of *P. falciparum* in vitro and *P. yoelii* in mice [144,145].

1.4.2. Copper

Copper, like iron, is an essential element to all organisms, as it is required for several enzymatic processes such as the respiratory electron transport chain – where it is a cofactor for the enzyme, cytochrome c oxidase [146,147]. Additionally, it is also a cofactor of superoxide dismutase (SOD). Free ionic copper, however, is highly toxic and accordingly is mostly bound to proteins such as SOD. In its reduced state, Cu^+ , copper can catalyse the formation of destructive reactive oxygen species (ROS) via Fenton-like chemistry [148].

The human RBC features a copper concentration of 20 μM , however at least 70% is bound to SOD [149]. Cu/Zn SOD is endocytosed, with haemoglobin, from the RBC cytosol during the malaria blood stage – representing a potential mechanism for copper acquisition. *P. falciparum* does not encode its own Cu/Zn SOD homologue and perhaps utilises that of its host to convert destructive oxygen free radicals into hydrogen peroxide and water. Interestingly, both copper and SOD are reduced in the infected versus uninfected RBC, which suggests that Cu/Zn SOD is very likely degraded in the digestive vacuole and that there must be a copper efflux mechanism [149].

In yeast, copper uptake is facilitated by the high affinity copper transporters Ctr1 and Ctr3 [150,151]. The expression of these transporters is upregulated in copper-limiting conditions and is controlled by the transcription factor MAC1 [152]. Likewise, copper

uptake in mammalian cells is mediated by a Ctr1 orthologue – commonly referred to as hCTR1 in humans [153].

P. falciparum encodes two Ctr orthologues, PfCTR1 and PfCTR2. It has been suggested that copper uptake could be mediated by one, or both, of these transporters. PfCTR1 has been shown to bind Cu^+ in vitro and when heterologously expressed in *E. coli*, although its ability to transport copper was not examined [154]. It has been shown that PfCTR1 localises to the erythrocyte membrane during the early ring stage of the life cycle and then within the parasite in the later blood stages. PfCTR1 has been shown to be essential to the *P. falciparum* blood stages, however this is not the case for *P. berghei* where PbCTR1 is essential for transmission from mosquito vector to mouse, but not for the asexual or sexual blood stages [112]. This is also the case for PbCTR2, although its *P. falciparum* orthologue appears dispensable to the blood stages – otherwise PfCTR2 remains virtually unstudied. Intriguingly, orthologues of PfCTR1 and 2 are expressed in the hypnozoites of *P. cynomolgi* – a close relative of *P. vivax* that infects Old World monkeys [155]. This finding not only suggests that the two putative copper transporters play a role in the *Plasmodium* liver stages, but also implicates them as attractive radical cure targets for vivax and ovale malaria.

It has been shown that the P-type ATPase, PfCuTP (PlasmoDB), binds copper but not zinc or iron. Furthermore, it only binds copper in the presence of ascorbate, which suggests that it selectively binds Cu (I) over Cu (II). Rasoloson et al. demonstrate that during the *P. falciparum* blood stage, PfCuP-ATPase localises to the erythrocyte membrane, as well as within the parasite itself, which could implicate PfCuP-ATPase as a mediator of copper efflux [149]. Moreover, Kenthirapalan et al. showed that the *P. berghei* orthologue of the PfCuTP localised to intracellular vesicles – which they speculate could be part of the trans-Golgi network [112]. In addition, they showed that the orthologue in the related parasite *Toxoplasma gondii* localised in a similar manner.

The authors note that the vesicular localisation they show suggests that PfCuTP could play a role in copper storage and redistribution, rather than export.

It is noteworthy that PbCuTP is not essential to the parasite, however its absence did result in impaired activation and exflagellation of male gametes, as well as impairing the fertility of female gametes, thus suggesting that the ATPase is key for sexual development [112]. Furthermore, it was shown that treating gametocytes with the neocuproine emulated the knockout phenotype, further suggesting a key role for copper in malaria parasite sexual development. Interestingly however, exogenous treatment with copper did not rescue the KO phenotype or those treated with neocuproine.

1.4.3. Zinc

Zinc is an integral trace metal to many cellular processes, where it features – as a cofactor – in all classes of enzymes [156]. In the malaria parasite, zinc unsurprisingly features in countless proteins – many of which are required for life cycle progression and virulence [107,112]. Zinc is essential to *Plasmodium*, as evidenced by inhibition of the parasite by the zinc chelator, TPEN, and the fact that this inhibition can be overcome by concurrent treatment with an equivalent concentration of zinc [157]. It has been demonstrated that there is a rapid accumulation of labile zinc by *P. falciparum* in the blood stage – specifically prior to rupture. It was shown that the acquired zinc accumulates in both the cytosol and mitochondria of the parasite, but not the nucleus. Interestingly, the rapid increase in zinc concentration coincides with high levels of DNA replication, late in the 48-hour erythrocytic cycle.

The mechanism by which zinc uptake is achieved by the malaria parasite – at any life stage – is unclear, however it could be mediated by one (or more) of three putative zinc transporters encoded by *P. falciparum*. These are the CDF homologue, PfCDF, and the two ZIP homologues, PfZIPCO and PfZIP1.

In yeast, zinc uptake is mediated by the two ZIP homologues, Zrt1 and Zrt2 [158,159]. These transporters are transcriptionally regulated to respond to changes in cytosolic zinc concentration, whereby the expression of Zrt1 – a high affinity zinc transporter – is upregulated in response to reduced zinc concentration. ZIPs also enable zinc uptake in numerous other organisms [160].

Conversely, the CDFs Zrc1 and Cot1 act to sequester zinc (and cobalt) into the vacuole of the yeast, thereby providing a mechanism for zinc tolerance and thus allowing zinc to be stored [161,162]. A third ZIP transporter, Zrt3, facilitates the import of stored zinc from the vacuole to the cytosol of the yeast [163]. CDFs also play similar roles in preventing metal toxicity in other organisms [164,165].

As mentioned in the section on iron, disruption of PbZIPCO had no effect on the *P. berghei* blood or mosquito stages but did affect the ability of the parasite to develop in the liver [130]. The mutant phenotype observed was reversed when parasites were supplemented with zinc, suggesting that ZIPCO might play a role in zinc acquisition during the liver stages of the life cycle. This conclusion is further supported by ZIPCO appearing to localise to the parasite plasma membrane during the liver stages, whereby it could mediate zinc (and iron) uptake from the hepatocyte.

Disruption of PbZIP1 resulted in an impaired blood stage phenotype and completely arrested onward development through the mosquito vector. Its essentiality in the liver stages remains unclear. There is currently no data on the potential function of PbZIP1 (or its *P. falciparum* orthologue). Speculation on zinc (or iron) transport function is informed solely by its homology to ZIPs in better studied organisms.

CDFs in other organisms transport a range of divalent metal cations such as, zinc, iron, manganese, cobalt, and nickel [138]. Typically, they act to prevent metal toxicity by transporting metals out of the cell, or from the cytosol into an organelle – such as a vacuole. Like *P. falciparum*, *T. gondii* encodes a single CDF which has been shown to

confer tolerance to zinc toxicity by sequestering zinc from the cytosol into a vacuole [166]. PfCDF could therefore play a similar role to TgCDF. CDF appears dispensable to the blood stages of both *P. berghei* and *P. falciparum*, but disruption of the encoding gene reduces viability in the sexual and insect life cycle stages of *P. berghei* [112]. PfCDF has been shown to localise within the parasite cytosol during the early blood stages and to multiple punctate foci within the cytosol during the schizont stage [167]. One might speculate that this localisation lends credence to the idea that PfCDF plays a role in preventing zinc toxicity, as it does in *T. gondii*, however this needs to be explored experimentally. This is also true for speculating on the role of PfCDF in the other life stages.

1.5. Aims

The work presented in this thesis aimed to characterise several of the putative transition metal transporters introduced in the above sections. These include the putative copper transporters PfCTR1 and 2, the putative zinc transporter, PfCDF and PfCRT – which were all heterologously expressed in yeast to study their effect on the growth phenotypes of metal-sensitive yeast strains, when cultured in differing concentrations of iron, copper, zinc or cobalt. Furthermore, reverse genetics approaches were undertaken which aimed to generate parasite lines that featured mutated putative transporter genes, to enable the study of protein essentiality, substrate specificity and sub-cellular localisation. Finally, the effects of iron on artemisinin action (current frontline antimalarial drug) were also explored.

2. Materials & Methods

All reagents used in this work were purchased from Sigma Aldrich unless stated otherwise.

2.1. Molecular biology

The plasmids used in work described in this thesis are detailed in Table 2.1 and are broadly split into two categories; those that were used to transform yeast and those that were used to transfect blood stage *Plasmodium falciparum* parasites. Several of the plasmids described therein were synthesised by Genscript. These plasmids were reconstituted in molecular biology grade water before initially being propagated in various competent *Escherichia coli* strains (Table 2.2).

Table 2.1. Plasmids

Name	Organism	Description	Selection	Size (bp)
p413GPD	Yeast	Yeast expression vector (empty)	Histidine	5876
p413GPD-hCTR1	Yeast	Human copper transporter	Histidine	6443
p413GPD-PfCTR1	Yeast	Several versions each expressing yeast codon optimised PfCTR1 variants $\pm$ signal peptide and <i>Plasmodium</i> -human chimeras	Histidine	6580 (variable)
p413GPD-PfCTR2	Yeast	Several versions each expressing yeast codon optimised PfCTR2 variants - $\pm$ signal peptide and <i>Plasmodium</i> -human chimeras	Histidine	6355 (variable)

p413GPD-PfCTR1-eGFP	Yeast	PfCTR1 featuring a C-terminal eGFP tag	Histidine	7309
pUGPD	Yeast	Yeast expression vector (empty)	Uracil	5528
pUGPD-AtIRT1	Yeast	Iron transporter from <i>Arabidopsis thaliana</i>	Uracil	6546
pUGPD-PfCRT	Yeast	Yeast codon optimised Chloroquine resistance transporter (PfCRT)	Uracil	6803
pUGPD-PfCRT-eGFP	Yeast	PfCRT featuring a C-terminal eGFP tag	Uracil	7532
pYES	Yeast	Inducible yeast expression vector	Uracil	5787
pYES-PfCRT	Yeast	Inducible expression of two versions of PfCRT - HB3 & Dd2	Uracil	7207 (variable)
pYES-CCC1	Yeast	Yeast vacuolar iron transporter CCC1	Uracil	6993
p415GPD	Yeast	Yeast expression vector (empty)	Leucine	8646
p415GPD-Zrc1	Yeast	Yeast vacuolar zinc/cobalt transporter	Leucine	9975
p415GPD-PfCDF	Yeast	Yeast codon optimised PfCDF	Leucine	10317

p415GPD-PfCDF-eGFP	Yeast	PfCDF featuring a C-terminal eGFP tag	Leucine	11014
pDC2-GFP	<i>P. f.</i>	Episomal GFP expression	Blasticidin-S	7862
pUF1-Cas9	<i>P. f.</i>	Episomal Cas9 and gRNA expression	DSM-1	11096
pL6-PfDMT	<i>P. f.</i>	Donor plasmid for CRISPR-Cas9 - 3x versions -	WR99210	7685
pARL-FIKK.cKo	<i>P. f.</i>	Donor plasmid used to construct pARL-PfDMT	WR99210	8616
pARL-PfDMT	<i>P. f.</i>	Donor plasmid to modify PfDMT locus by selection-linked integration for conditional mutagenesis	WR99210	9707
pSLI-CDF-mNeonGreen	<i>P. f.</i>	Donor plasmid to introduce C-terminal fluorescent tag to PfCDF by selection-linked integration	WR99210	7241

2.1.1. Transformation of *E. coli*

All plasmids used for yeast transformations were propagated in DH5 α competent *E. coli* cells. The bacteria were transformed with 5 ng of plasmid by heat-shocking at 42°C for 30 s after 25 min on ice. This was followed by recovery in SOC outgrowth media (New England Biolabs (NEB)) for 1 h at 37°C, shaking at 250 rpm. The bacteria were then pelleted by centrifugation at 13,000 x g, resuspended in Luria-Bertani (LB) broth and

inoculated onto LB-agar plates containing 100 μ M ampicillin (LB-amp). The plates were then incubated at 37°C for 14-16 h (stationary phase of growth). Single colonies were then picked and inoculated in LB-amp broth and cultured for a subsequent 12-16 h, shaking at 250 rpm. Stocks were made from these cultures by adding 50% (v/v) glycerol to the culture in a ratio of 70:30 – culture to glycerol. The glycerol stocks were then stored at -80°C. The plasmids pDC2-GFP, pUF1-Cas9 and pARL-FIKK.cKo were transformed into XL10-Gold following the same procedure described in the paragraph above. The three variants of both pL6-PfDMT and pARL-PfDMT, as well as the two pSLI-CDF variants were transformed into Stellar cells by heat-shocking at 42°C for 45 s – otherwise the procedure was the same as for the other two bacterial strains used.

Table 2.2. *Escherichia coli* strains

Strain	Supplier	Genotype
DH5α	NEB	<i>fhuA2Δ(argF-lacZ)U169 phoA glnV44 Φ80Δ(lacZ)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17</i>
XL10-Gold	Agilent	TetrD(mcrA)183 D(mcrCB-hsdSMR-mrr)173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac Hte
Stellar™	Takara	F-, endA1, supE44, thi-1, recA1, relA1, gyrA96, phoA, Φ 80d lacZ Δ M15, Δ (lacZYA-argF) U169, Δ (mrr-hsdRMS-mcrBC), Δ mcrA, λ -

2.1.2. Plasmid preparations

Depending on the required yield of a given plasmid, transformed *E. coli* were either cultured in 1 - 5 mL of LB-amp broth and plasmid was extracted by miniprep (NEB), or in 150-250 mL and extracted by maxiprep (Qiagen) for low (5-10 μ g) and high yields (250-

1000 µg), respectively. Minipreps were performed exactly according to the instructions provided by the kit manufacturer. Final elutions were undertaken using the elution buffers provided. Maxipreps were used to prepare sufficient plasmid DNA for multiple *P. falciparum* transfections. The instructions provided by the kit manufacturer were followed exactly apart from final elution that was undertaken into cytomix (KCl, 120 mM; CaCl₂, 0.15 mM; KH₂PO₄, 10 mM; HEPES 25 mM; EGTA 2 mM; MgCl₂, 5 mM; pH 7.6), as the transfection protocol requires the DNA to be resuspended in this buffer. The yield and purity of plasmid DNA was determined using a Nanodrop spectrophotometer (ThermoFisher).

2.1.3. Restriction digest

Plasmid DNA was digested using a variety of restriction enzymes. All enzymes used were manufactured by NEB. For each digest reaction, one unit of each enzyme was used to digest 1 µg of plasmid. One of three reaction buffers (NEB) was used for each reaction – 2.1, 3.1 or CutSmart – based on the recommendations for each enzyme. When performing double digests, if it was not possible to choose a buffer with optimal cutting efficiency for both enzymes, sequential digests were performed. The reactions were incubated at 37°C for 1 h, followed by 20 min at 65°C, if recommended for the enzyme being used. The products of a restriction digest were separated by electrophoresis on a 1% (w/v) agarose gel made in TAE buffer (Tris-acetate, 40 mM; EDTA, 1 mM; pH 8.3) treated with ethidium bromide (500 ng/mL) and visualised under ultraviolet light. Band sizes were estimated using the GeneRuler 1kB DNA ladder (ThermoFisher). If required, individual bands were excised from the gel and purified using the Monarch Gel Extraction kit (NEB) – following the manufacturer's instructions exactly.

2.1.4. Polymerase chain reaction

Polymerase chain reaction (PCR) was used to confirm successful transformations and transfections of yeast and parasites, respectively, as well as to amplify sections of DNA for cloning. Three different PCR protocols were followed depending on which polymerase was used and then variations therein depending on the annealing temperatures of the primers (Table 2.3) and the extension time required to produce the expected amplicon.

PCR reactions (20 μ L) using the Phusion™ High-Fidelity DNA Polymerase (ThermoFisher) were prepared that featured: 0.02 units/ μ L of polymerase; 0.5 μ M of each primer; deoxynucleotide triphosphates (dNTPs) 200 μ M each; Phusion™ HF Buffer at 1x concentration; and 1-20 ng of template DNA. The reaction conditions were as follows; initial denaturation at 98°C for 30 s, followed by 35 cycles of denaturation at 98°C for 10 s; annealing at 50-60°C for 30 s; extension at 72°C for approximately 30 s per 1 kb of expected amplicon and then (after 35 cycles) a final extension at 72°C for 10 min.

When using OneTaq (NEB), 50 μ L reactions were prepared that featured: 0.025 units/ μ L of polymerase; 0.2 μ M of each primer; deoxynucleotide triphosphates (dNTPs) 200 μ M each; OneTaq standard reaction buffer at 1x concentration; and 1-20 ng of template DNA. The reaction conditions were as follows; initial denaturation at 94°C for 30 s, followed by 35 cycles of denaturation at 94°C for 15 s; annealing at 50-60°C for 30 s; extension at 68°C for approximately 60 s per 1 kb of expected amplicon and then (after 35 cycles) a final extension at 68°C for 10 min.

Hot Start Taq DNA polymerase (NEB) was used with the *Mycoplasma* Detection Kit (Southern Biotech), which was used to routinely screen *P. falciparum* cultures for *Mycoplasma* contamination. Fifty microlitre reactions were set up that featured 0.04

units/ μ L of *Taq* and the volumes of buffer, master mix and water specified in the manufacturer's instructions.

A variety of PCR primers were used and are detailed in Table 2.3. Invariably, PCR products were separated by electrophoresis on a 1% (w/v) (in TAE) agarose gel treated with ethidium bromide (500 ng/mL) – as described for restriction digests.

Table 2.3. Primers

#	Name	Sequence (5'-3')	Length (Nt)	T _m (°C)
1	p413 F	GATGCAACCTGCCTGGAGTA	20	57
2	p413 R	AGCGTCCCAAACCTTCTCA	20	57
3	PfCTR 1 gr F	GCGTGACATAACTAATTACATGACTCG AGGAAAAATGAAATCTGATAACTCAAT CTG	57	61
4	PfCTR 1 gr R	CACCAGAACTTAGTTTCGACGGATTCT AGAGGATCCTTAACAACCACAAC	50	58
5	hCTR1 gr F	GCGTGACATAACTAATTACATGACTCG AGGAAAAATGAAATCTGATAACTCAAT CTG	57	61
6	hCTR1 gr R	CACCAGAACTTAGTTTCGACGGATTCT AGAGGATCCTTAACAACCACAAC	50	58
7	p413G PD gr F	GGAGCGGGCGCTAGGGCGGCGCGCA GATCTTCACGCTGCGCGTAAC	46	64

8	p413G PD gr R	ACACCAGAACTTAGTTTCGACGGATGT CGACCTCGAGTCATGTAATTA	48	62
9	pUGP D F	TGACCCACGCATGTATCTATCTC	23	58
10	pUGP D R	GCGCCATTCGCCATTCAG	18	56
11	PfCRT- GFP gr F	GAATTGACCAACGTAGATTCCATCATC ACTCAAGCCATGGTTTCTGTTATTAAAG	55	59
12	PfCRT- GFP gr R	CTATAGGGCGAATTGGAGCTCCACCG CGGTGTTATTTAGAAGCTTGTGATGGC AA	55	61
13	pYES F	GGATCGGACTACTAGCAGCTG	21	62
14	pYES R	GCGTCCCAAAACCTTCTC	18	62
15	CCC1 gr F	CTATAGGGAATATTAAGCTTGGTACCA GTCGACAAAAATGTCCATTGTAGCACT AAAGAAC	61	61
16	CCC1 gr R	TAAGCGTGACATAACTAATTACATGAT GCTTAACCCAGTAACTTAACAAAGAAC	54	59

17	p415 F	AGCTGGAGCTCTCGAGTTTATC	22	58
18	p415 R	GCAACTGACTGAAATGCCTC	20	58
19	PfCDF gr F	CACCAGAACTTAGTTTCGACGGATTCT AGATAAAAATGCCAACTGAAATG	50	60
20	PfCDF gr R	TGTAAAGCCTGGGGTGCCTAATGCGG CCGCGGATTTATTTAGAAGCTTGTGAT GGC	56	61
21	PfCDF- GFP F	CACCAGAACTTAGTTTCGACGGATTCT AGAAAAAATGGTTGATAAAATGTCAA GATTG	59	61
22	PfCDF- GFP R	TGTAAAGCCTGGGGTGCCTAATGCGG CCGCTTATGTGTCACCCTTCAAAGTAG AAC	56	60
23	18S F	AAATTGTTGCAGTTAAAACG	20	58
24	18S R	AAAAAGCAAAAGCAAAAGTG	20	58
25	PfCDF F1	TGGTGGATAAGATGTCGCGT	20	58
26	PfCDF F2	ATGCCACCGAAATGAACTC	20	58
27	PfCDF R1	GTGCACCTATAATTTAGCCCT	22	59

28	PfCDF R2	GCACCTATAATTTTCAGCCCTATG	23	60
29	pSLI F1	TGGTTGGACAAGGAACAGGA	20	61
30	pSLI F2	GCAAAACCAATGGCAGCAAA	20	62
31	pSLI R1	CATCAGAGCAGCCGATTGTC	20	61
32	pSLI R2	CTGTTGTGCCCAGTCATAGC	20	61
33	CDF int F1	TCCAAAATGCCCACCGAAAT	20	63
34	CDF int F2	AAATGCCCACCGAAATGAAC	20	63
35	CDF int R1	CATCAGAGCAGCCGATTGTC	20	62
36	CDF int R2	CTGTTGTGCCCAGTCATAGC	20	63
37	pDC2 F	TGGTCTGCTAGTTGAACGCT	20	59
38	pDC2 R	ACGGGAACCTACAAGACACGT	20	59

39	pARL F1	GTTGTCACTGAAGCGGGAAG	20	64
40	pARL F2	TTGGGTGGAGAGGCTATTCG	20	63
41	pARL R1	GAATCCAGAAAAGCGGCCAT	20	62
42	pARL R2	CGGCCATTTTCCACCATGAT	20	62
43	Frag 1 F	CTAACGTAACAGACTTAGGAGGAGATC TGAAAAGGATTTTACTGAGAG	48	55
44	Frag 1 R	CTCTTCCTTCTCCGTCGACGACCGCAT AATCCGGTACATCGTATG	45	59
45	Frag 2 F	CATACGATGTACCGGATTATGCGGTCG TCGACGGAGAAGGAAGAG	45	56
46	Frag 2 R	CTAAAAGAATATAAAATATATAAATATA TATATATATAC	39	50
47	Frag 3 F	GTATATATATATATATTTATATATTTTAT ATTCTTTTAG	39	52
48	Frag 3 R	GAAAAACGAACATTAAGCTGCCATATC	27	55

2.1.5. DNA Cloning

All the DNA cloning described in this work was either undertaken by NEBuilder cloning, yeast gap-repair cloning or restriction digest and ligation. Additionally, some of the constructs used were synthesised and cloned into vectors by Genscript – where this was done, it is explicitly stated.

Six of the yeast expression vectors used were assembled by yeast gap-repair cloning [168]. The required linearised vector backbone was unvaryingly retrieved by restriction digesting the corresponding circular vector with two different enzymes, thus releasing the existing insert. The new target insert was amplified by gap-repair PCR from a plasmid template, using primers that were roughly 50 nucleotides in length. For each primer, roughly twenty nucleotides shared homology with the target sequence and 30 nucleotides shared homology with the vector sequence located immediately either side of the cut sites. The PCR products were then separated on an agarose gel by electrophoresis, excised from the gel and purified from the gel using the NEB kit – as described previously. If required – based on purity of DNA – the insert was then further purified using the Monarch PCR & DNA Cleanup Kit (NEB). Two hundred nanograms of purified grPCR products and restriction digest products were then co-transformed into yeast (see 2.2.1). In theory, for the transformants to successfully grow on selective media, they would require the successful recombination of linearised vector and insert. Individual colonies were screened by PCR – as previously described – to establish if cloning had been successful.

The three versions of pARL-PfDMT were produced by NEBuilder cloning, using the HiFi DNA Assembly kit (NEB). Three DNA fragments were amplified by PCR and cloned into the pARL-FIKK.cKo backbone. Fragment 1 featured the N-terminal coding region of PfDMT and an artificial intron featuring a LoxP Cre recombinase cut site, followed by a haemagglutinin (HA) tag and was amplified from a DNA construct synthesised by Genscript using primers 43 and 44 (Table 2.3). Fragment 2 featured the sequence for

the viral self-cleaving peptide, T2A, followed by a G418-resistance cassette and was amplified from pARL-FIKK.cKo using primers 45 and 46 (Table 2.3). Three versions of fragment 3 featured further PfDMT coding sequence (resuming from fragment 1), each containing one of three mutations, S433S (silent), S433M, S433Stop, followed by a HA tag. These were amplified from constructs synthesised by Genscript using primers 47 and 48. The cloned plasmids were sequenced by Genscript to confirm successful cloning.

To generate the pYES vector, pYES-PfCRT was linearised using *Sall* and *XhoI*, thus giving rise to complementary overhangs. The overhangs were then religated using T4 ligase (NEB) – following the manufacturer’s instructions – thus re-circularising the vector minus the PfCRT insert.

2.2. Yeast culture

Numerous yeast strains were used for work described in this thesis and these are detailed in Table 2.4. Yeast strains were retrieved from glycerol stocks and initially inoculated onto SD-agar and cultured for 48-72 h at 30°C. Auxotrophic selection of individual yeast strains was achieved through the omission of one or more of three essential amino acids from the media - leucine, tryptophan, histidine – or uracil (a nucleoside).

Table 2.4. Yeast strains

Strain	Description	Disruptions	Supplier
Δctr1ctr3	Sensitive to copper-limited conditions due to disruption of plasma membrane copper transporters Ctr1 and Ctr3	ctr1::URA3::KanR ctr3::TRP1	Dennis J. Thiele - Duke University
ΔCCC1	Sensitive to iron toxicity due to disruption of vacuolar iron transporter CCC1	ccc1::TRP1	Jerry Kaplan - University of Utah
Δfet3fet4	Sensitive to iron-limited conditions due to the disruption of plasma membrane iron transporters fet3 and fet4	fet3::HIS3 fet4::LEU2	David Eide - University of Wisconsin- Madison
Δzrc1cot1	Sensitive to zinc and cobalt-replete conditions due to disruption of vacuolar zinc/cobalt transporter Zrc1 and Cot1	zrc1::HIS3 cot1::URA3	Jerry Kaplan - University of Utah

2.2.1. Yeast transformation

A day prior to transformation, single yeast colonies were picked from synthetic defined (SD) agar (Yeast Nitrogen Base, 0.67% (w/v); Yeast Synthetic Drop-out medium (- His, Leu, Trp and Ura), 0.14% (w/v); Agar, 2% (w/v); D-glucose, 2% (w/v)) plates, on which yeast from a glycerol stock had been cultured, inoculated into 5 mL YPD (yeast extract,

1% (w/v); peptone, 2% (w/v); D-glucose, 2% (w/v)), and cultured for 20-24 h (depending on strain) at 30°C in a shaking incubator at 200 rpm. For each transformation, 1 mL of YPD culture was pelleted by centrifugation at 8,000 x g. The pellet was then washed with distilled water and resuspended in lithium acetate (LiAc) buffer (LiAc, 100 mM; Tris-HCl, 10 mM; EDTA, 1 mM; pH 7.5). One to two hundred nanograms of plasmid (typically 0.5 – 1 µL in TE) was added to a transformation cytomix mix (LiAc, 100 mM; PEG 3350 33% (w/v); salmon sperm DNA, 1.4 mg/mL – denatured at 95°C immediately prior to use) on ice, in which the yeast were resuspended and heat shocked at 42°C for 1 h. The yeast were then pelleted, resuspended in YPD and incubated at 30°C for 1 h; before being pelleted again, resuspended in SD media and inoculated onto SD agar. All plasmids used for yeast transformation feature an auxotrophic marker which allows successful transformants to be selected for through omitting the amino acid or nucleoside encoded by said marker from the medium. Following transformation, the yeast were then cultured at 30°C for 48-72 h. Single colonies were picked and inoculated in 5 mL SD media, cultured for an additional 24 h and then glycerol stocks were prepared in exactly the same way as described for bacterial cultures in the previous section (2.1.1).

Plasmids were extracted from transformed yeast using a modified miniprep protocol using the Monarch Plasmid DNA Miniprep Kit (NEB) [169]. Three millilitres of yeast culture were pelleted by centrifugation and resuspended in 200 µL of Resuspension Buffer (B1). The resuspension was added to 100 µL of acid-washed glass beads and vortexed for 5 min. The resuspension was then added to 200 µL of Plasmid Lysis Buffer (B2), mixed and incubated at room temperature for 1 min. The lysate was then neutralised by adding 400 µL of Plasmid Neutralization Buffer (B3), mixed, incubated for 2 min and then centrifuged at 13,000 x g for 5 min. The supernatant was removed and added to a spin column and centrifuged for 1 min. The column was then washed (twice) with 400 µL of Plasmid Wash Buffer 2. Finally, the plasmid DNA was eluted in 50 µL of

TE buffer (pre-warmed to 55°C). The yield and purity of Plasmid DNA was determined using a Nanodrop spectrophotometer – as was done for bacterial minipreps.

Transformation success was confirmed by PCR using extracted plasmid DNA as template. Twenty nanograms of plasmid DNA was used as template and the Phusion polymerase protocol was invariably followed. Specific primers with different annealing temperatures were used for different yeast strains, as is detailed in Table 2.3.

Yeast genomic DNA was extracted using the Yeast DNA Extraction Kit following the manufacturer's instructions (Thermo Scientific).

2.2.2. Growth assays

Yeast growth assays were used to determine the effect of the episomal expression of a putative transporter from *P. falciparum* on the growth phenotype of the yeast. The growth phenotype was invariably compared with the same yeast strain that had been transformed with a plasmid lacking the insert and the same yeast strain that was episomally expressing an endogenous functional transporter, or a functional transporter from a model organism.

Transformed yeast were cultured in SD media for 16-24 h at 30°C. Yeast cell density of the culture was measured using either an Infinite F50 (Tecan) or a GloMax Discover (Promega) and was adjusted to an OD_{600nm} of 1.0 by diluting the culture by the appropriate factor in SD media.

For growth assays performed on solid media a four-point, ten-fold dilution series, in SD, SGR (Yeast Nitrogen Base, 0.67% (w/v); Yeast Synthetic Drop-out medium (- His, Leu, Trp and Ura), 0.14% (w/v); Agar, 2% (w/v); Raffinose, 2% (w/v); Galactose, 1% (w/v)) YPEG (Yeast Extract, 1% (w/v); Peptone, 2% (w/v); Ethanol, 2% (v/v); Glycerol, 2% (v/v)), was set up for each yeast culture. Five microlitres of each dilution was spotted

onto media featuring different concentrations of a transition metal. The plates were then cultured for 48-72 h at 30°C and imaged using a digital camera.

For growth assays performed in liquid media, each yeast culture was diluted 1:100, in media (SD, SGR, or YPEG), in a well of a clear, flat-bottomed, 96-well cell culture plate. As with the solid media assays, different wells feature different concentrations of a particular transition metal. For each yeast strain, three technical replicates were included. The culture plate was incubated at 30°C. The yeast cell density (OD_{600nm}) of culture in each well was measured periodically for 24-96 h using a Tecan plate reader.

2.2.3. Live cell fluorescent microscopy

For the yeast strains expressing eGFP-tagged proteins, such as, PfCTR1, PfCRT, and PfCDF, live cell imaging was undertaken by fluorescence microscopy. Yeast was cultured for 10-16 h (mid log phase) before being pipetted onto a glass slide and covered with a glass cover slip. The slide was imaged using a Nikon Epifluorescent Ni-E microscope at a magnification of x60.

2.3. In vitro culture of *P. falciparum* blood stages

The following strains of *P. falciparum* were used for the *in vitro* culture of blood stage parasites – 3D7, NF54, and NF54-DiCre [170–173]. *P. falciparum* was cultured in human red blood cells (RBCs) and RPMI 1640 supplemented with 35 mM HEPES, 0.2 mM hypoxanthine, 2 mM L-glutamine, 0.5% (w/v) AlbuMAX™ (ThermoFisher) and 50 µg/mL gentamycin, and were maintained at 37°C. Parasites were cultured in a gas mixture of 5% CO₂, 3% O₂, 92% N₂,

Human RBCs were purchased from the NHS Blood and Transplant service. The RBCs were pelleted by centrifugation at 2000 x g and washed twice in an equal volume of incomplete culture media (lacking albumax), before being resuspended in an equal volume of media and stored at 4°C.

Cryopreserved parasites were thawed by incubating at 37°C for 2-3 min. Twelve percent (w/v) NaCl was added dropwise to the thawed parasites in a ratio of 1:5 – NaCl to parasites. After 5 minutes of incubation at room temperature 10 mL of 1.8% (w/v) NaCl was added dropwise and then pelleted by centrifugation at 600 x g. The supernatant was then removed and a further 10 ml of 1.2% (w/v) NaCl + 0.2% (w/v) D-glucose was added dropwise, before being centrifuged and the supernatant removed. The pellet was then washed twice with incomplete culture media and resuspended in complete media and RBCs to a haematocrit of 2-5%. The culture was then gassed (see previous paragraph) and incubated at 37°C.

Following thawing, it typically took 1-4 48 h life cycles for the culture to reach a parasitaemia of 1%. Media was changed every 48 h by centrifuging the culture at 600 x g, removing the supernatant, and resuspending the pelleted RBCs in new media. Cultures were maintained at approximately 1-5% parasitaemia and 2-5% haematocrit. The parasitaemia was estimated by smearing a 50:50 mixture of RBCs and culture media on a glass slide and staining with Giemsa. Prior to application of stain, the smear was fixed with 100% methanol. Giemsa was diluted 1 in 5 in phosphate-buffered saline (PBS) and filtered before covering the blood smear for at least 10 mins. The stain was then washed off with tap water; dried and viewed under a compound microscope (Olympus) at x100 magnification. The parasitaemia was estimated by counting at least 1000 RBCs from at least 4 randomly selected fields of view and is the percentage of all RBCs that are infected with a malaria parasite (of any life cycle stage). This was typically done every time the media was changed.

To eliminate all life cycle stages other than ring-stage parasites and thus synchronise the culture, parasites were treated with sorbitol. The culture was pelleted by centrifugation at 600 x g for 5 min and resuspended in 10 volumes of 5% (w/v) D-sorbitol [174]. The suspension was then incubated at 37°C for 15 min and subsequently pelleted by

centrifugation. The pellet was then washed twice and resuspended in culture media. To keep parasites synchronised, cultures were treated with sorbitol once every three cycles.

Parasites were prepared for cryopreservation at -80°C by resuspending infected RBCs in an equal volume of freezing solution (glycerol, 28% (v/v); mannitol, 3% (w/v); NaCl, 0.65% (w/v)).

2.3.1. *P. falciparum* transfections

Transfection of blood stage *P. falciparum* was undertaken by electroporation with the Bio-Rad Gene Pulser and followed one of two protocols; 1. Transfection of ring-stage parasites; 2. Pre-loading uninfected RBCs [175,176].

2.3.1.1. Transfection of ring-stage parasites

Forty-eight hours before transfection the culture was synchronised by sorbitol lysis. The culture media was changed 24 h prior to transfection and the parasitaemia was adjusted to 1-2% by adding the appropriate amount of fresh (less than a week old) RBCs – to give rise to a culture with approximately 5% rings in the following cycle. The cell culture media was changed 3-4 h prior to transfection and the parasitaemia was adjusted to 5% if required. One hundred microlitres of infected RBCs were harvested for each transfection by centrifugation at 600 x g for 5 min and washed once in cytomix. Fifty to one hundred micrograms of plasmid DNA was used for each transfection and was resuspended in 300 µL of cytomix. The iRBCs were resuspended in the 300 µL of DNA in cytomix and transferred to a 2 mm electroporation cuvette (Bio-Rad). The iRBCs were then electroporated with the following settings: exponential decay program, voltage of 310 V, capacitance of 950 µF, and maximum resistance. Immediately following electroporation, 1 mL of pre-warmed cell culture media was added to the cuvette, in which the electroporated cells were resuspended and transferred into 4 mL of cell culture media and incubated at 37°C for 1 h. The electroporated cells were then pelleted by centrifugation at 600 x g for 5 min, and the cells were resuspended in 5 mL of fresh cell

culture media and transferred to a T25 cell culture flask. The cells were then gassed and incubated at 37°C. One day post transfection, drug pressure was applied, by replacing the cell culture media with media featuring one of the following drugs: blasticidin-S – 2 µg/ml; WR99210 (Jacobus) – 2.5 nM; DSM-1 – 1.5µM; G418 – 250 µg/ml. The media was then changed every day for six days and every other day thereafter. Five- and ten-days post drug pressure 100 µL of fresh RBCs was added to the culture. At two- and three-weeks post drug pressure, one third of the total culture was discarded and replaced with 100 µL of fresh RBCs. Smears were undertaken twice a week to check for transgenic parasites.

2.3.1.2. Pre-loading RBCs transfection

Cell culture media was changed 48 and 24 h prior to transfection. The parasitaemia was checked by blood smear and adjusted to give approximately 5% schizonts for the transfection. Three hundred microlitres of fresh RBCs – per transfection – were pelleted by centrifugation at 600 x g and washed with cytomix. Fifty to one hundred micrograms of plasmid DNA were resuspended in 100 µL of cytomix, added to the washed RBCs and transferred to a 2 mm electroporation cuvette. The electroporation parameters were the same as for the ring-stage protocol. The electroporated RBCs were recovered for 30 min in complete cell culture media at 37°C and then subsequently pelleted by centrifugation at 600 x g. The supernatant was then removed and replaced with 4.5 mL of fresh culture media and 0.5 mL of the schizont culture. Media was changed after 24 h and drug pressure was applied after 48 h. Drug pressure and maintenance of the culture was then as described in the previous section.

2.3.2. Selection-linked integration

SLI plasmids feature a second drug resistance cassette, which allows for the genomic integration of the plasmid, or part of the plasmid, by homologous recombination, to be readily selected for [177]. Following successful transfection of the SLI plasmid, as confirmed by appearance of WR99210-resistant parasites in the culture, treatment of the

culture with WR99210 is replaced by treatment with G418 to select for parasites that have integrated the plasmid into their genome. WR99210-resistant parasites were cultured to a parasitaemia of 1-5%. The iRBCs were then pelleted by centrifugation, the WR99210-treated media was removed and replaced with G418-treated media. The media was changed every day until G418-resistant parasites appeared in the culture – blood smears were checked every other day.

2.3.3. Genotyping transgenic parasites

G418-resistant parasites were cultured to a parasitaemia of at least 5% and then synchronised by sorbitol lysis. Genomic DNA was extracted from a culture that mainly featured late-stage trophozoites using the DNeasy® Blood & Tissue Kit (Qiagen). Saponin was added to 10 mL of culture to a final concentration of 0.1% (v/v), centrifuged at 2,500 x g for 3 min and the pellet resuspended in PBS. The resuspension was then centrifuged again, the supernatant was removed, and the pellet was resuspended in 200 µL phosphate-buffered saline, 200 µL of buffer AL and 20 µL proteinase K and incubated at 70°C for 10 min. Two hundred microlitres of 100% (v/v) ethanol was then added and the mixture was added to a spin column. The subsequent spins and washes followed the kit manufacturer's instructions, and the final elution was into 50 µL of TE buffer.

The extracted parasite DNA was used as template for PCR. Primers were designed to confirm a transfection success by detecting the presence of a particular plasmid and also to show successful integration of the plasmid into the genome by SLI.

2.3.4. Dose-response assays

Dose response assays were undertaken to determine the effect of various compounds on the growth of *P. falciparum* over a single 48 h cycle. The compounds tested were, the iron chelators, BPS and DFO, two putative DMT inhibitors and arsenic trioxide (ATO). Furthermore, ATO dose-responses were undertaken where cultures also featured sub-lethal doses of artesunate.

Highly synchronous ring-stage parasites were adjusted to a parasitaemia of 0.5% and a haematocrit of 4% and cultured in the presence of one of several compounds. For each compound, an eight-point, two-fold dilution series was set up across a plate. Each concentration featured three technical replicates. The cultures were incubated at 37°C in a gassed hypoxic chamber and frozen at -80°C after 48 h. The plates were then thawed and treated with the fluorescent DNA dye, PicoGreen (Life Technologies) – 1 in 200 in lysis buffer (Tris, 20mM; EDTA, 5mM; saponin, 0.008% (w/v); Triton X-100, 0.08% (v/v), pH 7.5), incubated for 10 min at room temperature and read using a fluorimeter at excitation 485nm, emission 538nm.

Mean parasite densities were calculated by comparing the amount of fluorescence detected compared with untreated controls and IC₅₀s were estimated by fitting a 4 parameter log fit curve to the means.

To assess whether artesunate and ATO had a synergistic, additive or antagonistic relationship when used to treat *P. falciparum*, combination indices (CIs) were calculated by dividing the IC₅₀ of drug A in combination by its IC₅₀ when used on its own plus the same for the other drug being tested [178].

2.4. Bioinformatics

Coding sequences for putative malarial transporters were retrieved from PlasmoDB [179]. Orthologues across the *Plasmodium* genus, the Apicomplexa and model organisms were identified by BLAST (NCBI). Multiple protein sequence alignments were performed using Clustal Omega [180]. Percentage sequence identities were calculated using Expasy SIM [181]. Transmembrane domains and signal peptides were identified using the DeepTMHMM prediction server [182]. Phylogenetic analysis was undertaken using the NGPhylogeny.Fr online platform [183].

2.5. Statistical analysis

All statistical analysis was performed using GraphPad Prism 9. Statistical differences between mean yeast cell densities were determined by two-way ANOVA with Tukey's multiple comparisons test.

3. Heterologous expression of malarial transition metal transporters in yeast

3.1. Introduction

Heterologous expression of transport proteins in model systems allows them to be studied in relative isolation and has aided in the characterisation of proteins from numerous organisms – including *Plasmodium* [184]. Whilst the majority of the proteins that comprise the *Plasmodium* transportome remain understudied, heterologous expression systems such as yeast and *Xenopus laevis* oocytes have been routinely utilised where characterisation has been undertaken [63,185–189].

The budding yeast, *Saccharomyces cerevisiae* is a unicellular, eukaryotic model organism, with a well-annotated genome and importantly a well-characterised transportome [190,191]. It is also easily cultured and genetically tractable, which makes the studying of transporters far less time-consuming and laborious than in blood-stage malaria parasites [184]. Furthermore, like *Plasmodium* trophozoites, yeast possess an intracellular food vacuole – which is utilised for digestion of substrates, storage and detoxification, as is the case in *Plasmodium*.

As yeast is comparably far better studied than *Plasmodium* (and indeed perhaps any eukaryote other than human), cell lines with various transporters disrupted or knocked-out are readily available [190,191]. To study whether a putative transporter might transport a specific substrate, one would typically express that transporter in a line that lacks some ability to transport that substrate. This is usually undertaken by disrupting one or more endogenous transporters responsible for the import or export of said substrate and then attempting to rescue the altered phenotype by heterologous expression of the transporter of interest.

Yeast utilises an array of membrane transporters for transition metal homeostasis [192]. Extracellular ferric iron (Fe^{3+}) is reduced by the plasma membrane metalloreductases, Fre1 and Fre2, and Fe^{2+} is imported by the carriers Fet3 and Fet4 [193]. The vacuolar iron transporter CCC1 confers resistance to iron replete conditions by facilitating the sequestration of iron from the yeast cytosol into the vacuole [194]. CCC1 is an orthologue of the *Plasmodium* vacuolar iron transporter, PfVIT, which was shown capable of complementing CCC1 in a yeast line with CCC1 disrupted and functionally knocked out [134].

In yeast, copper uptake is facilitated by the high affinity copper transporters Ctr1 and Ctr3. The expression of these transporters is upregulated in copper-limiting conditions and is controlled by the transcription factor MAC1 [195]. Likewise, copper uptake in mammalian cells is mediated by a Ctr1 orthologue – commonly referred to as hCTR1 in humans [196].

As discussed in the opening chapter, Bakouh et al. demonstrated that the chloroquine resistance transporter, PfCRT transported iron when expressed in *X. laevis* oocytes [136]. Here we used the same iron-sensitive yeast strain as was used by Slavic et al. [134] to explore whether PfCRT could confer tolerance to iron. Furthermore, to test whether PfCRT could facilitate the acquisition of iron in iron-limiting conditions, we expressed PfCRT in a yeast strain lacking iron uptake function, as well as validating the *Arabidopsis thaliana* iron transporter, AtIRT, as a positive control for these experiments.

To interrogate the potential function of the putative copper transporters PfCTR1 and PfCTR2, they were expressed in a yeast line lacking the high affinity copper transporters Ctr1 Ctr3. Yeast must acquire copper to be incorporated into cytochrome oxidase for respiration, with a high affinity copper uptake mechanism being a requirement for growth in non-fermentable media.

3.1.1 Aims & objectives

The work in this chapter aimed to interrogate the potential function of the putative transporters PfCTR1, PfCTR2 and PfCRT utilising heterologous expression in yeast.

Objectives

- Express PfCTR1 and PfCTR2 in $\Delta ctr1 ctr3$ yeast and assess ability to confer tolerance to low copper conditions.
- Express PfCTR-hCTR1 chimeras and assess ability to confer tolerance to low copper conditions – as in objective 1.
- Generate PfCTR1 mutants featuring random point mutations that confer tolerance to low copper conditions.
- Validate AtIRT1 as a positive growth control for yeast growth assays.
- Express PfCRT in iron sensitive yeast strains assess ability to confer tolerance to both iron-replete and low iron conditions.

Figure 3.1. The putative *Plasmodium falciparum* copper transporters, PfCTR1 and 2.

(a) Organisation of PfCTR1 and PfCTR2 features three predicted transmembrane domains and a signal peptide. **(b)** Multiple protein sequence alignment indicating conserved amino acid residues within the second and third transmembrane regions across related parasite species, yeast and human. Pv = *Plasmodium vivax*; Pk = *P. knowlesi*; Pb = *P. bergeri*; Tg = *Toxoplasma gondii*; Sc = *Saccharomyces cerevisiae*; Hs = *Homo sapiens*. Asterisk indicates a conserved residue across all aligned species; colon indicates aligned amino acids with strongly similar properties; period indicates aligned amino acids with less strongly similar properties – all scored on the Gonnet PAM 250 matrix [198].

3.2.2. Recomplementing yeast Ctr1 and Ctr3 with PfCTRs

As previously discussed, the high affinity copper transporters Ctr1 and Ctr3 facilitate copper uptake by yeast and are required for growth when cultured in the absence of a fermentable carbon source, such as glucose. To examine whether the putative copper transporters PfCTR1 and PfCTR2 could facilitate copper uptake in yeast, they were expressed in the $\Delta ctr1 ctr3$ line and assayed for growth in non-fermentable media.

Yeast codon optimised coding sequences for PfCTR1 and PfCTR2, with and without the predicted signal peptide domain, were cloned into the yeast expression vector p413GPD (Figure 3.2a) and transformed into $\Delta ctr1 ctr3$ yeast. Transformation success was determined by PCR (Figure 3.2b). In addition, yeast were transformed with p413GPD and p413GPD featuring the human copper transporter hCTR1 as negative and positive controls respectively.

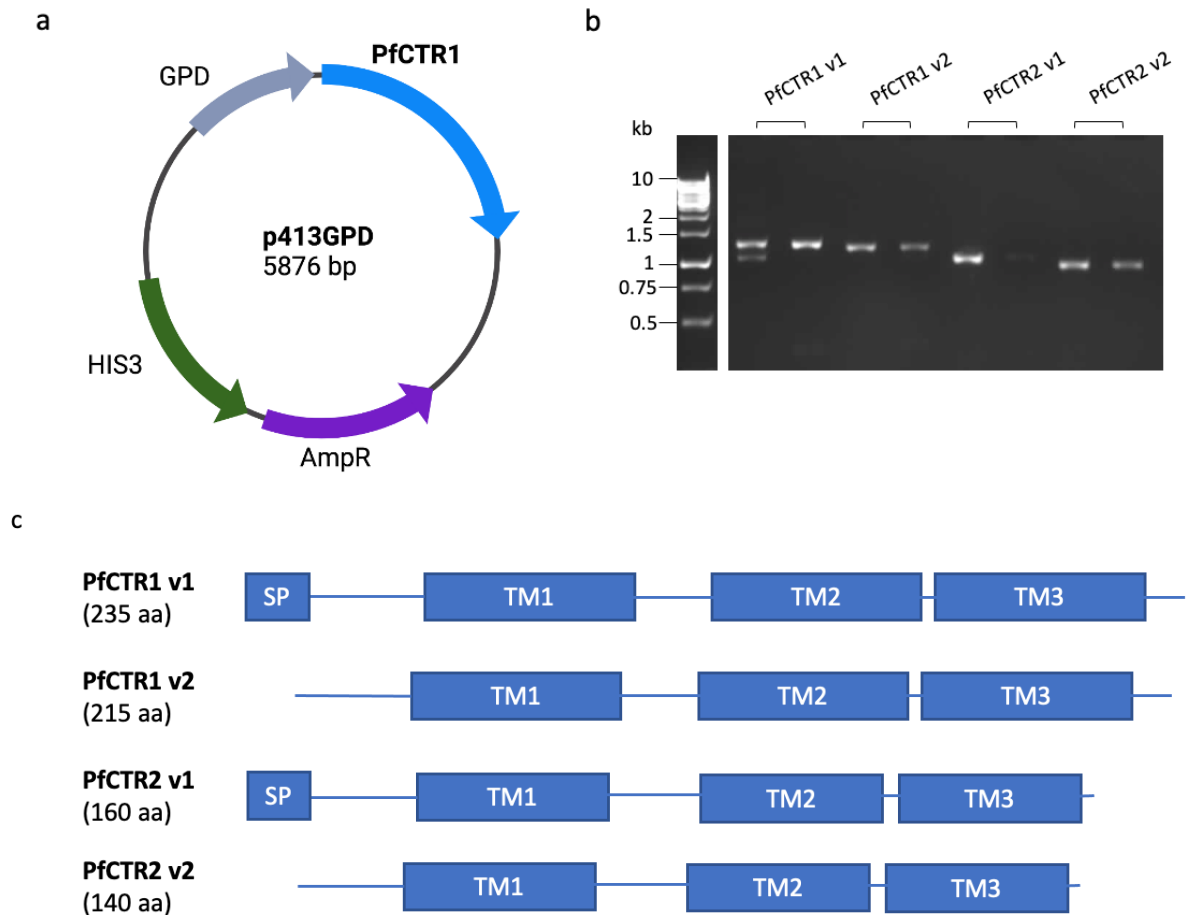


Figure 3.2. Expression of PfCTRs in Δ ctr1ctr3 yeast. **(a)** The PfCTRs were episomally expressed from p413GPD under the control of the GPD promoter. p413GPD selection in yeast and bacteria is enabled by the HIS3 (histidine) and AmpR (ampicillin) cassettes respectively. **(b)** PfCTR1 and 2 with and without the predicted signal peptide sequence were transformed into Δ ctr1ctr3 yeast. Transformation success was established by PCR amplification of insert, using the primers 1 and 2, from extracted plasmid DNA. The DNA ladder is in kilobases **(c)** Schematic shows arrangement and respective lengths of PfCTRs.

The transformed yeast were spotted onto SD-agar (positive growth control), as well as the non-fermentable media YPEG-agar $\pm$ 100 μ M CuSO₄. After 72 h of culture at 30°C, comparable levels of growth can be seen for all six yeast lines on SD and YPEG supplemented with CuSO₄ (Figure 3.3a). On YPEG alone – copper limiting conditions –

the hCTR1 line grew well, however the four PfCTR lines had a comparable growth phenotype to the negative control – yeast transformed with the empty vector.

Furthermore, the same trend can be seen when the same yeast were cultured in liquid media, whereby the positive control had a significantly higher cell density after 100 h of culture than the four PfCTR lines and the negative control (Figure 3.3b). In addition, the four PfCTR lines did not have a significantly higher yeast cell density after 100 h of culture than the empty vector control (Figure 3.3c-e).

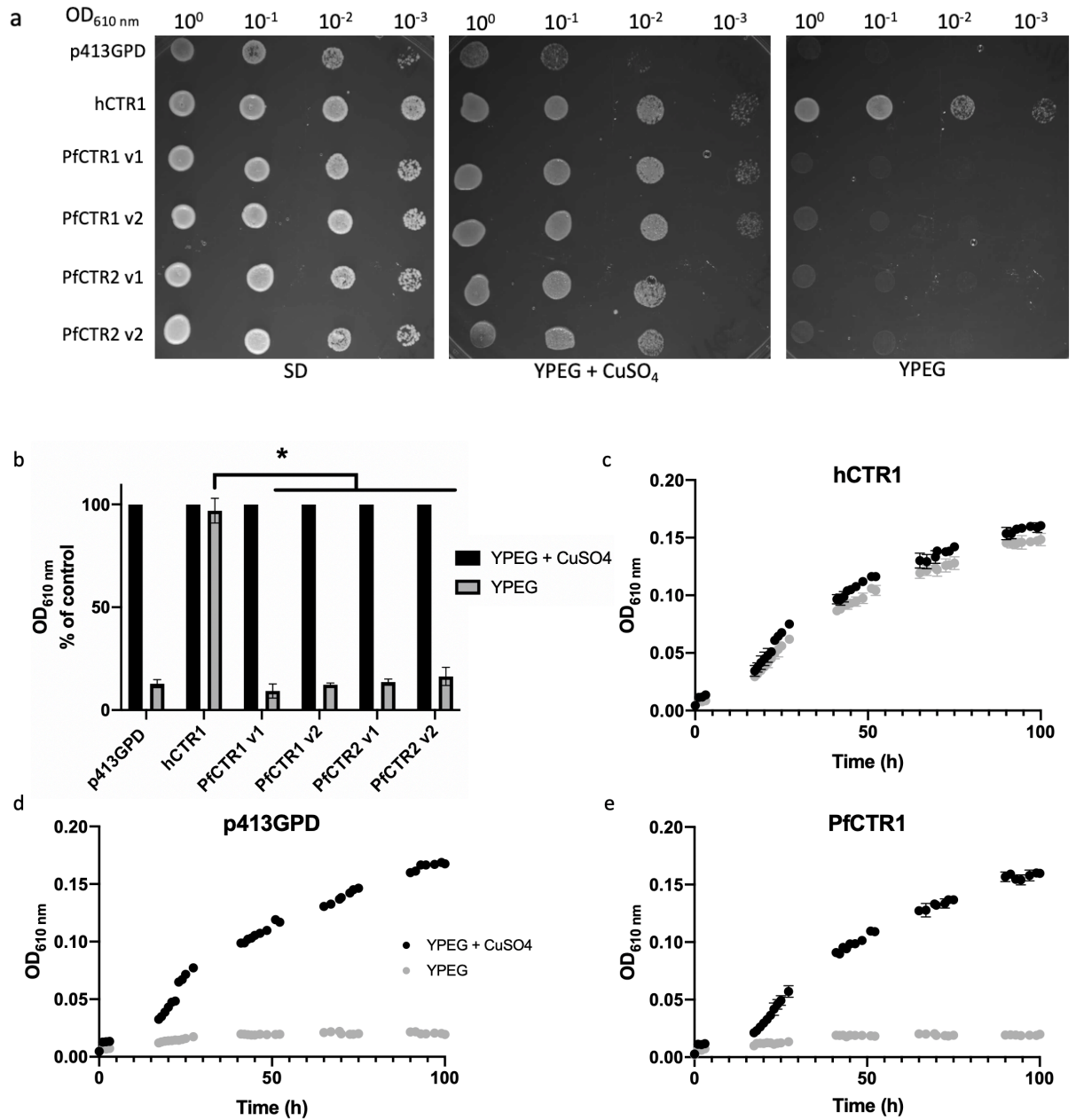


Figure 3.3. PfCTR1 and PfCTR2 growth assays in liquid and solid media. (a) $\Delta ctr1 ctr3$ yeast transformed with either, p413GPD (empty vector), p413GPD containing one of the four PfCTRs, p413GPD-hCTR1 were cultured in SD to a cell density of 1 (OD_{610nm}) and a four-point, ten-fold dilution series was set up. Yeast was spotted onto SD-agar lacking histidine, as well as YPEG $\pm$ CuSO₄ (100 μ M). The plates were imaged after 72 h of culture at 30°C. (b) The transformants described in a were diluted 1:100 in YPEG broth, $\pm$ CuSO₄, and cultured for 100 h at 30°C. The data shown are yeast cell densities after 96 h of culture and are means calculated from three biological replicates ($\pm$ SEM). Differences between mean

cell densities of yeast cultured in YPEG was assessed by one-way ANOVA with Tukey's multiple comparisons test. Asterisk indicates statistical significance ($P < 0.05$) (**c-e**)

Representative growth curves for PfCTR1 v1, p413GPD (empty vector), and hCTR1.

3.2.3. Recomplementing yeast Ctr1 and Ctr3 with PfCTR chimeras

As yeast expressing the four PfCTR variants did not appear to rescue the growth phenotype of $\Delta ctr1 ctr3$ in YPEG, we sought to explore whether this could be achieved by expressing a chimeric PfCTR-hCTR1 protein, as hCTR1 does clearly rescue the growth phenotype of $\Delta ctr1 ctr3$. Five chimeric sequences, each featuring different combinations of PfCTR and hCTR1 amino acid sequence were constructed and cloned into p413GPD.

The chimeras, PfCTR1 v3, v4 and v6 replaced the *P. falciparum* amino acid sequence with that of hCTR1 at the N-terminus, N-terminus including the first transmembrane region, or the C- terminus and second and third transmembrane regions, respectively (Figure 3.4a). The same approach was applied in creating PfCTR2 v3 and v4. $\Delta ctr1 ctr3$ yeast were transformed with p413GPD containing one of the five chimeras, as described previously for PfCTR1 and 2. Transformation success was confirmed by PCR (Figure 3.4b).

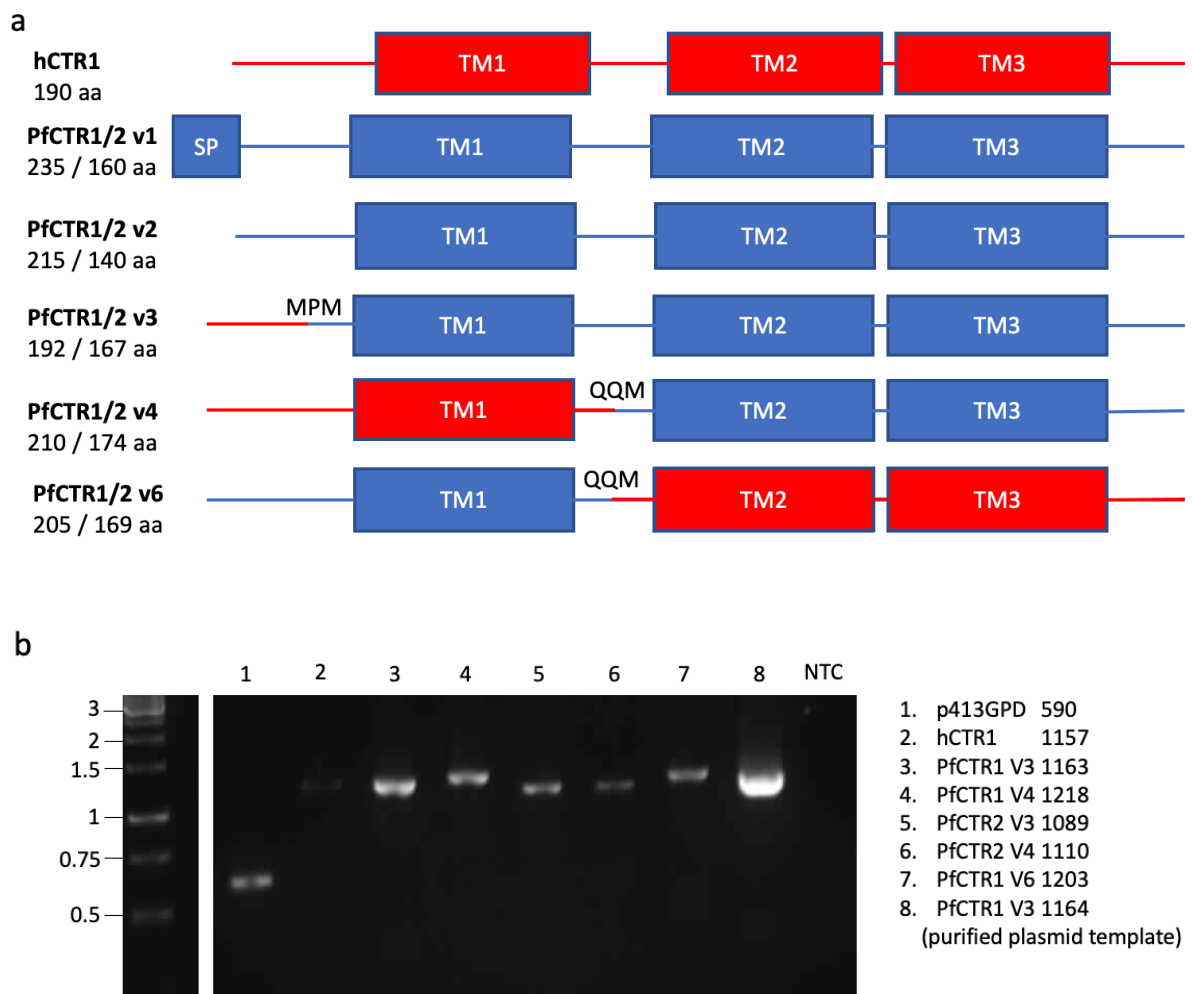


Figure 3.4. Expression of PfCTR-hCTR1 chimeras in $\Delta ctr1 ctr3$ yeast. (a) Arrangement of the human CTR orthologue hCTR1 (red), alongside human-*Plasmodium* chimeras. **(b)** The vector p413GPD featuring one of each chimeric insert was transformed into $\Delta ctr1 ctr3$ yeast. Transformation success was confirmed by PCR amplification of insert, with primers 1 and 2, from extracted plasmid DNA. NTC = no template control. The DNA ladder is in kilobases.

As was done previously, the transformed yeast were cultured on YPEG-agar $\pm 100 \mu\text{M}$ CuSO_4 and SD-agar for 72 h at 30°C . Comparable levels of growth can be observed for all seven lines across the SD and YPEG + CuSO_4 plates (Figure 3.5a). As seen previously, yeast transformed with p413GPD-hCTR1 clearly rescued the growth phenotype of $\Delta ctr1 ctr3$ when cultured on YPEG, in copper-limiting conditions, however the growth of the yeast transformed with the five chimeras was comparable to that of the

empty vector control. Furthermore, when cultured in liquid media, all of the chimeric yeast lines had a significantly lower cell density than the hCTR1 line, however there was no significant difference between any of the chimeric yeast lines and the empty vector control (Figure 3.5b).

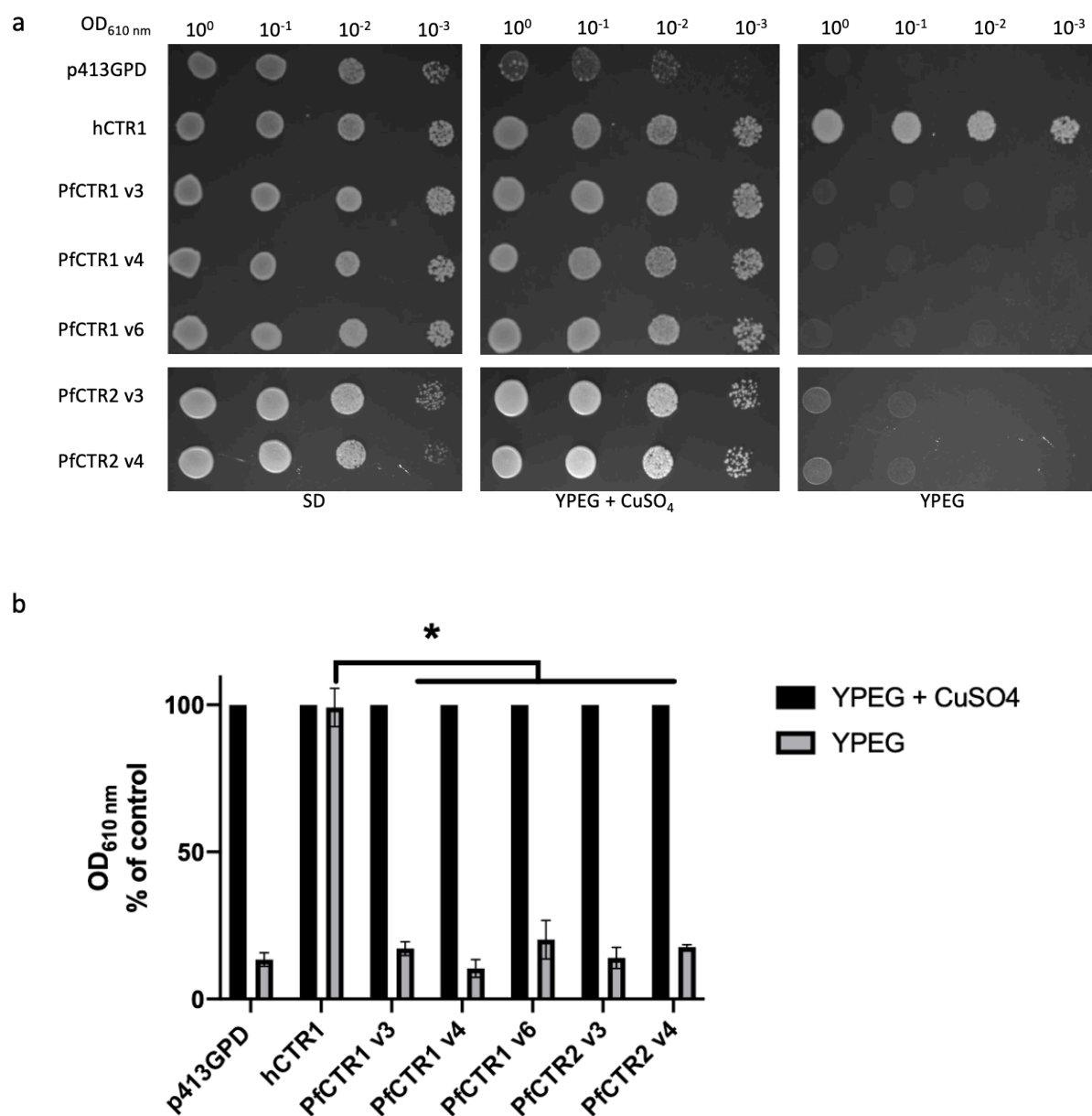


Figure 3.5. PfCTR-hCTR1 chimera growth assays in solid and liquid media. Δ ctr1ctr3 yeast transformed with either, p413GPD (empty vector), p413GPD containing one of the five PfCTR-hCTR1 chimeras, and p413GPD-hCTR1 were cultured in SD to a cell density of 1

(OD_{610nm}) and a four-point, ten-fold dilution series was set up. Yeast were spotted onto SD-agar lacking histidine, as well as YPEG $\pm$ CuSO₄. The plates were imaged after 72 h of culture at 30°C. **(b)** The transformants described in **a** were diluted 1:100 in YPEG $\pm$ CuSO₄, and cultured for 100 h at 30°C. The data shown are yeast cell densities after 96 h of culture and are means calculated from two biological replicates ($\pm$ SEM). Differences between mean cell densities of yeast cultured in YPEG was assessed by one-way ANOVA with Tukey's multiple comparisons test. Asterisk indicates statistical significance ($P < 0.05$)

3.2.4. Screening of random PfCTR1 mutants by replica plating

We next sought to determine whether the generation of random point mutations in the PfCTR1 coding sequence might result in amino acid changes that could enable copper transport function and thus confer phenotypic rescue to Δ ctr1ctr3. To do this we utilised yeast gap-repair cloning (Figure 3.6a), whereby PCR primers were designed to amplify the PfCTR1 coding sequence adding 30 base pair regions of homology to p413GPD at the 5' and 3' ends of the amplicon (Figure 3.6b). The low-fidelity DNA polymerase OneTaq was used to enable the generation of random point mutations in the resultant amplicons. The amplicon was then cotransformed with linearised p413GPD (Figure 3.6c) into Δ ctr1ctr3, where the two products would undergo homologous recombination and thus form the circular expression vector p413GPD-PfCTR1. The yeast were cultured in SD for 72 h at 30°C. In addition, the same approach was utilised for hCTR1 and empty p413GPD to provide positive and negative controls respectively. Transformation success was confirmed by PCR (Figure 3.6d).

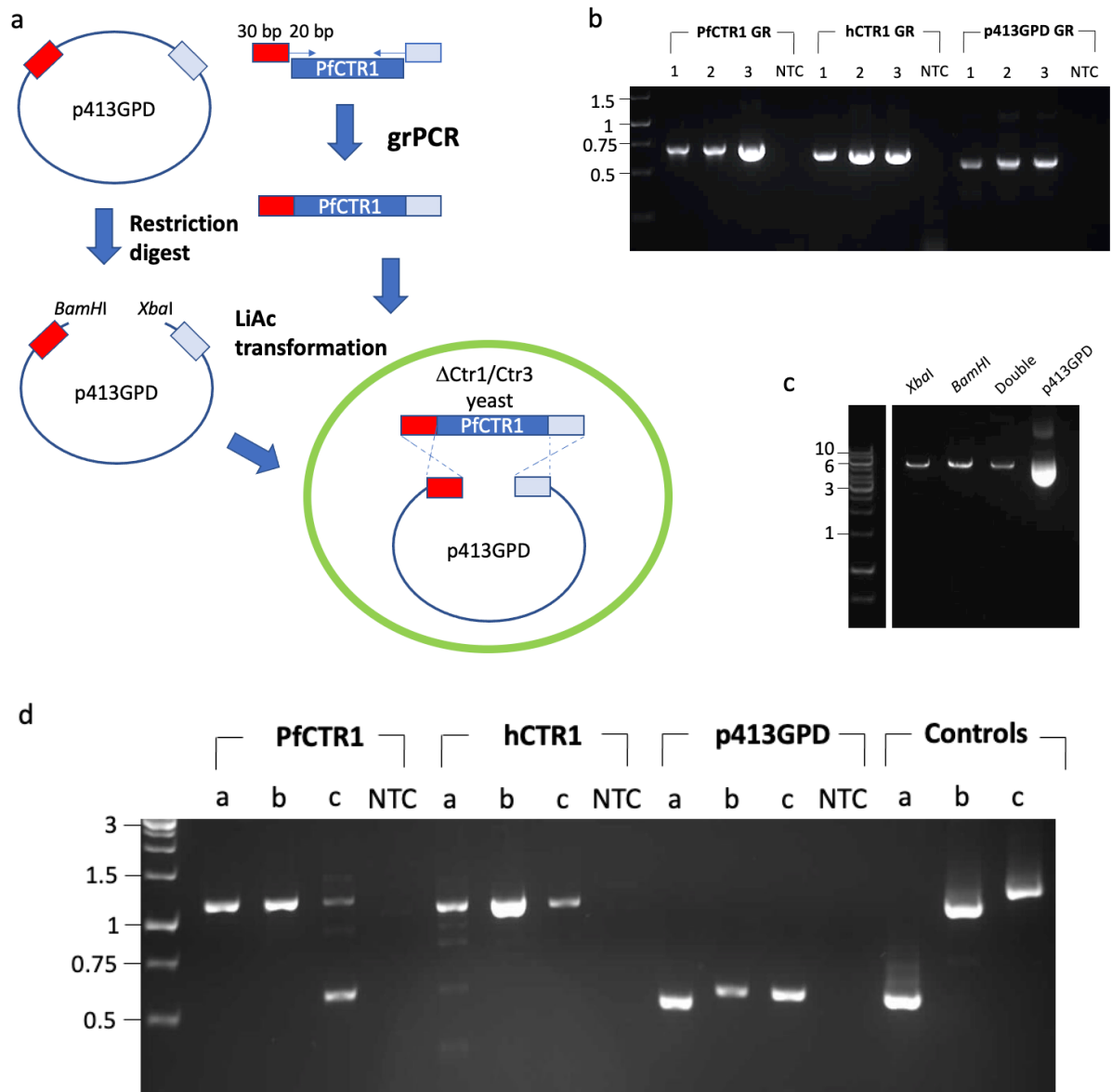


Figure 3.6. Generation of random PfCTR1 mutants using a low fidelity polymerase. (a)

Yeast gap repair cloning strategy to generate PfCTR1 with random point mutations. **(b)**

PfCTR1, hCTR1 and p413GPD were amplified from purified p413GPD (containing either PfCTR1, hCTR1, or empty vector) by gap-repair PCR using primers 3-8 and column purified. Expected band sizes, 1. PfCTR1 = 628 bp, 2. hCTR1 = 573 bp, 3. p413GPD = 515 bp. **(c)**

The vector p413GPD was digested with *XbaI* and *BamHI*, excised from the gel and column purified. **(d)** The purified grPCR amplicons and linearised p413GPD were cotransformed into Δ ctr1ctr3 yeast (as detailed in **a**). Transformation success was confirmed by PCR

amplification of the cloned insert, using primers 1 and 2, from extracted plasmid DNA.

Plasmid DNA was extracted from yeast cultures grown from three colonies per transformation. PCR reactions using purified p413GPD (a), p413GPD-hCTR1 (b), and p413GPD-PfCTR1 (c) as template were used as controls. Expected band sizes, 1. PfCTR1 = 1234 bp, 2. hCTR1 = 1157 bp, 3. p413GPD = 590 bp. DNA ladder in kilobases.

The yeast were replicated on YPEG agar $\pm$ CuSO₄ (100 μ M) – as well as SD-agar using sterilised velvet (Figure 3.7). After 96 h of growth at 30°C, the yeast colonies replicated well on the SD-agar growth control plate and YPEG-agar + CuSO₄. Approximately 20% of the hCTR1 (positive control) colonies replicated on the YPEG-agar, however zero colonies replicated on either the empty p413GPD negative control YPEG-agar plate or that of PfCTR1.

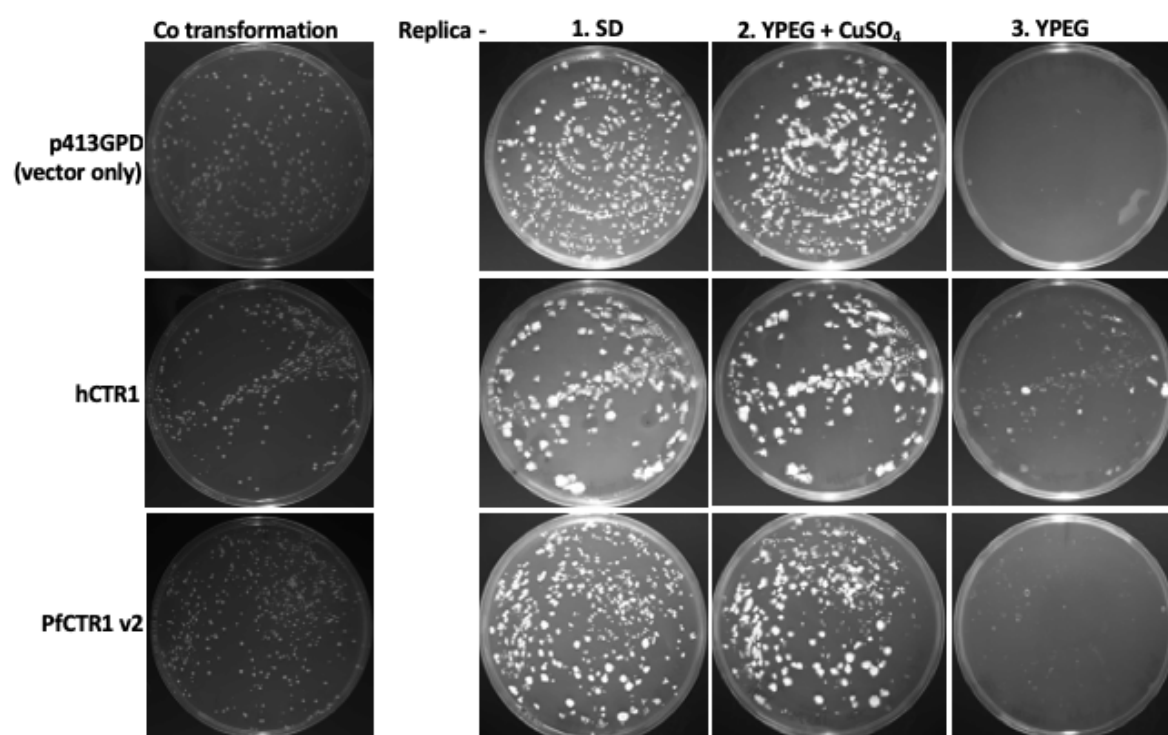


Figure 3.7. Screening of PfCTR1 mutants by replica plating. Δ ctr1ctr3 yeast were transformed with p413GPD-PfCTR1 produced by yeast gap repair cloning that utilised a low fidelity polymerase to generate point mutations in the coding region of PfCTR1. The transformed yeast were replicated onto SD-agar, YPEG-agar $\pm$ CuSO₄ (100 μ M) using

sterilised velvet. The replicas were cultured for a further 96 h at 30°C. Yeast transformed with hCTR1 and empty p413GPD were replicated as well as positive and negative controls respectively.

3.2.5. Expressing AtIRT in Fet3/Fet4 yeast

High affinity iron uptake in yeast is facilitated by the Fet3/Fet4 system, which is comprised of two transporters Fet3 and Fet4. To test the iron uptake capacity of putative iron transporters from *Plasmodium* we used $\Delta\text{fet3fet4}$ yeast – which is a line with both Fet3 and Fet4 disrupted and thus cannot be cultured in iron replete conditions. Importantly, for such experiments to be undertaken a positive growth control yeast line had to be produced.

The coding sequence for the *Arabidopsis thaliana* iron transporter, AtIRT was yeast codon optimised and cloned into the yeast expression vector, pUGPD (Figure 3.8a). pUGPD-AtIRT was transformed into $\Delta\text{fet3fet4}$ with transformation success being determined by PCR (Figure 3.8b).

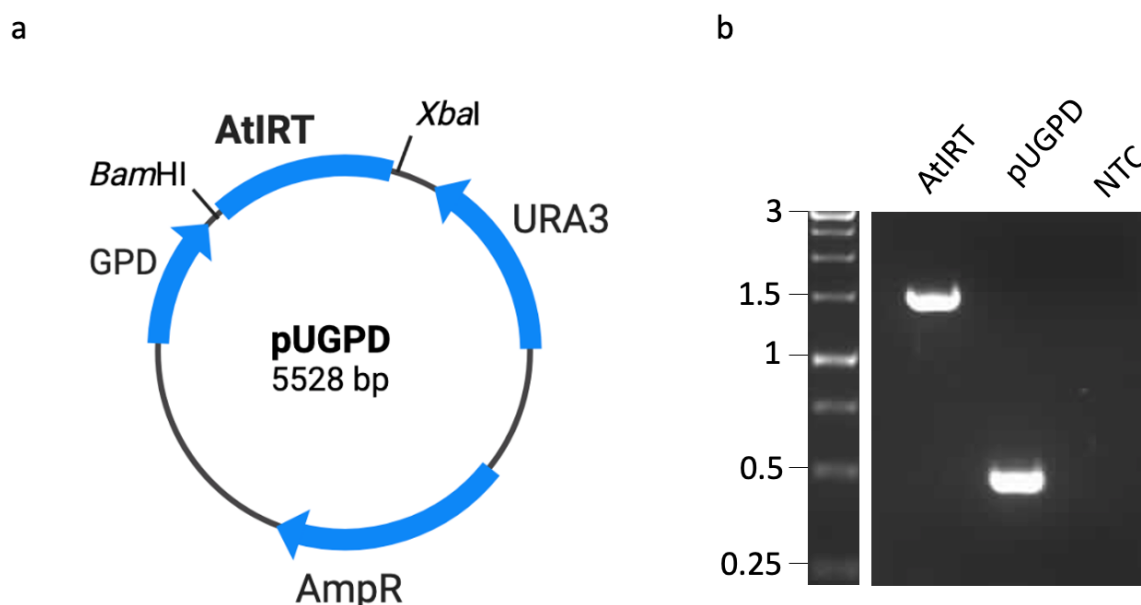


Figure 3.8. Expression of AtIRT in Δ fet3fet4 yeast. (a) Schematic representation of the yeast expression vector pUGPD. The *Arabidopsis thaliana* iron importer AtIRT is expressed under the control of the GPD promoter. pUGPD selection in yeast and bacteria is enabled by the URA3 (uracil) and AmpR (ampicillin) cassettes respectively. (b) Δ fet3fet4 yeast were transformed with pUGPD-AtIRT and pUGPD. Transformation success was confirmed by PCR amplification of the plasmid, using primers 9 and 10, from extracted plasmid DNA. Expected band sizes, 1. AtIRT = 1500 bp, 2. pUGPD = 482 bp. NTC = no template control. The DNA ladder is in kilobases.

Growth assays on solid and in liquid media were performed on/in SD. On SD-agar supplemented with 100 μ M FeSO₄ and 100 μ M ascorbate (a reducing agent), comparable growth was seen for Δ fet3fet4 transformed with pUGPD-AtIRT and empty pUGPD (Figure 3.9a). In non-supplemented media and thus iron-limited conditions, higher levels of growth were observed for yeast transformed with pUGPD-AtIRT than was seen for those transformed with empty pUGPD.

To control for residual iron in SD media, the Fe^{2+} chelator bathophenanthrolinedisulfonic acid (BPS) was also added, at increasing concentration (1, 5 and 10 μM). With this supplementation, the growth of $\Delta\text{fet3fet4}$ transformed with empty pUGPD was seemingly reduced, as the concentration of BPS increased. With that being said, it was also seen that increasing the concentration of BPS also resulted in reduced growth of $\Delta\text{fet3fet4}$ transformed with pUGPD-AtIRT. Additionally, the same yeast cultures were grown in SD and SD supplemented with FeSO_4 (100 μM) and ascorbate (100 μM), or BPS (1, 5 and 10 μM) for 24 h at 30°C. After 24 h of culture, $\Delta\text{fet3fet4}$ transformed with pUGPD-AtIRT had a significantly higher cell density – in iron-limited conditions – than the negative empty pUGPD control. This was seen for all four concentrations of BPS.

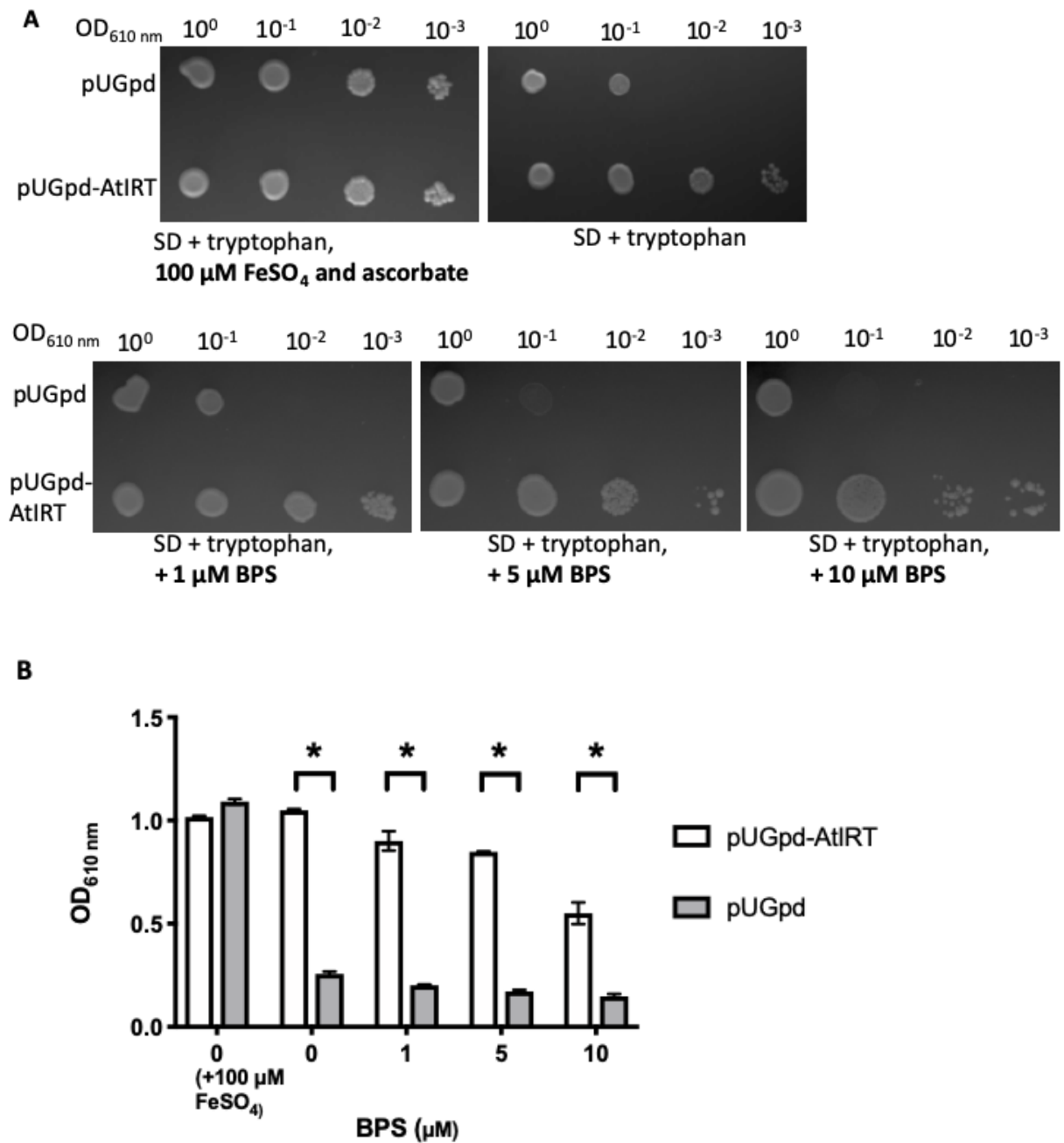


Figure 3.9. AtIRT growth assays in solid and liquid media. (a) Δ fet3fet4 yeast

transformed with pUGPD and pUGPD-AtIRT were cultured in SD broth to a cell density of 1 (OD_{610nm}) and a four-point, ten-fold dilution series was set up. Yeast was spotted onto SD-agar lacking uracil and featuring different concentrations of FeSO₄ and BPS. Cultures that were supplemented with iron were also supplemented with an equal concentration of ascorbate – this was ensure the iron in the media remained in its reduced state (Fe²⁺). The

plates were imaged after 48 h of culture at 30°C. **(b)** The transformants described in **a** were diluted 1:100 in SD broth and cultured for 24 h at 30°C. The data shown are mean cell densities ($\pm$ SEM) calculated from five technical replicates. Statistically significant differences between means at each BPS concentration were assessed using the Student's t-test (two tailed, equal variance). Asterisk symbolises a statistically significant difference ($P < 0.05$).

3.2.6. Expression of PfCRT in Δ fet3fet4 and Δ CCC1 yeast

The chloroquine resistance transporter has been implicated in the transport of a number of substrates, in addition to chloroquine, including iron [136]. To test whether PfCRT could confer iron tolerance to yeast, it was expressed in two different iron-sensitive yeast lines. They were, Δ fet3fet4 (described above) and Δ CCC1 – with the latter lacking the vacuolar iron transporter CCC1 and is thus sensitive to low micromolar concentrations of iron.

The coding sequence of PfCRT was yeast codon optimised, cloned into the yeast expression vector pUGPD and transformed into Δ CCC1 and Δ fet3fet4 yeast.

Transformation success was established by PCR (Figure 3.10).

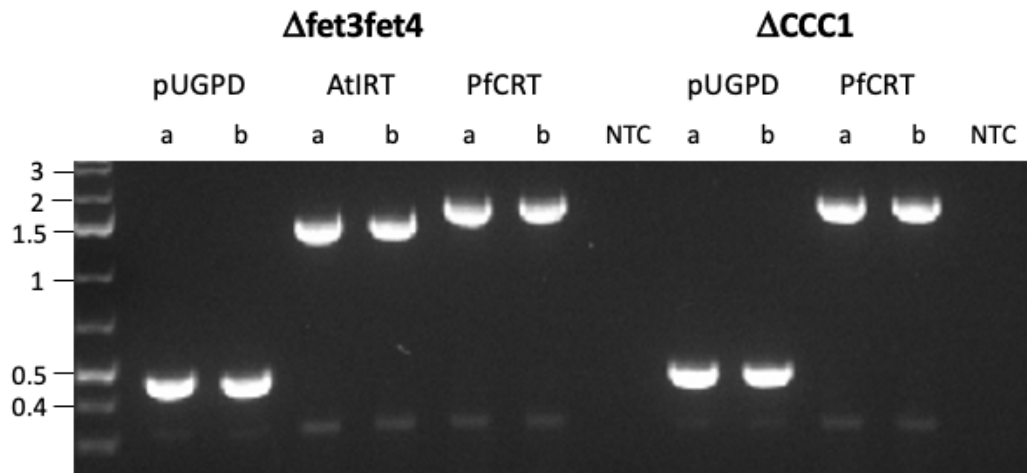


Figure 3.10. Transformation of Δ fet3fet4 and Δ CCC1 yeast with pUGPD-PfCRT.

Confirmation by PCR for successful transformations of Δ CCC1 and Δ fet3fet4 yeast with pUGPD, pUGPD-PfCRT, and pUGPD-AtIRT (Δ Fet3/Fet4 only) using primers 9 and 10.

Expected band sizes = 1. pUGPD = 482 bp, 2. AtIRT = 1500 bp, 3. PfCRT = 1748 bp. NTC = no template control. The DNA ladder is in kilobases.

To determine whether PfCRT could confer phenotypic rescue to Δ fet3fet4 yeast – those lacking the capacity for high affinity iron uptake and thus struggle to grow in iron limiting conditions – pUGPD-PfCRT was transformed into Δ fet3fet4 and cultured in iron-limiting (0 μ M) and iron replete (100 μ M) conditions (Figure 3.11a). Furthermore, the pUGPD and pUGPD-AtIRT yeast lines from the previous section were used as negative and positive growth controls respectively. After 24 h of culture at 30°C on SD-agar that was supplemented with 100 μ M of FeSO₄ (and ascorbate) yeast transformed with pUGPD-PfCRT had comparable levels of growth to both control lines. However, on SD-agar that was not supplemented with FeSO₄, yeast transformed with pUGPD-PfCRT had levels of growth that were comparable to the negative empty vector control, whereas both of these lines had much lower levels of growth than the AtIRT positive control.

To determine whether PfCRT could confer tolerance to Δ CCC1 in iron-replete conditions, Δ CCC1 transformed with pUGPD-PfCRT was cultured on SD-agar featuring three different concentrations of iron sulphate (0, 1 and 3 mM) (Figure 3.11b). In addition, Δ CCC1 was transformed with empty pUGPD, which was used as a negative growth control. A recomplemented CCC1- Δ CCC1 yeast line was used as a positive control. After 48 h of culture at 30°C, all three yeast lines had comparable growth on SD-agar with no added iron. On SD-agar that was supplemented with 1 mM of iron, yeast transformed with pUGPD-PfCRT appeared to have increased levels of growth compared to the negative empty vector control but noticeably less growth than the recomplemented CCC1- Δ CCC1 line. However, at 3 mM iron yeast transformed with pUGPD-PfCRT had comparable levels of growth to the empty vector control (pUGPD). It is noteworthy that these growth assays only represent a single experiment, and when cultured in SD media over 72 h, the difference in growth phenotype at 3 mM iron was not observed (n = 3), thus suggesting PfCRT does not confer iron tolerance to Δ CCC1 (Figure 3.11c).

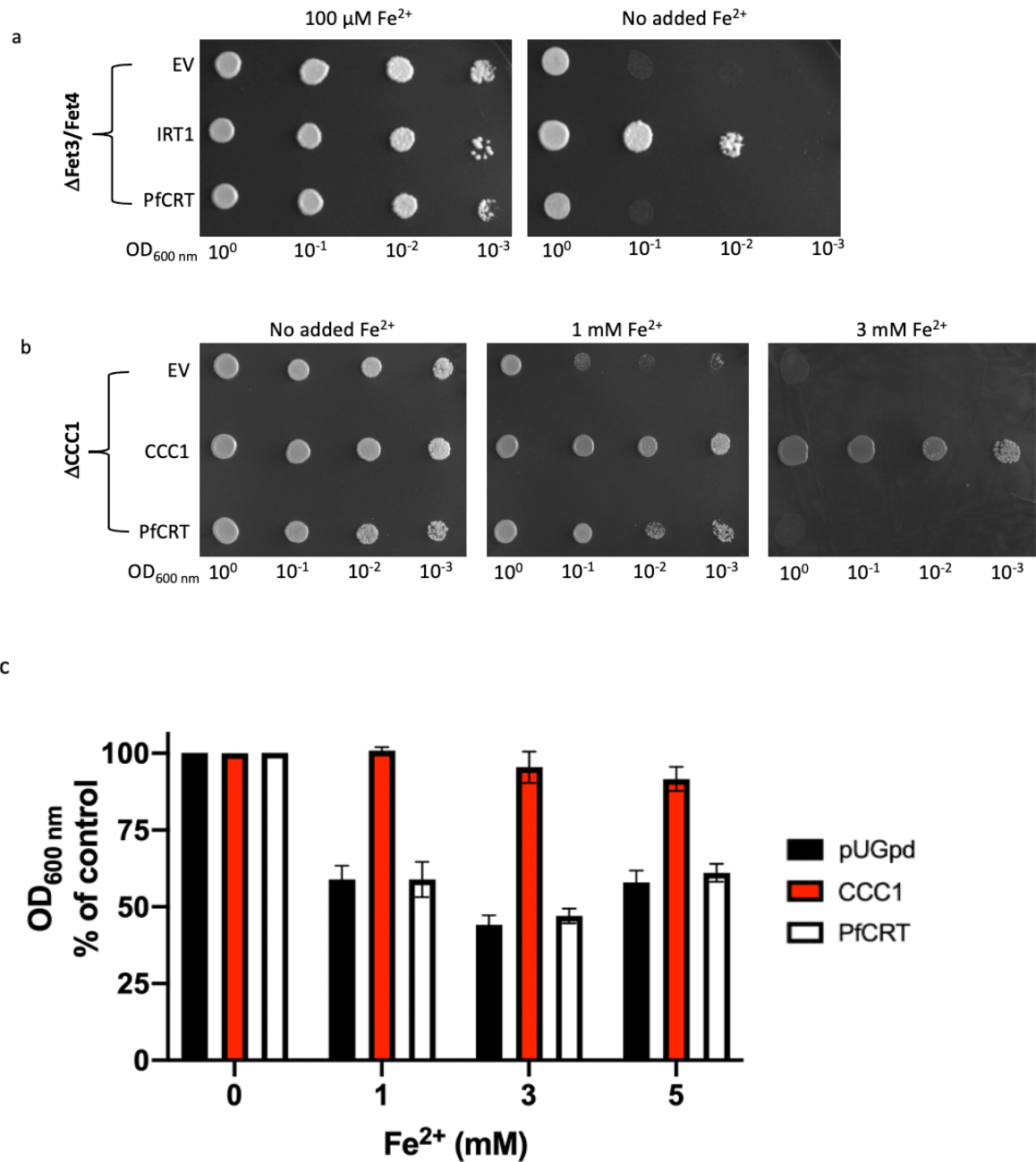


Figure 3.11. PfCRT growth assays on solid and in liquid media. (a) $\Delta\text{fet3fet4}$ yeast transformed with either pUGPD (empty vector), pUGPD-PfCRT, or the pUGPD-AtIRT line described in section 3.2.5. were cultured in SD to a cell density of 1 ($\text{OD}_{600\text{nm}}$) and a four-point, ten-fold dilution series was set up. Yeast was spotted onto SD-agar that lacked uracil and featured either 100 or 0 μM FeSO_4 . Each culture also featured an equal concentration of ascorbate to that of FeSO_4 . The plates were imaged after 48 h of culture at 30°C. Displayed assays are representative of two independent experiments. **(b)** ΔCCC1 yeast transformed

with either pUGPD (empty vector), pUGPD-PfCRT, or the recomplemented CCC1- Δ CCC1 line were cultured in SD to a cell density of 1 (OD_{600nm}) and a four-point, ten-fold dilution series was set up. Yeast was spotted onto SD-agar lacking uracil and featuring different concentrations of $FeSO_4$ – 0, 1 and 3 mM. Each culture also featured an equal concentration of ascorbate to that of $FeSO_4$. The plates were imaged after 48 h of culture at 30°C. Displayed assays only represent a single experiment **(c)** The transformants described in **a** were diluted 1:100 in SD broth and cultured for 72 h at 30°C. The data shown are mean cell densities ($\pm$ SEM) calculated from three independent experiments.

Despite not conferring any noticeable growth phenotype to either of the two yeast strains PfCRT was expressed in, we reasoned that this might be due to errant localisation of PfCRT in the yeast. To explore this, we sought to generate a yeast line that expressed a PfCRT fusion protein that featured an enhanced green fluorescent protein (eGFP) at its C-terminus, which could then be visualised by fluorescence microscopy. eGFP was amplified from the yeast expression vector p415GPD-PfCDF-eGFP and cloned onto the 3' end of PfCRT, within the pUGPD-PfCRT vector, by yeast gap-repair cloning (Figure 3.12a). Cloning success was determined by PCR amplification of the coding region of the pUGPD vector, which had been extracted from transformed Δ fet3fet4 yeast (Figure 3.12c). The yeast was cultured to a cell density of 1.0 (OD_{600nm}) and imaged. Δ fet3fet4 transformed with pUGPD and p415GPD-PfCDF-GFP were used as positive and negative fluorescence controls respectively. No fluorescent signal was detected in yeast expressing PfCRT-eGFP (data not shown).

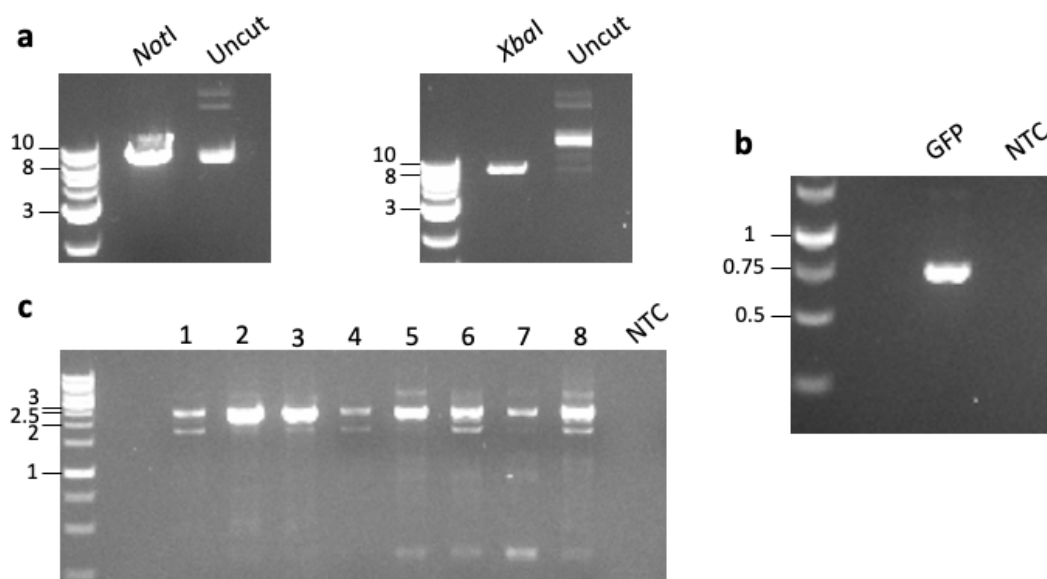


Figure 3.12. Generation of enhanced green fluorescent protein (eGFP) tagged PfCRT.

(a) PfCRT-GFP was produced by gap repair cloning. pUGPD-PfCRT was sequentially digested with *NotI* and *XbaI* and column purified after gel extraction. (b) eGFP was amplified by PCR from PfCDF-eGFP using primers 11 and 12 and column purified. Expected band size = 750 bp. (c) Digested pUGPD-PfCRT and eGFP were cotransformed into Δ fet3fet4 yeast, cultured for 72 h at 30°C with selection by omitting uracil from the media. Transformation success was confirmed by PCR amplification of insert from extracted plasmid DNA. Expected band size = 2434 bp. DNA ladders in kilobases.

As we could not detect any expression of PfCRT, we decided to transform Δ CCC1 with two versions of the pYES-PfCRT plasmid that have been used extensively by others to express PfCRT in various yeast strains (Figure 3.13b) – these were a kind gift of Prof. Paul Roepe [199]. The two plasmids featured the two PfCRT variants, HB3 and Dd2, which are representative of PfCRT coding sequences from chloroquine sensitive and resistant isolates respectively. In addition, Δ CCC1 was also transformed with pYES (empty vector), which was generated by removing the PfCRT insert from pYES-PfCRT

by restriction digest and re-ligating the backbone; and pYES-CCC1, as a positive control (Figure 3.13c-g). Expression from the pYES vector is induced by the sugars galactose and raffinose. Accordingly, growth assays were performed in SGR rather than SD media.

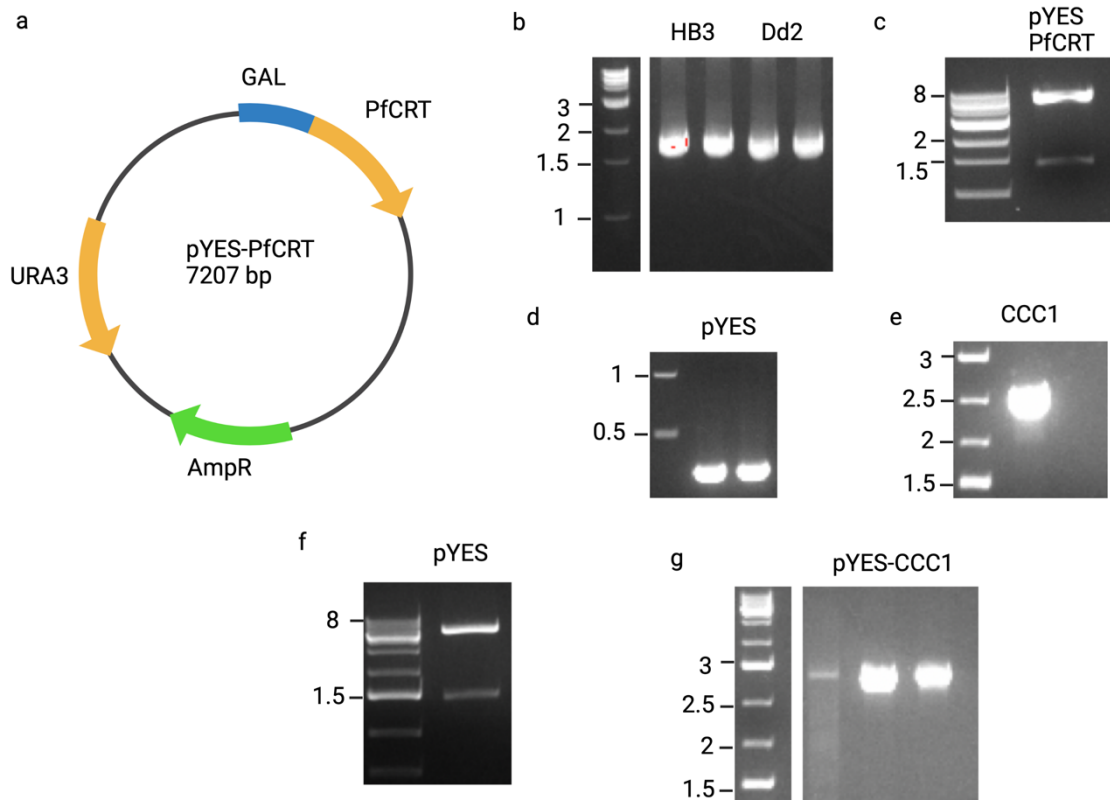


Figure 3.13. Transformation of Δ CCC1 with pYES-PfCRT. (a) Schematic representation of the inducible yeast expression vector pYES. PfCRT is expressed under the control of the GAL promoter when cultured in media containing the sugars galactose and raffinose. pYES selection in yeast and bacteria is enabled by the URA3 (uracil) and AmpR (ampicillin) cassettes respectively. (b) Δ CCC1 yeast was transformed with two variants of pYES-PfCRT – HB3 and Dd2. Transformation success was confirmed by PCR using primers 13 and 14 – expected band size 1736 bp. (c) pYES-PfCRT was digested with *Xho*I and *Sal*I to release the vector backbone, which was religated to create the pYES vector. (d) pYES was successfully transformed into Δ CCC1, two colonies were screened by PCR using primers 13

and 14 – expected band size = 316 bp. **(e-g)** The yeast iron transporter CCC1 coding sequence was amplified from extracted genomic DNA using primers 15 and 16 – expected band size = 2551, and cotransformed into Δ CCC1 yeast with digested pYES. Transformation success was confirmed by PCR using primers 13 and 14 – expected band size = 2867 bp. DNA ladders in kilobases.

To determine whether either PfCRT could confer tolerance in iron-replete conditions, Δ CCC1 transformed with pYES-PfCRT-HB3, or pYES-PfCRT-Dd2 were cultured on both SD and SGR-agar that was supplemented with either 0, 1 or 5 mM FeSO₄ and an equal volume of ascorbate (Figure 3.14). Additionally, Δ CCC1 transformed with pYES was used as a negative control. Δ CCC1 transformed with pYES-CCC1 was to be used as a positive control, however it clearly displayed a comparable growth phenotype to the negative control in both SD and SGR and at all concentrations of iron that were tested. We therefore, used the Δ CCC1-CCC1 line used in previous experiments as a positive growth control. After 48 h of growth at 30°C all lines other than Δ CCC1-CCC1 had comparable growth in SD and SGR at all iron concentrations tested.

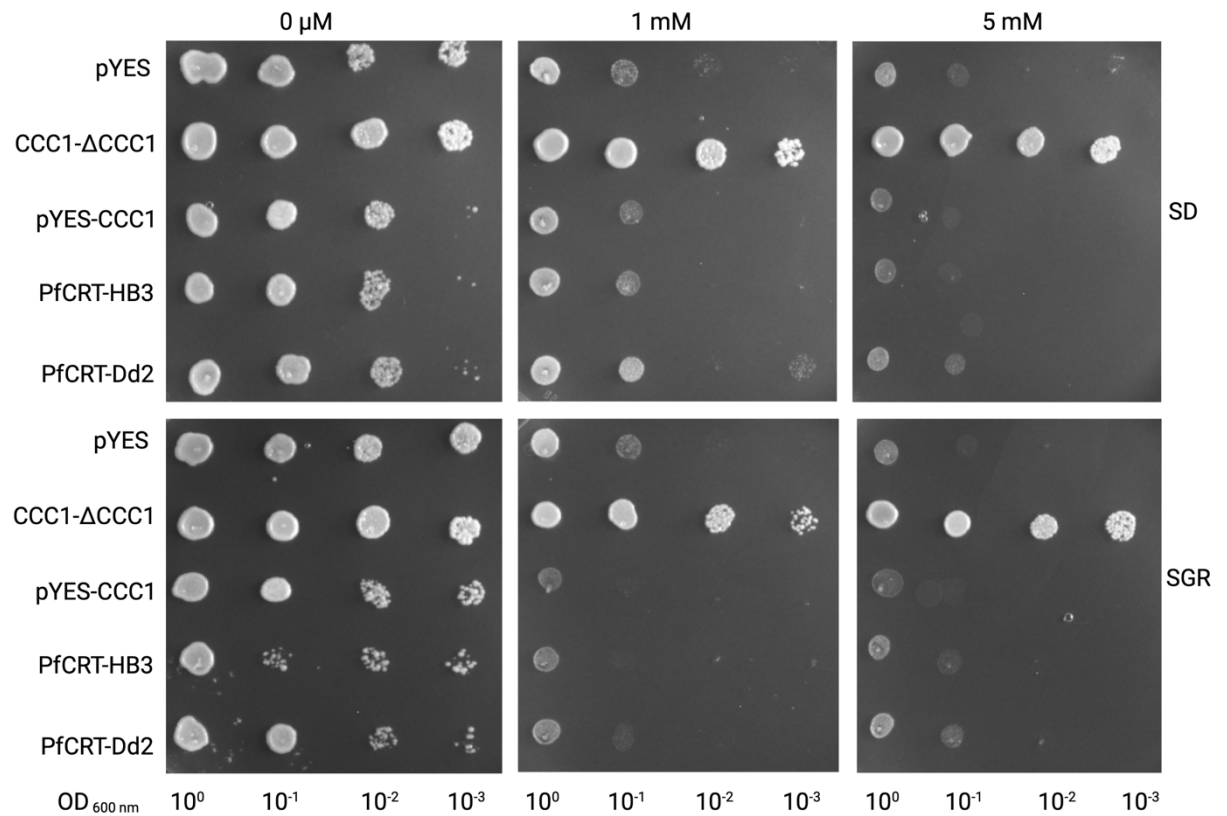


Figure 3.14. Growth assays with PfCRT variants in iron. Δ CCC1 yeast transformed with either pYES (empty vector), pYES-CCC1, pYES-PfCRT-HB3, pYES-PfCRT-Dd2, or the recomplemented CCC1- Δ CCC1 line were cultured in SD to a cell density of 1 (OD_{600nm}) and a four-point, ten-fold dilution series was set up. Yeast was spotted onto SD-agar, or SGR-agar lacking uracil and featuring different concentrations of FeSO_4 – 0, 1 and 5 mM and an equal volume of ascorbate. Each culture also featured an equal concentration of ascorbate to that of FeSO_4 . The plates were imaged after 48 h of culture at 30°C. Displayed assays only represent a single experiment.

3.3. Discussion

Transition metal homeostasis is essential to the survival of *Plasmodium*. Both copper and iron are required for numerous cellular processes yet become toxic to the parasite at high concentrations. As such, the parasite must have evolved a multitude of mechanisms to facilitate this homeostasis. One such mechanism that has already been mentioned is the sequestration of cytosolic iron by PfVIT which provides resistance against the toxic effects of iron [134]. Here we examined the putative roles of PfCTR1 and PfCTR2 as copper transporters, as well as the putative role of PfCRT as a transporter of iron.

3.3.1. PfCTR1 and PfCTR2

When expressed in yeast lacking high affinity copper uptake capacity, neither PfCTR1 or PfCTR2, with or without their signal peptide, showed any effect on growth phenotype when compared to controls. Additionally, five PfCTR-hCTR1 chimeras also failed to confer any change to growth phenotype. Furthermore, an approach that used a low-fidelity polymerase to generate variants of PfCTR1 with random point mutations that could facilitate the growth of $\Delta\text{ctr1ctr3}$ in copper-limiting conditions failed to produce any hits. Together these data suggest that in the yeast system used in this work, neither PfCTR1 or PfCTR2 function as a transporter of copper.

It is entirely possible that in this yeast system, the PfCTRs are being expressed but not exported to and incorporated within the plasma membrane. To ascertain where the PfCTRs are being expressed within the yeast, an epitope or fluorescent tag could be cloned onto the coding sequence. Sub-cellular localisation could then be determined by fluorescence microscopy. This would be undertaken in parallel with hCTR1 to establish whether different localisation patterns could account for the apparent lack of functional PfCTRs. As hCTR1 does confer functional rescue to $\Delta\text{ctr1ctr3}$, one would presume that it is expressed and trafficked to the plasma membrane. This, however, might not be the

case for the PfCTRs in this system. Furthermore, although any signal at all would confirm that the proteins are being expressed, in the absence of this confirmation, expression could be shown by Western blot.

Whilst hCTR1 was not predicted to contain a signal peptide, PfCTR1 and 2 both were. Accordingly, in addition to the yeast codon optimised, full length PfCTR1 and 2, Δ ctr1ctr3 yeast was also transformed with the PfCTRs minus the signal peptide sequence. It has been noted that an increase in transport activity has resulted from N-terminal truncation of transporters when expressed in heterologous systems such as yeast [134,200]. With that being said however, Δ ctr1ctr3 yeast transformed with PfCTRs that featured an N-terminal truncation that removed the predicted signal peptide grew no better than any other PfCTR line, or indeed the empty vector negative control.

It has been shown that the uptake of copper in human cells is mediated by hCTR1 – used in this work as a positive control [201]. Structural analysis of hCTR1 revealed that it is comprised of three transmembrane domains and forms a homotrimer – this creates a membrane channel which mediates copper acquisition [197]. It is possible that both PfCTR1 and PfCTR2 are required for copper uptake in yeast, as they could form either a heterodimer or trimer. To test this both proteins would be expressed in Δ ctr1ctr3 from the same promoter as a fusion protein separated by a self-cleaving peptide, or from two different episomes with different auxotrophic selection cassettes.

It has been suggested that the MX3M motif in the second transmembrane domain is involved in copper binding and is thus essential to transport function [197]. We saw this motif to be well-conserved across the *Plasmodium* genus. Additional important residues were identified in hCTR1, with these being H 139 and the GX3G motif in the third transmembrane domain. It was suggested that these were important to structural integrity. Interestingly, in *Plasmodium* and fellow Apicomplexan *T. gondii*, H 139 is replaced with asparagine (N). Additionally, while the GX3G motif appears to be well-

conserved among the *Plasmodium* and *T. gondii* orthologues, one can see that in PfCTR2 the second glycine (G) in this motif is replaced with alanine (A) – which may well influence function.

PfCTR1 is likely essential to the blood stages of *P. falciparum*, as evidenced by it being refractory to knockout as part of a transposon mutagenesis screen [202]. With that being said, to confirm its apparent essentiality it would be worth attempting to knock out the gene, either by introducing a premature stop codon or replacing the entire locus with a drug-selectable marker with CRISPR-Cas9 – as has now been achieved for numerous *P. falciparum* genes. The same screen suggested that PfCTR2 might not be essential to the asexual blood stages and would therefore likely be amenable to knock out.

Furthermore, similar experimental genetic approaches could also be utilised to add either an epitope or fluorescent tag to the PfCTRs to allow their respective sub-cellular localisation to be determined. It is noteworthy that Choveaux et al. showed PfCTR1 to localise to the erythrocyte plasma membrane in the early blood stages of *P. falciparum* and then later the parasitophorous vacuole membrane using a monoclonal antibody that was specific for part of the N-terminal domain of the protein [154].

If it is determined that either PfCTR1 or PfCTR2 are essential to the blood (or liver) stages of *P. falciparum*, they will no doubt be regarded as attractive drug targets.

Moreover, due to the apparent relatively high expression of their *P. cynomolgi* orthologues in the dormant hypnozoite liver stage, these proteins are intriguing radical cure targets for vivax malaria [155]. Of the drugs currently available, only two – primaquine and tafenoquine – target the dormant hypnozoite stage seen in ovale and vivax malaria and both are from the same drug class and have contra-indications – as discussed in the opening chapter.

PfCTR1 shares a 40% amino acid sequence similarity with its human orthologue hCTR1. For any inhibitor to be explored as a potential antimalarial drug that targets PfCTR1, it

would have to selectively bind to PfCTR1 over hCTR1. To determine drug specificity, one could attempt to replace the entire PfCTR1 coding sequence with a *P. falciparum* codon optimised coding sequence for hCTR1 and if it were to successfully complement the parasite line, comparative inhibition assays could be performed.

3.3.2. PfCRT

PfCRT failed to confer iron tolerance to the iron-sensitive yeast line Δ CCC1, nor did it enable growth of Δ fet3fet4 – a yeast line sensitive to iron-limiting conditions – when cultured in the absence of iron. Additionally, endogenous tagging with green fluorescent protein did not yield any information on the sub-cellular localisation of PfCRT in the yeast as no fluorescent signal was detected. Furthermore, PfCRT-HB3 and PfCRT-Dd2 when expressed from the inducible pYES vector also did not confer tolerance to Δ CCC1.

The studies detailed in this chapter aimed chiefly to examine the direction by which PfCRT might transport iron. Where the Δ fet3fet4 yeast lacks high affinity iron uptake capability, complementation by PfCRT would suggest that it functions by importing iron from outside of the cell into the cytosol. If this were true, it might suggest that in the malaria parasite PfCRT could be involved with iron acquisition. On the other hand, if PfCRT were to have complemented the Δ CCC1 system, this would indicate that it conferred tolerance against iron toxicity to the yeast by facilitating iron efflux, either from the cytosol to out of the cell, or to an intracellular compartment such as the vacuole. Likewise, this might suggest a similar function in the malaria parasite, as is the case for PfVIT [134]. A second transporter serving that function could go some way in explaining the apparent inessentiality, and therefore redundancy, of PfVIT. However, this is all highly speculative as PfCRT did not complement either yeast line in the experiments detailed here.

As was the case for PfCTR1, the lack of localisation data for PfCRT in yeast suggests that either PfCRT is not being expressed – this could be tested by Western blot – or

PfCRT is being expressed but not reaching either the plasma membrane or the vacuolar membrane. It is also possible that if the protein is localising to the vacuolar membrane, the lack of signal could be because the C-terminal GFP is situated on the luminal side of the membrane and the low pH of vacuolar lumen affects fluorescence.

Whilst poor localisation or lack of expression would unequivocally prevent PfCRT from conferring any kind of tolerance to iron in either of the iron-sensitive yeast lines used in this work, it must also be considered that PfCRT is not actually an iron transporter. As discussed, Bakouh et al. showed PfCRT to be capable of transporting both ferrous and ferric iron in the *X. laevis* model, however the findings of this single report are yet to be backed up when expressing PfCRT in any other system (including yeast) or by any data from the parasite itself [136]. Experiments using the *X. laevis* system demonstrated that PfCRT is capable of transporting haemoglobin-derived short peptides [137]. In addition, proton leak experiments in the parasite itself corroborated these findings, showing several peptides and peptide mimics to be substrates of PfCRT. Furthermore, Sanchez et al. generated PfCRT conditional knock-down mutants which they showed accumulated short peptides in the vacuole, thus further lending credence to the theory that the physiological role of PfCRT is to expedite the redistribution of host-derived peptides from the digestive vacuole to the rest of the parasite [203].

4. Characterisation of the *Plasmodium falciparum* cation diffusion facilitator

4.1. Introduction

As the malaria parasite progresses through its lifecycle it interchanges among several distinct ecological niches, each with differing levels of essential micronutrients such as transition metals. Accordingly, the parasite must both be able to acquire the nutrients it requires from its host, and at appropriate concentrations, and expel or sequester the excess to prevent toxicity.

In the red blood cell (RBC), the malaria parasite faces a total iron concentration of 15 mM, of which the labile pool has been shown to increase during the intraerythrocytic cycle [157,204]. As discussed in the opening chapter, the iron concentration of the *Plasmodium*-infected hepatocyte is dynamic – which is, in part, due to modulatory effects of hepcidin [127]. With that being said, the uninfected hepatocyte has a total iron concentration of around 5 μ M [205]. Good estimates of total iron (and other metals) are yet to be determined for the midgut and salivary glands of the mosquito vector [206]. The total zinc concentration of the RBC is around 250 μ M and this has been shown to rapidly increase prior to parasite egress [157].

As already discussed, transporters facilitate the uptake and expulsion of metals in *Plasmodium* and several putative transporters remain to have function ascribed. In other organisms, cation diffusion facilitators (CDFs) – a class of transporter – provide an efflux mechanism for transition metals such as iron, zinc, cobalt, and manganese and thus enable cells to tolerate these essential but potentially toxic metals [138]. The *P. falciparum* genome is predicted to encode a single, previously uncharacterised CDF whose potential function is explored in this chapter.

CDFs have been identified across all major phyla and many of which have been well-characterised with *A. thaliana*, yeast and humans possessing nine, four and ten respectively [207–209]. The CDF family was first described by Nies and Silver upon noticing sequence similarities between two bacterial proteins and two from yeast that all seemed to respond to increases in the concentration of zinc and other transition metal cations [210,211]. Zinc and cadmium were shown induce a transcriptional upregulation of CzcD in *Alcaligenes eutrophus* – an orthologue of the yeast CDFs Zrc1 and Cot1 [211,212]. When that work was published, it had been revealed that disruption of Zrc1 and Cot1 sensitised the yeast to zinc and cobalt respectively [213,214]. Likewise, their overexpression conferred increased tolerance to the same metals [215]. It was later established that Zrc1 facilitates the transport of zinc from the yeast cytosol into the vacuole, thus providing a tolerance mechanism [161]. In addition to Zrc1 and Cot1, the yeast CDFs Zrg17 and Msc2 form a heteromer that mediates zinc uptake into the endoplasmic reticulum [216].

CDFs represent one of four main families of zinc transporters, with the others being ZIPs (ZRT-, IRT-like proteins), P-type ATPases and RND (resistance, nodulation, and division) multi-drug efflux proteins – with the latter exclusively being seen in prokaryotes [138]. In mammalian cells zinc transport is exclusively mediated by ZIPs and CDFs – with the latter commonly being referred to as ZnTs in the mammalian literature. ZIPs and CDFs have opposite and complementary functions, whereby ZIPs function by importing zinc into the cytosol, whereas CDFs facilitate its efflux, either into intracellular compartments, such as the endoplasmic reticulum or Golgi, or out of the cell all together [209]. This enables careful control of cytosolic zinc concentration, to prevent toxicity, whilst expediting the much-needed supply of zinc to various organelles.

Of course, as already discussed, many CDFs can transport various other transition metals in addition to zinc, including iron, cobalt, and manganese. Several key functional amino acid residues have been identified that determine substrate specificity across

various CDF orthologues. Notably, mutating certain residues in Zrc1 has been shown to alter substrate specificity to include iron or manganese [162,217]. Phylogenetic analysis by Montanini et al. revealed that the CDF substrate specificity can be predicted by where they fall in one of three clades – these being zinc, zinc/iron, and manganese [165]. Subsequent work further divided these groups to account for the ability of various CDFs to transport other metals such as copper, cobalt, cadmium and nickel [164,218,219].

4.1.1 Aims & objectives

The work in this chapter aimed to characterise the potential function of the sole predicted CDF from *P. falciparum* utilising heterologous expression in yeast and reverse genetic approaches in the asexual blood stage parasite.

Objectives

- Align the PfCDF amino acid sequence with orthologues with established function and perform phylogenetic analysis.
- Assess ability of PfCDF to confer tolerance to yeast strains that are sensitive to zinc, cobalt and iron when heterologously expressed.
- Establish sub-cellular localisation of PfCDF when expressed in yeast
- Generate PfCDF mutant parasite lines using the selection-linked integration method that enable determination of essentiality and sub-cellular localisation in the *P. falciparum* blood stages.

4.2. Results

4.2.1. The cation diffusion facilitator PfCDF

Plasmodium falciparum encodes a single cation diffusion facilitator (PF3D7_0715900), herein referred to as PfCDF [220]. A single CDF is also encoded by all the human malaria parasites, *P. berghei*, many other *Plasmodium* spp., as well as the related Apicomplexans *Toxoplasma gondii* and *Cryptosporidium parvum*. PfCDF is 556 amino acids in length, encoded across two exons, and like most of its orthologues is predicted to feature six transmembrane domains (Figure 4.1c). Multiple protein sequence alignments between PfCDF and selected orthologues showed 36.7%, 37.7% and 29% sequence identities with TgCDF, Zrc1 and the human CDF, HsZnT1 respectively. Phylogenetic analysis revealed that PfCDF sat in a clade with its Apicomplexan orthologues, as well as well-characterised zinc or zinc/cobalt specific CDFs from model organisms (Figure 4.1a). With that being said, close examination of the second predicted transmembrane domain revealed the presence of an amino acid that has been shown to alter substrate specificity in yeast orthologues [162,217]. Specifically, as indicated in Figure 4.1b, this is F40S, which has been shown to enable iron transport in addition to zinc in yeast.

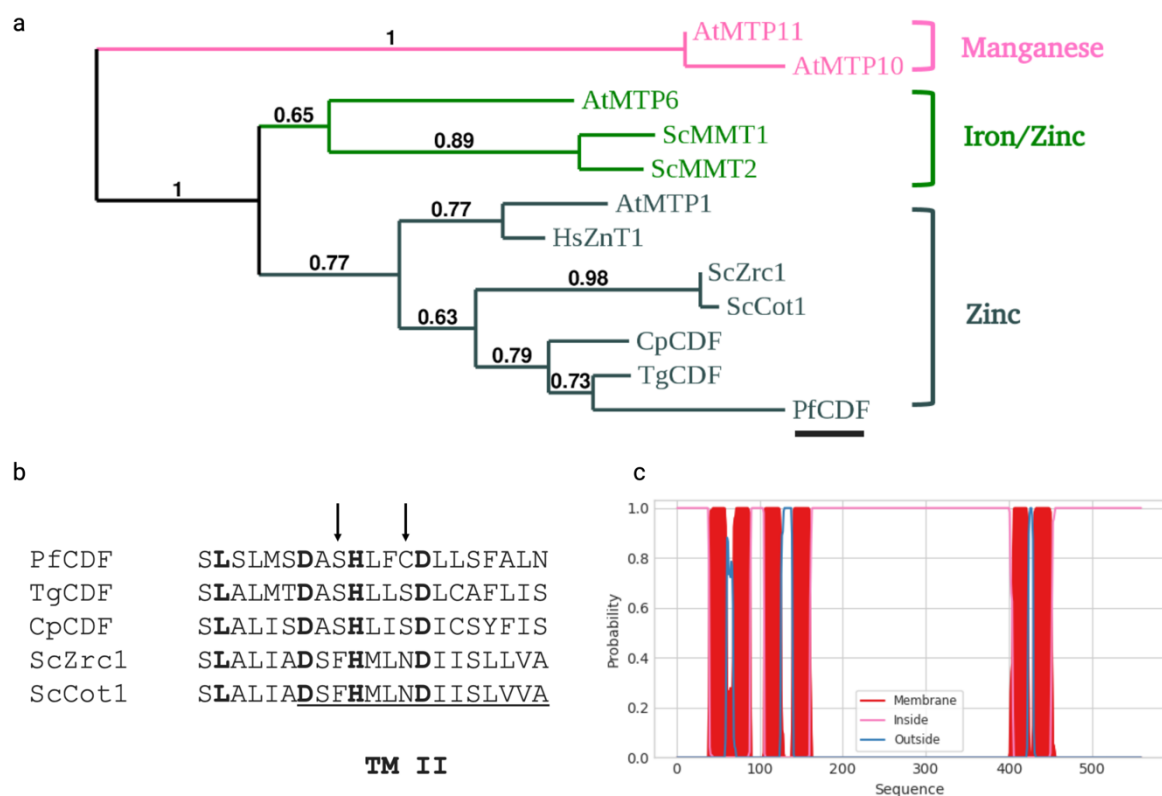


Figure 4.1. Substrate specificity predictions for PfCDF. (a) Phylogenetic analysis of PfCDF and functionally characterised orthologues show three distinct groups with different substrate specificities. The tree was constructed using Phylogeny.Fr. CDFs from the following organisms are represented; Tg = *Toxoplasma gondii*; Cp = *Cryptosporidium parvum*; Sc = *S. cerevisiae*; Hs = *Homo sapiens*; At = *Arabidopsis thaliana*. Bootstrap values are noted on nodes. (b) Multiple protein sequence alignment of the second predicted transmembrane domain (TM II) of PfCDF with related apicomplexans (*T. gondii* & *C. parvum*) and functionally characterised yeast CDFs. Conserved amino acid residues that have been shown to be required for zinc transport in other organisms are in bold. The two arrows indicate positions at which homologous residues to those in PfCDF in other organisms have been shown to alter substrate specificity to include Fe²⁺. (c) Transmembrane domain probability predictions based on amino acid sequence generated with DeepTMHMM [182].

4.2.2. Expression of PfCDF in a zinc-cobalt sensitive yeast strain

In yeast, the CDFs Zrc1 and Cot1 facilitate zinc and cobalt transport from the cytosol into the vacuole, thus enabling tolerance to zinc (and cobalt) replete conditions. As such, the mutant $\Delta zrc1cot1$ yeast line, which was created by disrupting the genes that encode Zrc1 and Cot1, is sensitive to high concentrations of zinc and cobalt. To explore whether PfCDF might act in a similar manner to Zrc1 and Cot1, a yeast codon optimised PfCDF coding sequence was cloned into the yeast expression vector p415GPD by yeast gap-repair cloning and transformed into $\Delta zrc1cot1$ (Figure 4.2). Transformation success was determined by PCR amplification of the PfCDF from extracted plasmid DNA. In parallel, the $\Delta zrc1cot1$ was reconstituted with Zrc1. This was achieved by cloning Zrc1, which had been amplified from extracted yeast genomic DNA, into p415GPD and subsequent transformation. The resultant line was used as a growth control. $\Delta zrc1cot1$ transformed with p415GPD was used as a negative control.

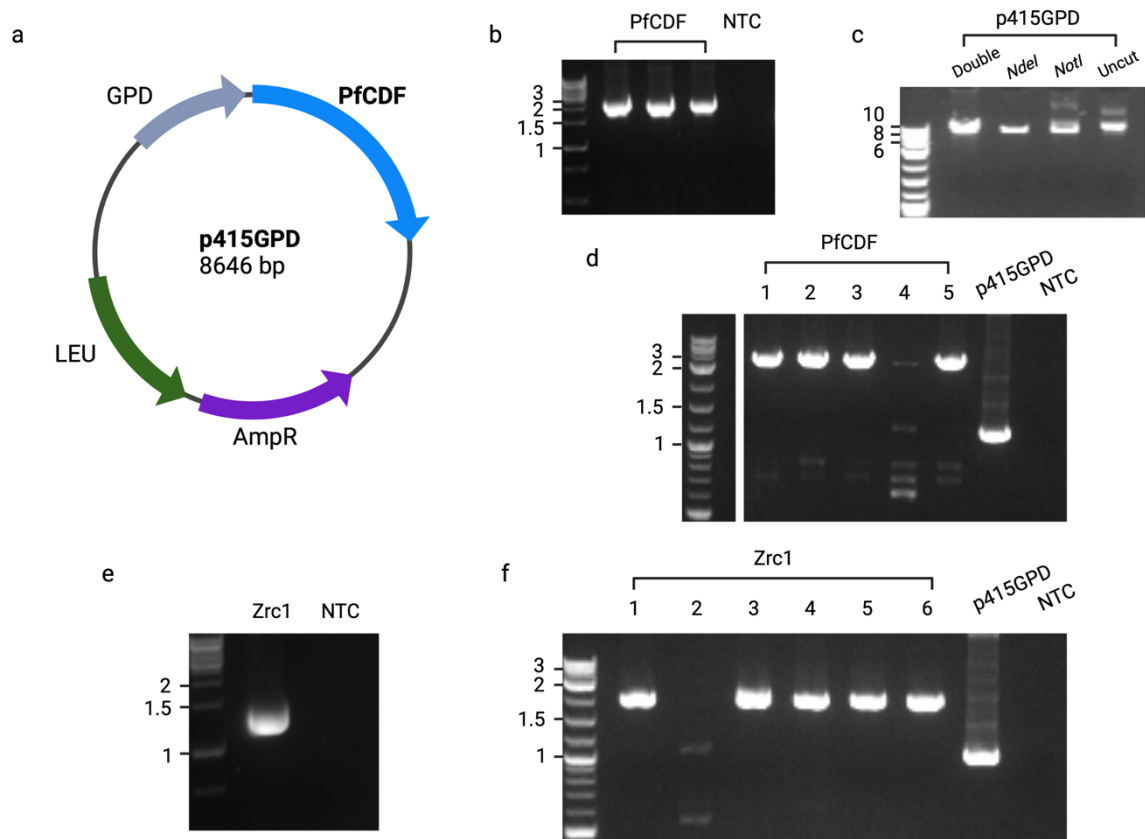


Figure 4.2. Cloning PfCDF into p415GPD and transformation into $\Delta zrc1cot1$ yeast (a)

PfCDF was episomally expressed from p415GPD under the control of the GPD promoter. p415GPD selection in yeast and bacteria is enabled by the LEU (leucine) and AmpR (ampicillin) cassettes respectively. p415GPD-PfCDF was constructed by yeast gap repair cloning. **(b)** The PfCDF coding sequence was amplified from the vector pUC57 by gap-repair PCR (grPCR) using primers 19 and 20, and column purified. Expected amplicon size = 2308 bp. **(c)** p415GPD was digested with *NdeI* and *NotI* and column purified **(d)** The grPCR and restriction digest products were transformed into $\Delta zrc1cot1$ and successful cloning was confirmed by extracting plasmid DNA from the transformed yeast after 72 h of culture and PCR amplification of the cloned insert using the primers 17 and 18. Five yeast colonies were screened and a parallel reaction that used purified p415GPD as template was included as a control. NTC = no template control. Expected band sizes 1. PfCDF = 3400 bp, 2. p415GPD = 1092 bp **(e)** The Zrc1 coding sequence was amplified from extracted yeast genomic DNA by grPCR and cloned into p415GPD by yeast gap-repair cloning as was done for PfCDF. Expected band size = 1329 bp. **(f)** Transformation and cloning success were established by

screening six transformed yeast colonies using primers – as was the case for PfCDF.

Expected band size = 2421 bp. DNA ladders in kilobases.

To explore subcellular localisation and confirm that PfCDF was being expressed in $\Delta zrc1cot1$, said strain was transformed with p415GPD-PfCDF-eGFP, which produced a PfCDF with a C-terminal green fluorescent protein tag (Figure 4.3). The transformed yeast were then imaged by fluorescence microscopy. Prior to imaging, the yeast were cultured in SD to a cell density of 0.5 (mid-log) to ensure that only actively dividing yeast were imaged so to limit the detection of autofluorescence. Fluorescent signal was observed only when using the green channel of the microscope. The punctate, intracellular signal pattern suggests that PfCDF is primarily being localised to membranous structures in the cytosol, rather than the vacuolar or plasma membrane.

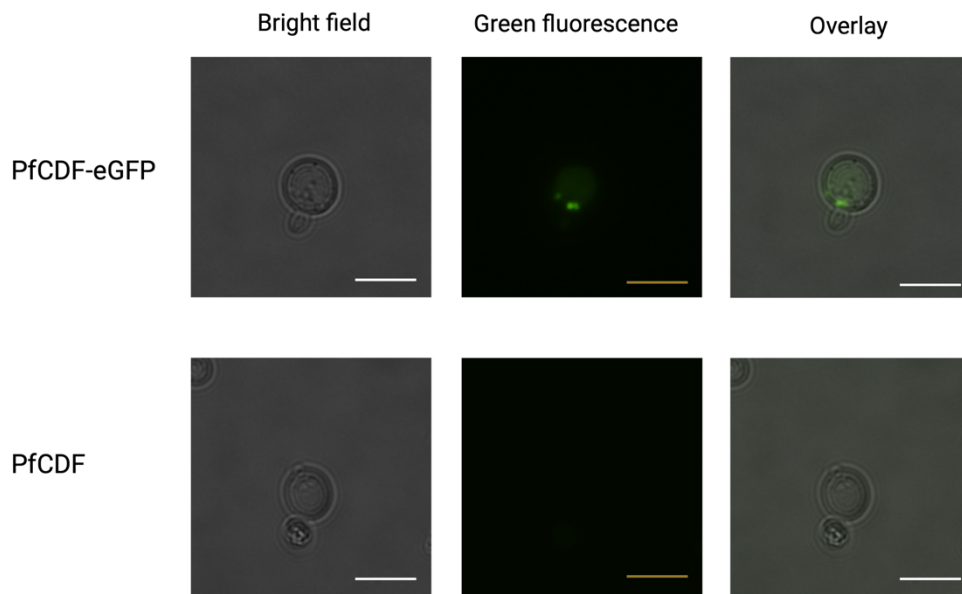


Figure 4.3. Expression of PfCDF-GFP in $\Delta zrc1cot1$ yeast. Yeast transformed with either p415GPD-PfCDF or p415GPD-PfCDF-eGFP were cultured to an OD_{600nm} of 0.5 and live cell imaged under 60x magnification. Scale bar = 5 μm .

$\Delta zrc1cot1$ transformed with p415GPD-PfCDF, p415GPD-Zrc1 or p415GPD (empty vector) were cultured in SD at 30°C and spotted onto SD-agar featuring three different concentrations of $ZnSO_4$ – 0, 75 and 150 μM . After 72 h of culture, comparable levels of growth can be seen for all three lines at 0 μM $ZnSO_4$ (Figure 4.4a). As the zinc concentration increases however, the growth of the Zrc1 recomplemented line is seemingly unaffected, however the growth of the PfCDF line is severely impaired and comparable to that of the empty vector control.

In liquid media the same strains, once again, showed comparable growth when cultured in 0 μM $ZnSO_4$ (Figure 4.4b). As expected, the Zrc1 line showed comparable levels of growth when cultured in all zinc concentrations that were tested. In 75 μM $ZnSO_4$ the PfCDF line reached a significantly lower cell density after 72 h of culture than the positive control and had comparable levels of growth to the negative control (Figure 4.4c). In 125 μM $ZnSO_4$ the PfCDF line, once again, reached a significantly lower cell density than the positive control, however it showed significantly greater levels of growth than the vector only control, thus conferring a partial tolerance to zinc (Figure 4.4d). Culture in 250 μM $ZnSO_4$ almost entirely ablated the growth of both the PfCDF and negative control strains, whilst the growth of Zrc1 was unaffected (Figure 4.4e).

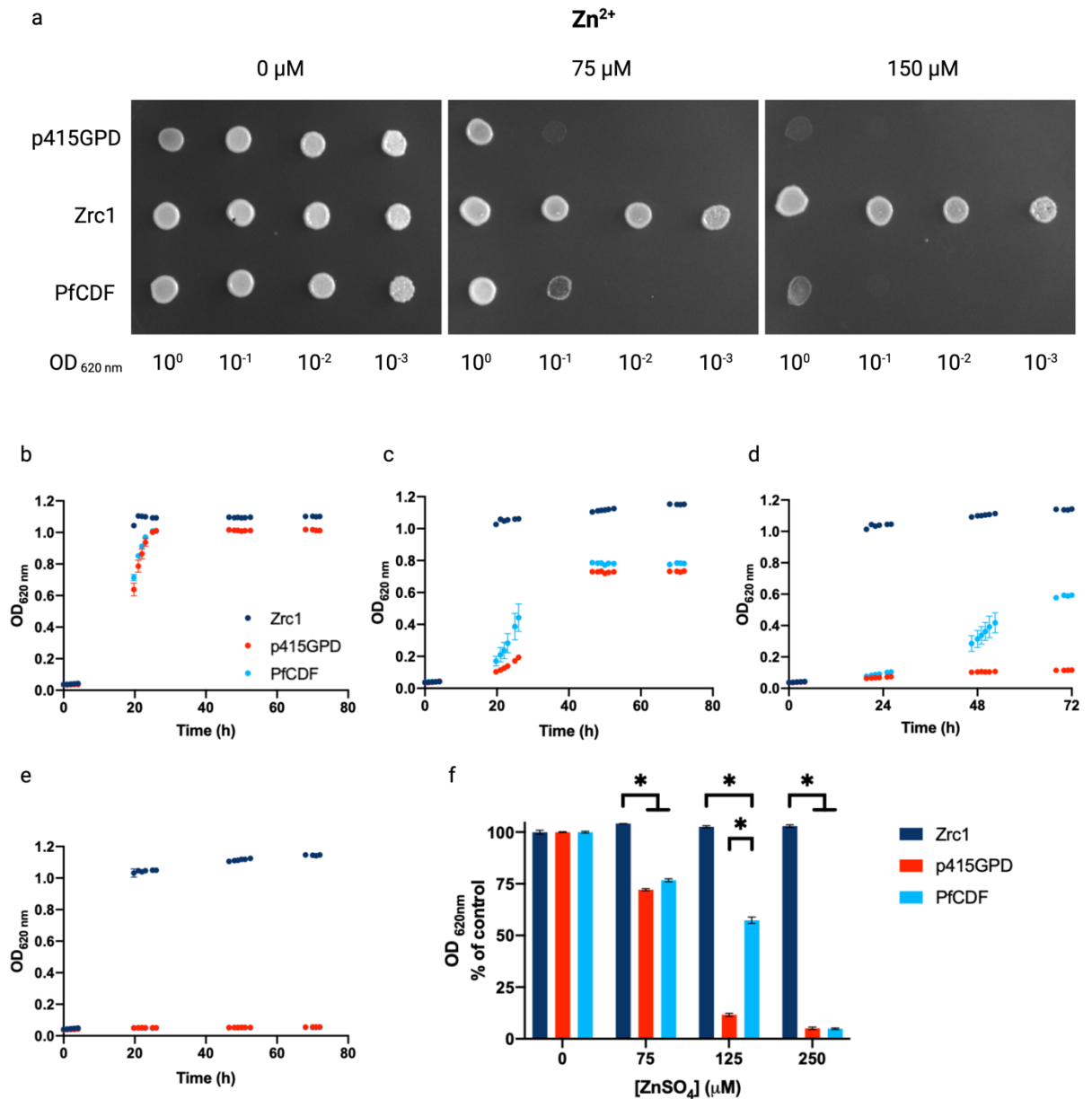


Figure 4.4. Zinc tolerance assays in solid and liquid media. (a) $\Delta zrc1cot1$ yeast featuring p415GPD-PfCDF, p415GPD-Zrc1, or p415GPD (empty vector) were cultured in SD to a cell density of 1 (OD_{620 nm}) and a four-point, ten-fold dilution series was set up. Yeast was spotted onto SD-agar that lacked leucine and featured either 0, 75, or 150 μ M ZnSO₄. The yeast was imaged after 72 h of culture at 30°C. (b-e) The yeast lines described in a were diluted 1:100 in SD media that featured either 0, 75, 125, or 250 μ M ZnSO₄ and cultured for 72 h at 30°C with cell density being measured periodically. Each data point represents the mean of three repeated measures of the same cultures in different wells of a culture plate, with each curve

being representative of three independent experiments. **(f)** Mean cell densities ($\pm$ SEM) for each yeast line after 72 h growth in different ZnSO_4 concentrations normalised to 0 μM ZnSO_4 . Differences between means at each concentration were assessed using two-way ANOVA with Tukey's multiple comparisons test. Asterisk indicates statistical significance ($P < 0.05$).

As $\Delta\text{zrc1cot1}$ is also sensitive to cobalt replete conditions, the same strains described in the previous paragraphs were used to test whether PfCDF could confer some level of tolerance to cobalt in addition to zinc. $\Delta\text{zrc1cot1}$ transformed with p415GPD-PfCDF, p415GPD-Zrc1 or p415GPD (empty vector) were cultured in SD at 30°C and spotted onto SD-agar featuring three different concentrations of $\text{Co}(\text{NO}_3)_2$ – 0, 75 and 150 μM (Figure 4.5a). After 72 h of culture comparable levels of growth can be seen for all three lines at 0 μM $\text{Co}(\text{NO}_3)_2$. As the cobalt concentration increases however, the growth of the Zrc1 recomplemented line is seemingly unaffected, however the growth of the PfCDF line is severely impaired compared and comparable to that of the empty vector control.

When cultured in liquid media, as was the case for zinc, the three lines reached comparable cell densities when no cobalt was added to the media. Supplementation with 75 and 150 μM $\text{Co}(\text{NO}_3)_2$ appeared to slightly reduce the cell density that all three yeast lines reached, but all by comparable relative amounts. Supplementation with 300 μM $\text{Co}(\text{NO}_3)_2$ caused a significant reduction in cell density after 72 h of culture for the PfCDF line compared to the positive control and had a growth phenotype that was comparable to that of the negative control line.

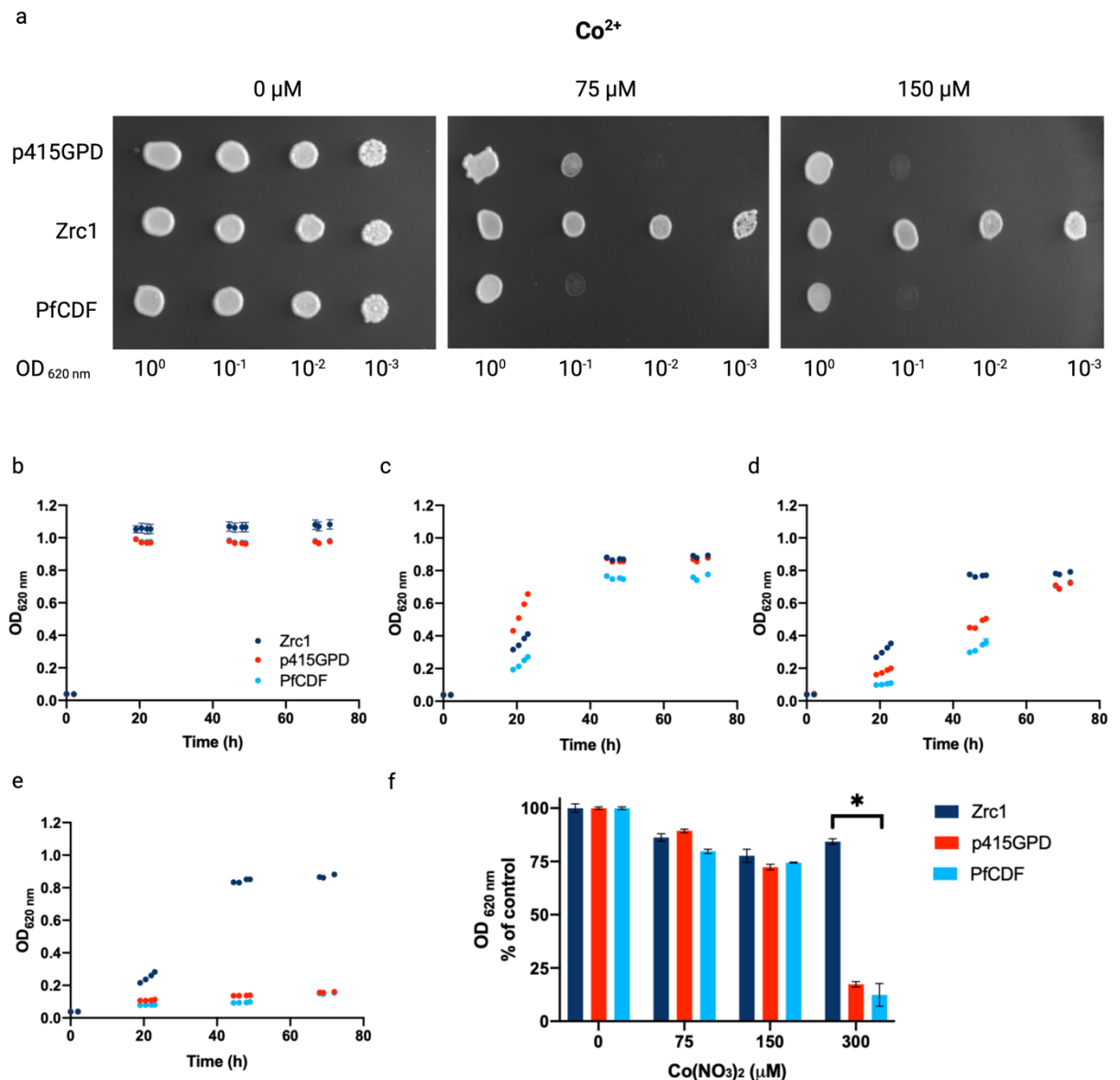


Figure 4.5. Cobalt tolerance assays in solid and liquid media. (a) Δ zrc1cot1 yeast featuring p415GPD-PfCDF, p415GPD-Zrc1, or p415GPD (empty vector) were cultured in SD to a cell density of 1 (OD_{620 nm}) and a four-point, ten-fold dilution series was set up. Yeast was spotted onto SD-agar that lacked leucine and featured either 0, 75, or 150 μ M Co(NO₃)₂. The yeast was imaged after 72 h of culture at 30°C. (b-e) The yeast lines described in a were diluted 1:100 in SD media that featured either 0, 75, 150, or 300 μ M Co(NO₃)₂ and cultured for 72 h at 30°C with cell density being measured periodically. Each data point represents the mean of three technical replicates, with each curve being representative of three independent experiments. (f) Mean cell densities ($\pm$ SEM) for each yeast line after 72 h

growth in different $\text{Co}(\text{NO}_3)_2$ concentrations normalised to 0 μM $\text{Co}(\text{NO}_3)_2$. Differences between means at each concentration was assessed using two-way ANOVA with Tukey's multiple comparisons test. Asterisk indicates statistical significance ($P < 0.05$).

4.2.3. Expression of PfCDF in an iron-sensitive yeast strain

Bioinformatic analysis identified an amino acid residue in PfCDF that has been shown to enable iron transport in Zrc1. To test whether PfCDF could confer iron tolerance to an iron sensitive yeast line, the yeast codon optimised PfCDF coding sequence was cloned into the yeast expression vector pUGPD and transformed into the iron-sensitive yeast line ΔCCC1 (Figure 4.6a). Transformation success was confirmed by PCR (Figure 4.6b).

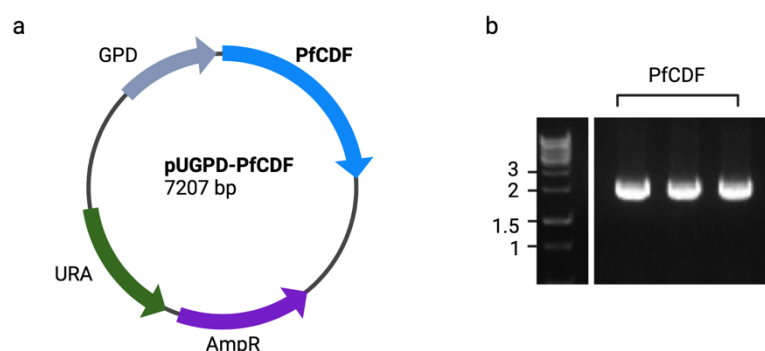


Figure 4.6. Expressing PfCDF in ΔCCC1 yeast. (a) PfCDF was episomally expressed from pUGPD under the control of the GPD promoter. pUGPD selection in yeast and bacteria is enabled by the URA (uracil) and AmpR (ampicillin) cassettes respectively (b) Transformation success for three yeast colonies confirmed by PCR using primers 9 and 10. Expected amplicon size = 2061 bp. NTC = no template control. DNA ladder in kilobases.

To establish whether PfCDF could confer tolerance to Δ CCC1 in iron-replete conditions, Δ CCC1 transformed with pUGPD-PfCDF was spotted onto SD-agar featuring three different FeSO₄ concentrations – 0, 1 and 3 mM (Figure 4.7a). In addition, Δ CCC1 was transformed with empty pUGPD, which was used as a negative growth control. A recomplemented CCC1- Δ CCC1 yeast line was used as a positive control. After 48 h of culture at 30°C, all three yeast lines had comparable growth on SD-agar with no added iron. On SD-agar that was supplemented with 1 mM of iron, yeast transformed with pUGPD-PfCDF had comparable levels of growth to the negative empty vector control and noticeably less growth than the positive control. Furthermore, this effect was more pronounced when cultured on SD-agar supplemented with 3 mM of iron. In liquid media the same trend was observed, whereby culture in both 1 and 3 mM FeSO₄ for 72 h significantly reduced the growth of the PfCDF and negative control lines in comparison to the recomplemented CCC1- Δ CCC1 line whose growth was seemingly unaffected (Figure 4.7b-f).

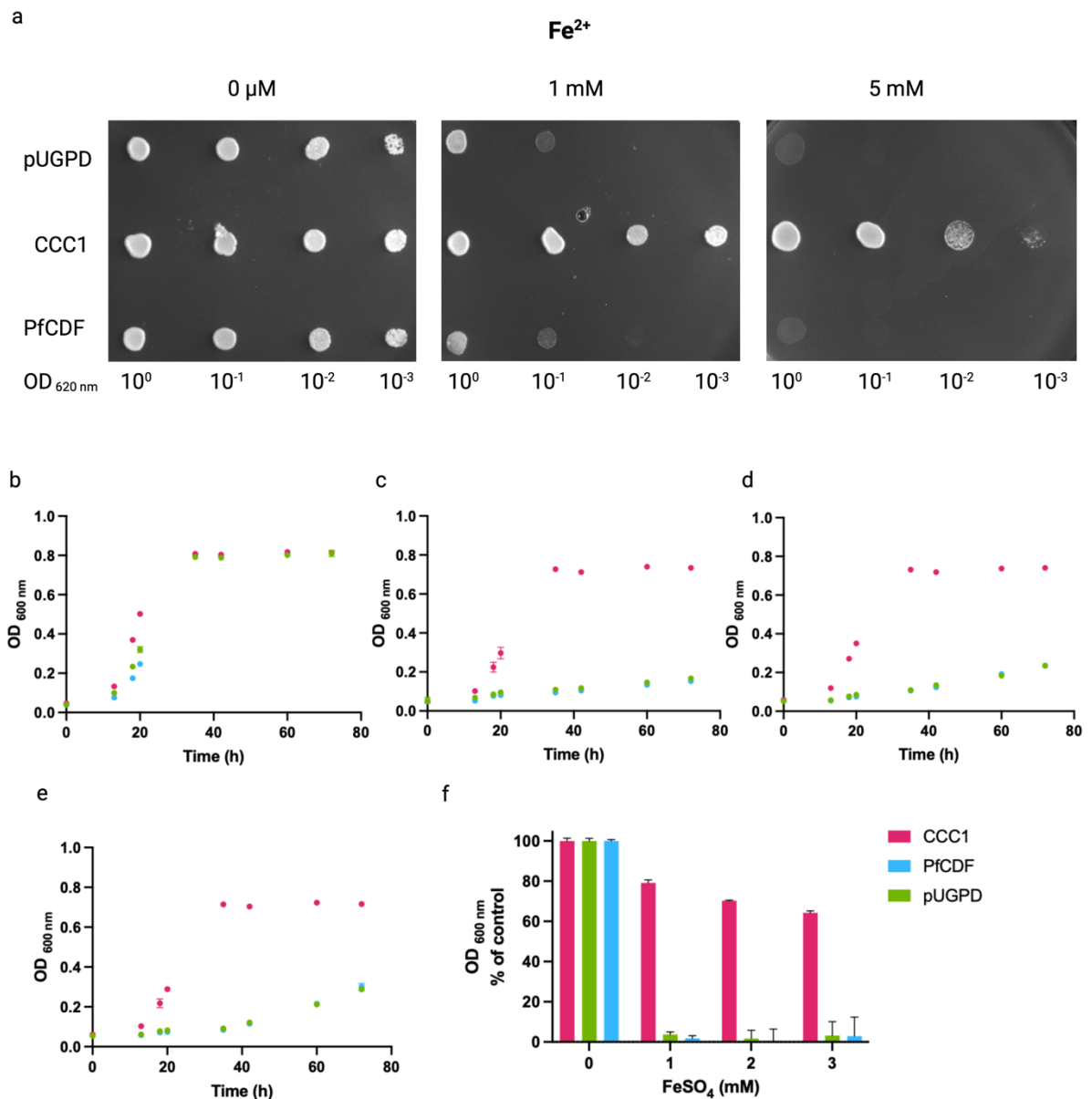


Figure 4.7. Iron tolerance assays in solid and liquid media (a) Δ CCC1 yeast transformed with was cultured in SD to a cell density of 1 (OD_{600 nm}). In parallel, Δ CCC1 yeast transformed with pUGPD (empty vector) and the recomplemented Δ CCC1-CCC1 (see previous chapter) were also cultured. For each line, a four-point, ten-fold dilution series was set up. Yeast was spotted onto SD-agar that lacked leucine and featured either 0, 1, or 5 mM FeSO₄ and ascorbate. The yeast was imaged after 72 h of culture at 30°C. **(b-e)** The yeast lines described in **a** were diluted 1:100 in SD media that featured either 0, 1, 2, or 3 mM FeSO₄ and ascorbate and cultured for 72 h at 30°C with cell density being measured periodically. Each data point represents the mean of three technical replicates, with each

curve being representative of three independent experiments. **(f)** Mean cell densities ($\pm$ SEM) for each yeast line after 72 h growth in different FeSO₄ concentrations normalised to 0 mM FeSO₄.

4.2.4. Reverse genetic approaches to study PfCDF in *P. falciparum*

Heterologous expression in metal sensitive yeast strains suggested that PfCDF might function as a transporter of zinc in *P. falciparum*. To test whether PfCDF plays a role in zinc homeostasis in the blood stage malaria parasite, we sought to generate a mutant parasite line that expressed a truncated and therefore non-functional PfCDF protein. The production of such a line would suggest that PfCDF is not essential to the *P. falciparum* blood stages. Furthermore, to explore the expression of PfCDF and specifically its sub-cellular localisation during the blood stages, we sought to generate a fluorescently tagged line.

Highly synchronous *P. falciparum* 3D7 ring-stage parasites were transfected by electroporation with one of the two selection-linked integration vectors pSLI-PfCDF.mNeon1.1 and pSLI-PfCDF.mNeon1.2 (Figure 4.8b). pSLI-CDF.mNeon1.1 featured the first 500 base pairs of the PfCDF coding sequence followed by an mNeonGreen fluorescent tag and a neomycin drug resistance cassette separated by a self-cleaving peptide (Figure 4.8a). The presence of the 500 bp homology region was to enable a single homologous crossover of the entire vector into the PfCDF locus of the parasite genome. This would result in the expression of an N-terminally truncated and functionally inert protein. pSLI-PfCDF.mNeon1.2 was exactly the same as pSLI-CDF.mNeon1.1 other than it featured the last 500 base pairs of PfCDF and thus would result in a full-length, tagged protein.

After 23 days of drug pressure with WR99210 to select for parasites that possessed vector, WR99210-resistant parasites were identified on a Giemsa-stained thin blood film for parasites transfected with pSLI-CDF.mNeon1.2. Drug pressure with WR99210 was

removed five days later and selection-linked integration of the vector was forced by treatment of the cultures with G418. After 40 days of culture under drug pressure with WR99210, parasite transfected with pSLI-CDF.mNeon1.1 were not detected, and the culture was abandoned. Successful uptake of the pSLI-CDF.mNeon1.2 plasmid was confirmed by PCR, however integration of the plasmid into the genome could not be confirmed (Figure 4.8c).

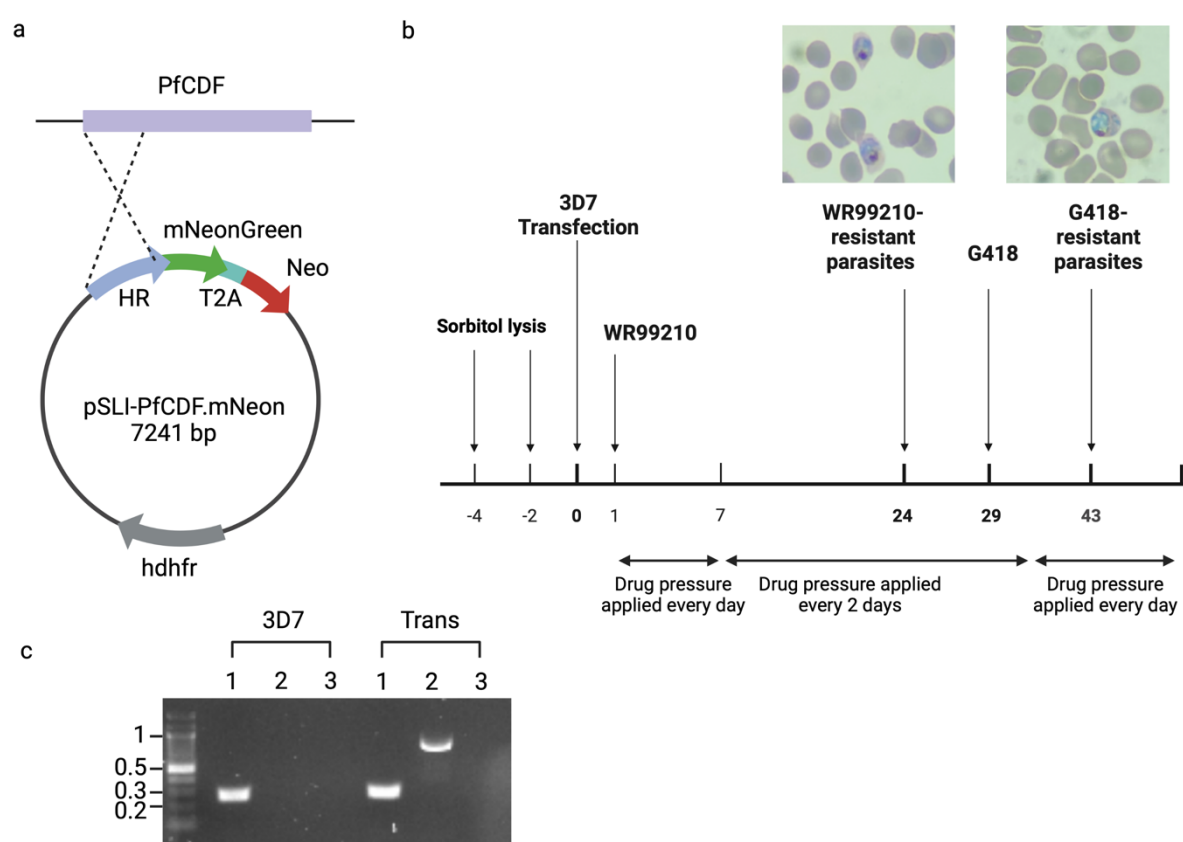


Figure 4.8. Mutagenesis of PfCDF by selection-linked integration. (a) The pSLI-PfCDF.mNG vector features a homology region (HR) that comprises the last 500 bp of the PfCDF coding sequence (minus the stop codon) linked to an mNeonGreen fluorescent tag. The tag is separated from a neomycin selection cassette by the self-cleaving peptide T2A. The drug selection cassette, human dihydrofolate reductase (hdhfr) allows for selection of the plasmid with WR99210 in blood stage *P. falciparum*. Targeted integration of the plasmid into the PfCDF locus of the *P. falciparum* genome is facilitated by a single crossover

recombination and is selected for using neomycin analogue G418. **(b)** Highly synchronous *P. falciparum* ring stage parasites were transfected with one of two versions of pSLI-PfCDF.mNeon by electroporation. pSLI-PfCDF.mNeon1.1 features a HR at the 5' end of the PfCDF gene, thus aiming to produce a N-terminally truncated protein. pSLI-PfCDF.mNeon1.2 features a HR at the 3' end of the PfCDF gene, thus aiming to produce a mNeonGreen-tagged full-length protein. Parasites were synchronised by sorbitol lysis 48 and 96 h prior to transfection. Seventy-five micrograms of each plasmid were used per transfection. A negative transfection control was established, whereby parasites were electroporated in the absence of any plasmid. For 18 h post electroporation, the transfected parasites were cultured in standard parasite culture media and after this point the media featured 2.5 nM WR99210 to select for the vector. Media was changed every 24 h thereafter for seven days and then every 48 h for 22 days. WR99210-resistant parasites were cultured in G418 (250 µg/ml) thereafter. **(c)** DNA was extracted from parasites transfected with pSLI-CDF.mNeon1.2 (Trans) and a wildtype control (3D7) and used as template for PCR. 1. Primers 25 and 27 to detect the PfCDF locus – expected amplicon = 270 bp, 2. Primers 29 and 30 to detect the plasmid – expected amplicon = 777 bp, 3. Primers 33 and 34 to demonstrate integration of plasmid into the genome. DNA ladder in kilobases.

As PfCDF is unessential to the blood stages of *P. falciparum*, but its orthologue in *P. berghei* has been shown to be important to the sexual, insect and liver stages of that parasite [221,222], we sought to generate a two NF54 lines that expressed a truncated and thus non-functional PfCDF and a full-length fluorescent tagged protein – as was aimed in 3D7. Unlike 3D7, NF54 produces sexual stages of *P. falciparum* in vitro and can therefore be used to infect a female *Anopheles* mosquito thus enabling study of proteins in the mosquito vector.

To generate transgenic NF54 parasites, uninfected, or “empty”, RBCs were transfected with the two vectors by electroporation and then highly synchronous late-stage parasites

were added to the RBCs, thus resulting in merozoites invading RBCs that had been “pre-loaded” with vector DNA (Figure 4.9a). Drug pressure with WR99210 to select for the vector was applied 48 h post transfection and was maintained until drug resistant parasites emerged and exceeded a parasitaemia of 1%. Drug pressure with WR99210 was then replaced with pressure from G418. Thirty-four days after commencing pressure with G418 there were no parasites detectable by Giemsa-stained thin blood film and accordingly the cultures were abandoned. The described transfection was repeated a second time which resulted in the same outcome.

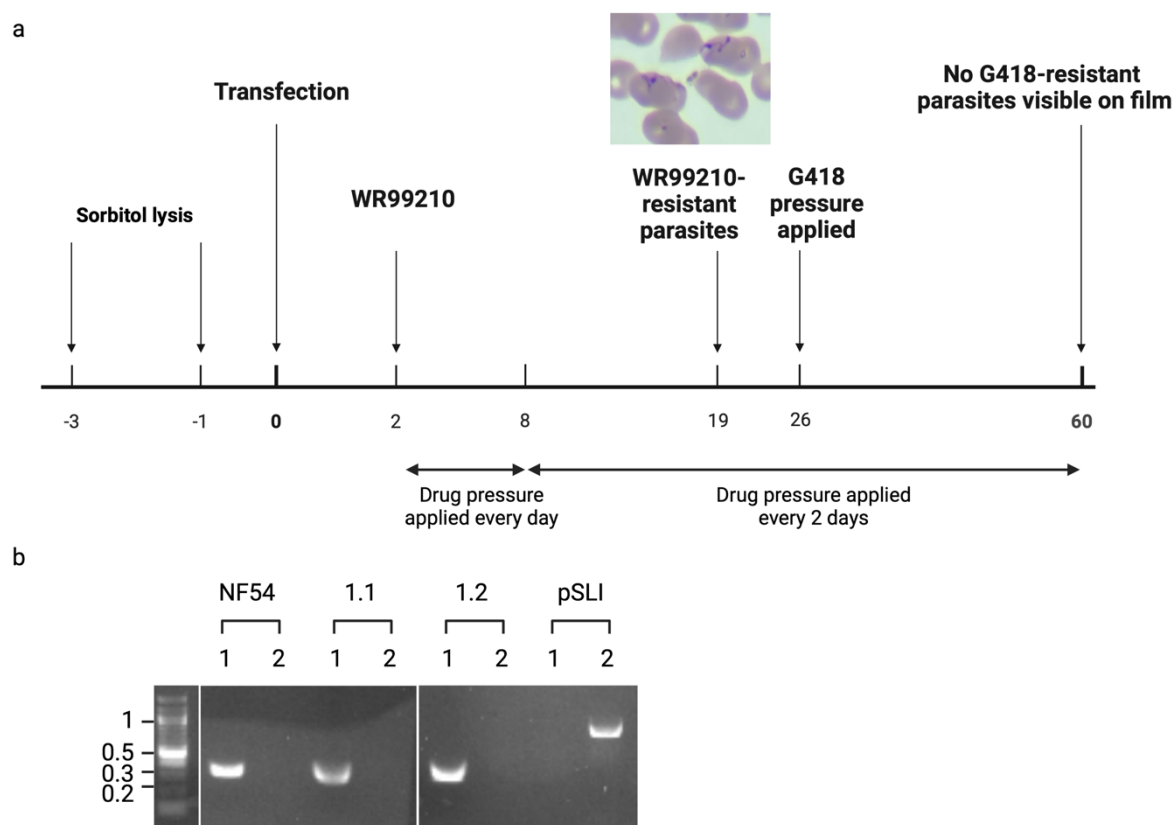


Figure 4.9 – Electroporation of uninfected RBCs with pSLI-PfCDF.mNeon and subsequent transfection of *P. falciparum* NF54. (a) Fresh human RBCs (O+) were transfected with seventy-five micrograms of each of the two pSLI-PfCDF.mNeon vectors and subsequently infected with highly synchronous late-stage *P. falciparum*. Parasites were synchronised 30 and 78 h prior to transfection and added to transfected RBCs. Drug pressure was applied 48 h after transfection with WR99210 (2.5 nM) to select for parasites

containing the vector. Media was changed every 24 h for a further 6 d and then every 48 h for 18 d. WR99210-resistant parasites were cultured in G418 (250 µg/ml) thereafter. **(b)** DNA was extracted from parasites transfected with pSLI-CDF.mNeon1.1 (1.1) and pSLI-CDF.mNeon1.2 (1.2) and a wildtype control (NF54) and used as template for PCR, purified pSLI-CDF.mNeon1.1 (pSLI) was also used as template. 1. Primers 25 and 27 to detect the PfCDF locus – expected amplicon = 270 bp, 2. Primers 29 and 30 to detect the plasmid – expected amplicon = 777 bp. DNA ladder in kilobases.

4.3. Discussion

Cation diffusion facilitators play key roles in enabling cells from organisms from right across the tree of life to regulate and detoxify transition metals. The work presented in this chapter demonstrates that the sole CDF expressed by *P. falciparum* likely functions to mediate the transport of zinc and potentially other metals such as iron. PfCDF was shown to confer partial zinc tolerance to a zinc sensitive yeast line, but not to cobalt or iron. These findings led to attempts to generate novel mutant parasite lines that would enable the study of the role of PfCDF in transition metal homeostasis in the parasite itself.

4.3.1. PfCDF expressed in yeast

As discussed in the previous chapter, heterologous expression experiments in yeast have enabled the phenotypic and functional characterisation of countless putative transporters, including several CDFs [184,223–225]. Notably, this now includes recent work undertaken on the CDF orthologue from *Toxoplasma gondii*, which was shown to confer tolerance to zinc when expressed in $\Delta zrc1cot1$ [166]. It is also notable that TgCDF was not examined for a potential ability to confer tolerance to cobalt or iron – as was done here for PfCDF.

Substrate specificity differs among CDFs but importantly only includes a subset of divalent metal cations. In some of the organisms that encode multiple CDFs, different CDFs often feature different substrate specificities – this is seen for example in *A. thaliana*, whereby different CDFs (or MTPs as they are often referred to in the plant literature) transport zinc, iron and manganese [226,227]. Furthermore, in such organisms redundancy is often observed among the CDFs, whereby multiple transporters have the same substrate specificity – such as AtMTP10 and 11 which both transport manganese [228]. Phylogenetic analysis revealed that PfCDF grouped with zinc-specific CDFs from

humans, yeast and *A. thaliana* and this prediction was backed up by the yeast data presented in this chapter.

As noted in the previous section, numerous amino acid residues in Zrc1 have been shown, when mutated, to alter substrate specificity. Notably, the F40S mutation, in particular, begged the question whether PfCDF might transport iron, addition to zinc. Expression of PfCDF in the iron-sensitive yeast line Δ CCC1 did not do anything to substantiate this theory. Lin et al. demonstrated that the N44I mutation in Zrc1 altered the substrate specificity of the protein from Zn^{2+} to Fe^{2+} and Mn^{2+} [162]. Then, using a similar approach to that employed in section 3.2.4, the same group undertook a screen that used an error-prone DNA polymerase to generate Zrc1 mutants that revealed several other mutations that enabled Δ CCC1 to tolerate iron replete conditions [217]. Notably, many of the mutations led to the apparent loss of zinc function. To further explore and better understand the roles of various amino acids on PfCDF substrate specificity, a similar approach could be undertaken. Furthermore, due to the phylogenetic predictions, experiments that tested tolerance to zinc, cobalt and iron were prioritised in this work, however future work could include exploring a potential role for PfCDF in manganese tolerance. It is noteworthy that no malarial protein has yet been identified to play a role in either the detoxification or acquisition of manganese.

C-terminal tagging of PfCDF demonstrated that PfCDF was being expressed in Δ zrc1cot1, however it was clear from the signal pattern that the protein was not solely being expressed on the plasma membrane or the membrane of the food vacuole. As PfCDF was shown to confer tolerance to zinc, presumably by expediting its elimination from the yeast cytosol, one might expect to observe localisation to either the plasma membrane or the vacuolar membrane – where the transporter would provide an efflux mechanism. In general, to gain an understanding of the sub-cellular localisation of a protein, co-localisation experiments should be performed. It is plausible that mislocalisation could have accounted for the partial rescue growth phenotype PfCDF

conferred to $\Delta zrc1cot1$ when cultured in 125 μM ZnSO_4 , whereby the protein being localised to the vacuolar membrane could have resulted in a growth phenotype akin to that of the Zrc1-recomplemented line. Issues with expressing functional protein in a heterologous system, including mislocalisation, are commonplace and have been overcome by modifying the amino acid sequence to remove N-terminal localisation domains, as well as expressing chimeric proteins that feature part of an endogenous host protein, and codon optimisation [134,229–232].

4.3.2. PfCDF in the malaria asexual blood stages

Large-scale knock-out screens have demonstrated that PfCDF is unessential to the asexual blood stages of *P. falciparum* and *P. berghei* [222,233]. However, it was shown that in *P. berghei*, disruption of PbCDF resulted in reduced, but not completely ablated, male sexual development, numbers of sporozoites in the salivary glands of the vector and prepatent liver development time [221]. Here we attempted to generate *P. falciparum* lines that expressed a severely N-truncated PfCDF, which would be functionally inert, on the 3D7 and NF54 genetic backgrounds. In general, these functional knockout lines would enable one to explore the effect of zinc, and other transition metals, on the growth phenotype of *P. falciparum* lacking functional PfCDF and therefore differences to the wild type would give hints on protein function. Unlike 3D7, NF54 readily produces the sexual life cycle stages and can therefore be used to infect anopheline mosquitos, thus enabling study of the effect of a gene knockout beyond the asexual blood stages. It is noteworthy that it is anecdotally more challenging to genetically manipulate NF54 than 3D7, which is why transfections were undertaken with both parasite lines. In addition to attempting to create lines that featured a truncated PfCDF, we also attempted to generate lines in which PfCDF featured a C-terminal fluorescent tag, which would have enabled localisation studies to have been performed. No such lines were generated. A recently published study showed PfCDF to localise within the cytosol during the asexual blood stages – as discussed above regarding

PfCDF localisation in yeast, due to the nature of PfCDF, it is likely found on membranous structures within the cytosol [167].

With regards to potential functional redundancy, while it is clear that there likely is not a second malarial CDF, the existence of other yet-to-be-identified zinc transporters that could function analogously is a possibility. The apparent inessentiality of PfCDF asks the question whether the malaria parasite has an absolute requirement for a zinc detoxification mechanism and if so, what is performing this role when PfCDF is functionally knocked-out. It is noteworthy however, that it has been shown that the vacuolar iron transporter PfVIT does not confer zinc tolerance to $\Delta zrc1cot1$ and thus seems unlikely to play any kind of role in zinc homeostasis [134]. Furthermore, considering that they primarily function as zinc importers in other systems, the two malarial ZIP homologues seem like unlikely candidates as potential zinc detoxifiers.

In aiming to generate PfCDF mutant parasite lines, parasites were transfected with a single plasmid that was selected for by with WR99210, and for the 3D7 transfections, this was successful as shown by PCR. It is unclear however, why following treatment with the second drug, and the emergence of G418-resistant parasites, successful integration of the plasmid could not be confirmed by PCR. Furthermore, for the NF54 transfections, plasmid DNA could not be detected following the emergence of WR99210-resistant parasites and unsurprisingly, treatment of the culture with the second drug G418 did not yield a population of parasites resistant to that drug. It is noteworthy that shortly after the second round of NF54 transfections we were notified by collaborators that the NF54 line that they had kindly gifted us to perform these experiments had been unknowingly contaminated with a WR99210-resistant line, thus explaining why WR99210-resistant parasites appeared but integration of a transfected vector into the genome was not possible.

5. On iron homeostasis in *Plasmodium falciparum*

5.1. Introduction

The role of iron in human malaria has long been hotly debated due to several observational studies that link host iron deficiency and some protection from falciparum malaria [234–236]. Furthermore, and intriguingly, a clinical trial conducted in Pemba – an island off the coast of Tanzania – in 2002-03 reported that routine supplementation with iron and folic acid in children resulted in an increase in severe malaria and mortality, which understandably led to the early cessation of the trial [237]. It is noteworthy that the Pemba trial was preceded by a randomised controlled trial on the Tanzanian mainland, which reported that supplementation with iron did not increase malaria risk in children [238]. Likewise, subsequent, albeit smaller, trials in different parts of Africa reported similar findings [239]. This was then corroborated by a meta-analysis [240].

Nevertheless, it is noteworthy that unlike the Pemba trial, other trials have featured measures to reduce malaria prevalence among study participants, such as insecticide-treated bed nets and preventative treatment. It is therefore possible that these measures play a role in reducing the previously seen risks of iron supplementation. With all considered, it remains somewhat unclear to what extent iron deficiency provides protection against malaria.

When considering malaria at the cellular level, it seems plausible that as is the case with other pathogens, a host environment that limits access to iron would be less hospitable to *Plasmodium*. It has been shown that *P. falciparum* shows a reduced invasion capacity and merozoite production, when cultured in vitro in RBCs that were provided by iron deficient donors compared to RBCs from iron replete donors [241,242]. Furthermore, Clark et al. showed that this effect can be reversed by the donor supplementing with iron, thus lending credence to the idea that iron deficiency may offer some protection against *P. falciparum* [241].

In the liver, supplementation with iron has been shown to enhance the development of the murine malaria parasite *Plasmodium yoelii* [243]. However, there are conflicting results on the effect of iron deficiency in the same model [244]. As discussed in previous chapters, the seminal work of Portugal et al. links ongoing blood stage malaria with liver hypoferraemia, showing a mechanism whereby hepcidin mediates a reduction in iron concentration in hepatocytes (and elsewhere) and thus prevents superinfection [127].

As has been stated numerous times throughout this thesis, it remains unclear how the malaria parasite acquires host iron when residing in the hepatocyte or the RBC. While early work suggested that iron was acquired by the blood-stage parasite bound to host transferrin via endocytosis, the dearth of data to support this hypothesis over the last thirty years indicates that this is unlikely the main route for iron import [121,125]. In other organisms, such as yeast, iron acquisition is facilitated by transporters [152]. The malaria parasite possesses a number of candidate proteins that might play a role in iron acquisition, and these were introduced in the opening chapter. These include the two ZIP orthologues, PfCRT and PfDMT. PfDMT – an orthologue of the human iron transporter DMT1 – is likely essential to the blood stage malaria parasite, based on it being refractory to knockout by piggy bac transposon mutagenesis and is otherwise largely uncharacterised [245]. Unpublished localisation data from David Sullivan's lab suggests that PfDMT is expressed on the parasite's plasma membrane, however more recent conflicting data exist suggesting that it is, in fact, located on the digestive vacuolar membrane of the parasite [126,246]. PbVIT is the only transporter that has been shown to provide an iron tolerance mechanism to the parasite – presumably by mediating the sequestration of iron into the endoplasmic reticulum and vacuole [134].

It is also well-established that iron is important for the activation of artemisinin, whose derivatives are used in combination with other drugs as part of artemisinin combination therapies (ACTs) – current frontline treatment for falciparum malaria. Artemisinin has also been shown to be highly efficacious in the treatment of several other diseases, such

as cancer [247]. Zhao et al. demonstrate a synergistic relationship between artemisinin and the arsenical, arsenic trioxide (ATO) – a drug routinely used for the treatment of acute promyelocytic leukaemia [248]. While the exact mode of action of ATO is not fully understood, it is established that arsenicals likely have several molecular targets and it is suspected that one of these is ferritin [249,250]. Ahmad et al. showed the arsenicals, arsenate, arsenite, monomethylarsonic acid, and dimethylarsinic acid to induce the release of iron from ferritin in vitro, thus raising the question, could this phenomenon result in synergy between arsenic species and artemisinin derivatives when treating malaria parasites [250].

5.1.1. Aims & objectives

The work presented in this chapter aimed to explore roles of the putative iron transporter PfDMT and iron homeostasis more broadly in the *P. falciparum* asexual blood stages. In addition, a potential synergistic relationship between artesunate and arsenic trioxide in treating *P. falciparum* was explored.

Objectives

- Generate mutagenic PfDMT parasite lines using CRISPR/Cas9.
- Construct DNA vectors that would enable conditional mutagenesis of PfDMT.
- Generate conditional-mutagenic PfDMT parasite lines using selection-linked integration.
- Establish dose-responses for various divalent chelators and DMT inhibitors in the *P. falciparum* asexual blood stages
- Assess whether a synergistic relationship exists between artesunate and arsenic trioxide in the *P. falciparum* asexual blood stages

5.2. Results

5.2.1 PfDMT mutagenesis approaches

To explore the potential role played by PfDMT in the blood stage parasite, we sought to generate transgenic parasite lines that featured mutations in the coding sequence of PfDMT. Firstly, to confirm the results of the piggy bac transposon mutagenesis screen that showed PfDMT to be refractory to disruption, we sought to generate a parasite line that expressed a truncated and therefore likely non-functional PfDMT [222]. In addition, to examine substrate specificity we sought to generate a line that featured a single amino acid change that might alter substrate specificity, as has been shown in a bacterial orthologue [251].

To generate these lines (along with a control line), we adapted the two vector CRISPR-Cas9 system utilised by Ghorbal et al [252]. The donor vector pL6-PfDMT was constructed by cloning a 400 bp region of the PfDMT gene into the pL6 vector and by cloning the gRNA sequence immediately downstream of the U6 promoter (Figure 5.1a). Both steps were done by Gibson assembly. The three versions of pL6-PfDMT featured the same gRNA sequence and were identical in every way other than specific mutations in the homology region – which were generated by site-directed mutagenesis. Each vector featured at least two silent mutations in the sequence being targeted by the gRNA, with one of these being in the protospacer-adjacent motif (PAM) to ensure that further targeting post modification did not occur. pL6-PfDMT.2 featured an S433stop mutation, whilst pL6-PfDMT.3 featured an S433M mutation (Figure 5.1c). pL6-DMT.1 featured a silent mutation at the same codon which would result in no change in amino acid sequence of the protein being expressed and was thus included as a control. The vector design, cloning and site-directed mutagenesis steps were all undertaken by Dr Ksenija Slavic.

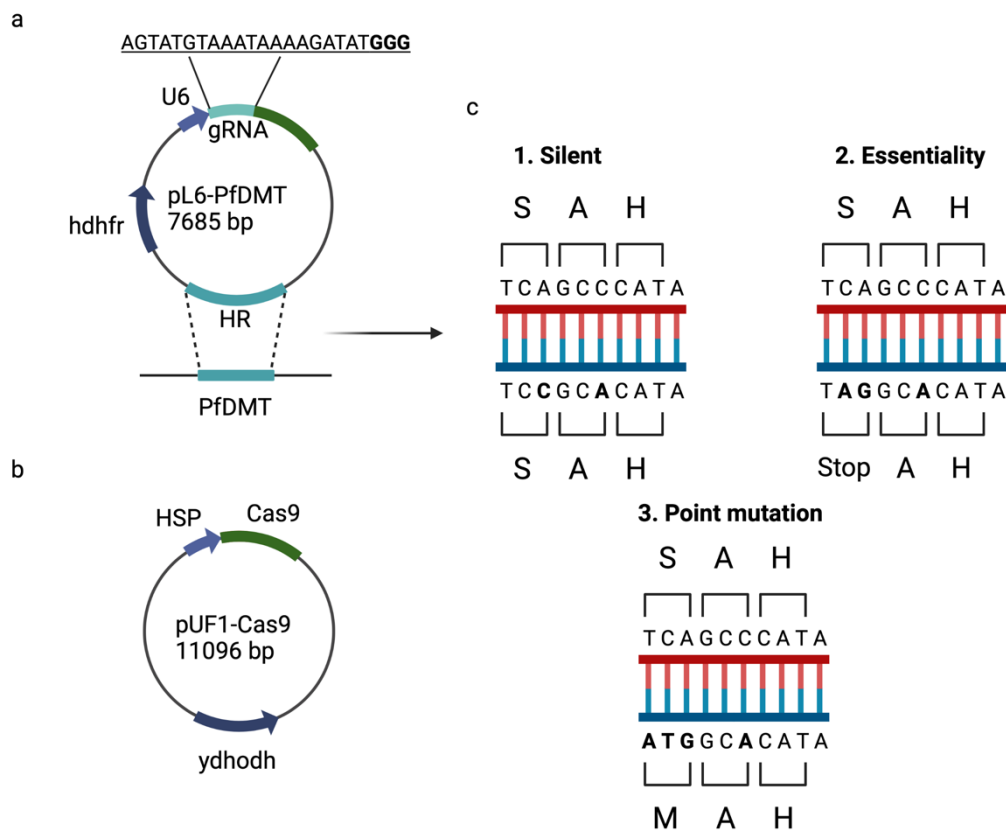


Figure 5.1. Approach to target PfDMT in blood stage parasites using CRISPR-Cas9. (a)

The plasmid pL6-PfDMT features the guide RNA (gRNA) and Cas9 binding cassette, expressed from the U6 promoter. The gRNA specifically targets the DNA sequence noted in the PfDMT locus of the *P. falciparum* genome, within the homology region (HR) on the plasmid. Protospacer motif in bold. The drug selection cassette, human dihydrofolate reductase (hdhfr) allows for selection of the plasmid with WR99210 in blood stage *P. falciparum*. **(b)** The plasmid pUF1-Cas9 encodes Cas9, which is expressed under the control of the HSP86 (heat shock protein) promoter. The yeast dihydroorotate dehydrogenase cassette enables selection with DSM-1. **(c)** Co-transfection of blood stage *P. falciparum* with pL6-PfDMT and pUF1-Cas9 enables expression of both Cas9 and gRNA and results in a targeted double strand break in the PfDMT locus. The HR on pL6-PfDMT enables homology-directed repair of the genome resulting in point mutations that featured in the HR. Three

plasmid variants (1, 2 and 3) featured point mutations that were to induce a premature stop codon and amino acid change (S433M) and a silent mutation as a control.

Blood stage *P. falciparum* 3D7 underwent two rounds of synchronisation by sorbitol lysis and was then transfected with one of the three pL6-PfDMT vectors and pUF1-Cas9 by electroporation (Figure 5.2a). Furthermore, parasites were transfected in parallel with pDC2-GFP as a transfection control, as well as pL6-PfDMT.1 only as a negative control. Additionally, parasites were also electroporated in the absence of either vector as a final control. This was to ensure that any parasites that emerged during drug treatment had emerged because they had been successfully transfected and not because they could tolerate WR99210 by some other mechanism. Twenty-seven days post transfection, blasticidin-S-resistant parasites, that had been transfected with pDC2-GFP, were detected by Giemsa-stained thin blood film. Successful uptake of pDC2-GFP was confirmed by PCR (Figure 5.2b) and detection of fluorescent parasites by fluorescence microscopy (data not shown). Puzzlingly, the parasitaemia of this transgenic culture did not exceed 1% and parasites became undetectable by blood film only ten days after emerging. After 60 days of continuous culture no parasites were detectable in any of the transfected lines. These transfections were then repeated and resulted in the same outcome.

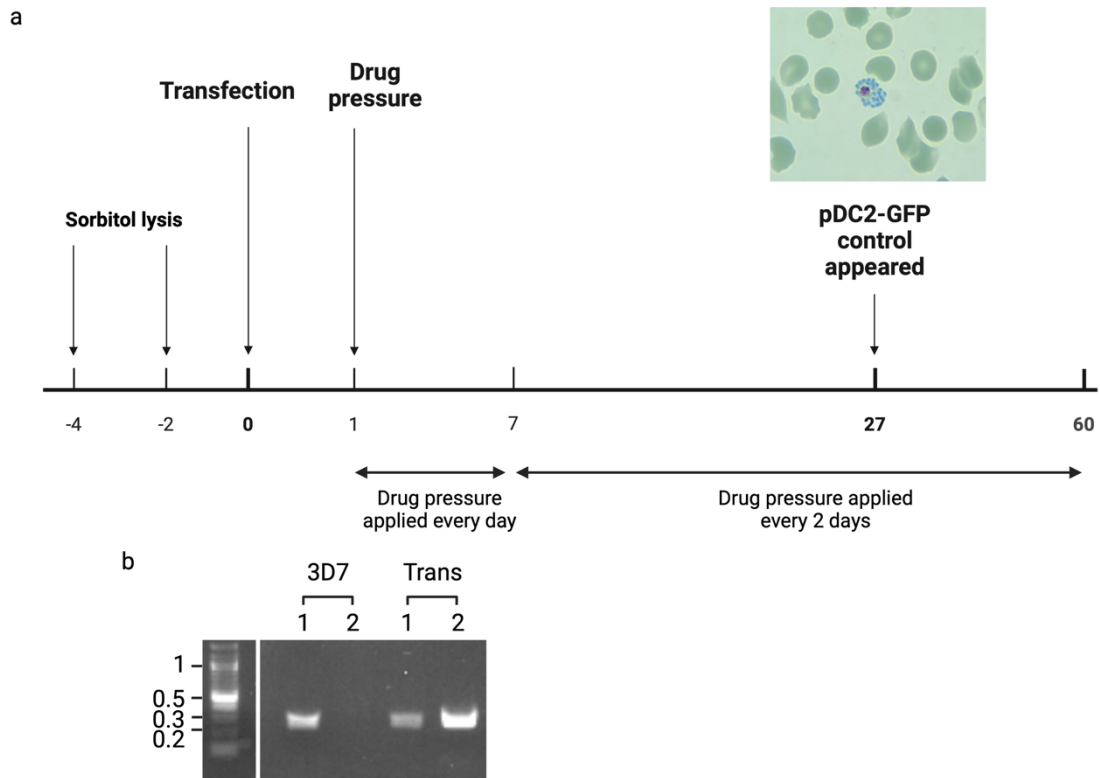


Figure 5.2. Transfection of ring-stage *P. falciparum* with pL6- Δ PfDMT and pUF1-Cas9.

(a) Highly synchronous *P. falciparum* ring-stage parasites were co-transfected with the plasmids pL6-PfDMT and pUF1-Cas9 by electroporation. Parasites were synchronised by sorbitol lysis 48 and 96 h prior to transfection. Seventy-five micrograms of each plasmid were used per transfection. In addition to the three co-transfections with pUF1-Cas9 and the three pL6-PfDMT variants, parasites were also transfected with pDC2-GFP in parallel. A negative transfection control was established, whereby parasites were electroporated in the absence of any plasmid. For 18 h following electroporation, the transfected parasites were cultured in standard parasite culture media and after this point the media featured 2.5 nM WR99210 and 1.5 μ M DSM-1 to select for the pL6-PfDMT and pUF1-Cas9 plasmids, or 2 μ g/mL Blasticidin-S to select for pDC2-GFP. Media was changed every 24 h thereafter for seven days and then every 48 h for 53 days. **(b)** DNA was extracted from parasites transfected with pDC2-GFP (Trans) and a wildtype control (3D7) and used as template for PCR. 1. Primers 23 and 24 to detect the 18S locus – expected amplicon = 264 bp, 2. Primers 37 and 38 to detect the plasmid – expected amplicon = 281 bp, 3. DNA ladder in kilobases.

To generate conditional PfDMT mutants, the DiCre system was used [253]. As can be seen in Figure 5.3a, to generate said mutants, parasites were transfected with a single vector that featured a region that was homologous to part of the targeted gene to enable modification of the locus by selection-linked integration. The approach aimed to modify the target gene to incorporate artificial intronic regions that contained LoxP sites within, that surround the subsequent section of the gene and a drug selectable marker.

Transgenic parasites would then be treated with rapamycin which enables the Cre recombinase to facilitate the excision of the sequence between the two LoxP sites and the subsequent recombination of surrounding sequence. Importantly, immediately downstream of the second LoxP site was the downstream section of target gene, which was identical to the excised portion of PfDMT but featured the desired mutations. The approach therefore enables the expression of specific genes and mutations therein to be exogenously controlled.

To generate conditional mutagenic parasites the vector pARL-DMT was constructed by joining fragments 1, 2 and 3 with the pARL-FIKKcKo vector backbone by NEBuilder cloning. Fragment 1 (F1) was synthesised and amplified from pUC57 by PCR with primers 43 and 44. Fragment 2 (F2) was amplified from the vector pARL-FIKKcKo (a gift from Dr Moritz Treeck) by PCR with primers 45 and 46. Three versions of fragment 3 (F3) were amplified from pUC57 by PCR with primers 47 and 48. The pARL-FIKKcKo vector backbone – or fragment 4 (F4) – was liberated by digestion with the restriction enzymes *Bgl*II and *Ava*I. All fragments were column purified and pARL-DMT.1, pARL-DMT.2 and pARL-DMT.3 subsequently assembled by NEBuilder cloning. As was the case for the three pL6-DMT vectors, one, two and three feature the following mutations respectively – S433S (silent), S433stop (essentiality), and S433M (point mutation). The vectors were used to transform competent *E. coli*, were cultured and vector maxi-preps were undertaken to generate vector DNA for subsequent transfections. All vectors were sequenced and showed that the assembly was successful.

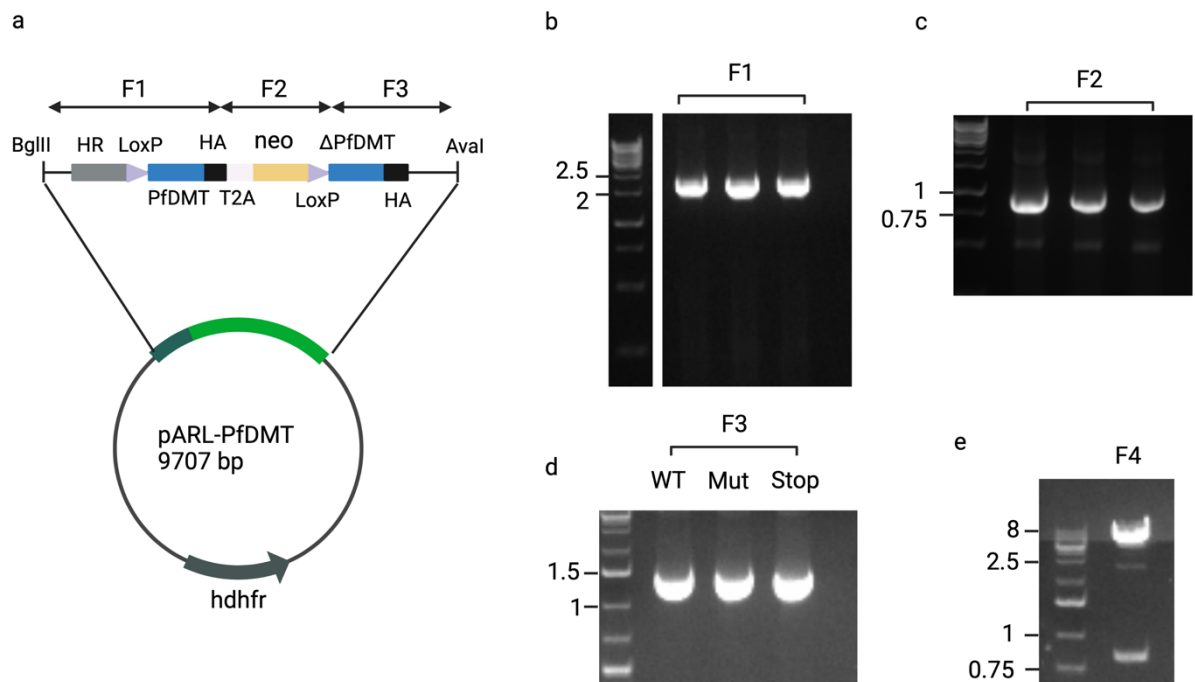


Figure 5.3. Conditional mutagenesis approach targeting PfDMT. (a) The vector pARL-PfDMT features an 887 bp homology region (HR) with the PfDMT locus; immediately downstream of this is a LoxP artificial intron, followed by the 3' end of the PfDMT coding sequence, linked to a haemagglutinin (HA) tag. This is followed by a self-cleaving peptide (T2A), a neomycin selection cassette (neo), a second artificial intro, followed by a recodonised version of the 3' end of PfDMT, followed by a HA tag. pARL-PfDMT was assembled by combining fragments 1, 2 and 3 (F1-3) with the pFIKK.cKo backbone (F4) by NEBuilder cloning. The hdhfr cassette allows for the vector to be selected for in the parasite with the drug WR99210 (b-e) PCR amplification of fragments 1, 2 and 3 using primers 43 and 44, 45 and 46, 47 and 48 respectively. Fragment 4 was produced by digesting pFIKK.cKo with *Bgl*II and *Ava*I. Expected band sizes for each fragment were as follows: F1 = 2245, F2 = 994, F3 = 1324, F4 = 5137. DNA ladder in kilobases.

A *P. falciparum* NF54 line that was generated to express the components of the Cre recombinase was transfected with the three pARL-PfDMT vectors described in the above section (Figure 5.4a) [254]. Highly synchronous ring stage parasites were

transfected and drug pressure with WR99210 was applied one day post transfection (Figure 5.4b). After 34 days of continuous culture under drug pressure, parasites that had been transfected with pARL-PfDMT.1 and pARL-PfDMT.2 were detectable by Giemsa-stained thin blood film. Six days later, the parasites had reached a parasitaemia of 1% and WR99210 was replaced with G418 to force the vector to integrate into the genome. After 22 days of culture under G418 pressure no parasites were detectable by blood film and the cultures were abandoned. Parasites transfected with pARL-PfDMT.3 did not emerge under WR99210 pressure. PCR was used to confirm initial uptake of the pARL-PfDMT vector (Figure 5.4c).

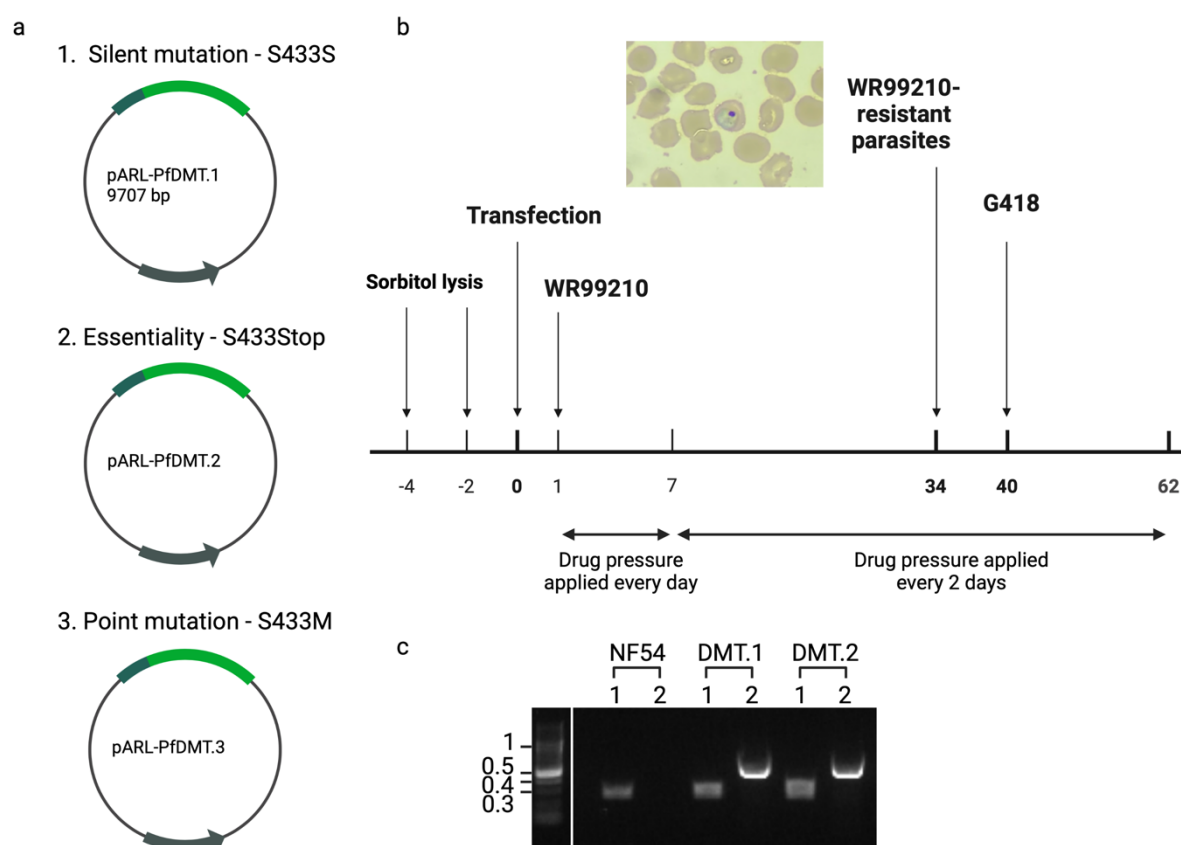


Figure 5.4. Transfection of ring-stage *P. falciparum* with pARL-DMT-DiCre. Highly synchronous *P. falciparum* ring-stage parasites were transfected with the plasmids pARL-PfDMT (1, 2 & 3) by electroporation. Parasites were synchronised by sorbitol lysis 48 and 96 h prior to transfection. Seventy-five micrograms of plasmid were used per transfection. A negative transfection control was established, whereby parasites were electroporated in the

absence of any plasmid. For 18 h post electroporation, the transfected parasites were cultured in standard parasite culture media and after this point the media featured 2.5 nM WR99210 to select for parasites that had taken up the plasmid. Media was changed every 24 h thereafter for seven days and then every 48 h until WR99210-resistant parasites appeared. G418 (250 ug/mL) pressure was then applied to attempt to force integration of the plasmid into the *P. falciparum* genome. Drug pressure with G418 was maintained for a further 22 days, with media being changed every 48 h. **(c)** DNA was extracted from parasites transfected with pARL-PfDMT.1 (DMT.1), pARL-PfDMT.2 (DMT.2) and a wildtype control (NF54) and used as template for PCR. 1. Primers 23 and 24 to detect the 18S locus – expected amplicon = 264 bp, 2. Primers 37 and 38 to detect the plasmid – expected amplicon = 564 bp, 3. DNA ladder in kilobases.

In parallel with the transfections described in the previous paragraph, NF54-DiCre parasites were transfected with the same three vectors using the pre-loading method described in the previous chapter (Figure 5.5). Briefly, vector DNA was introduced to uninfected RBCs by electroporation which subsequently were invaded by NF54-DiCre merozoites. Drug pressure with WR99210 was applied 48 h post transfection to select for transfectants. After 45 days of continuous culture under drug pressure no parasites were detectable by Giemsa-stained blood film.

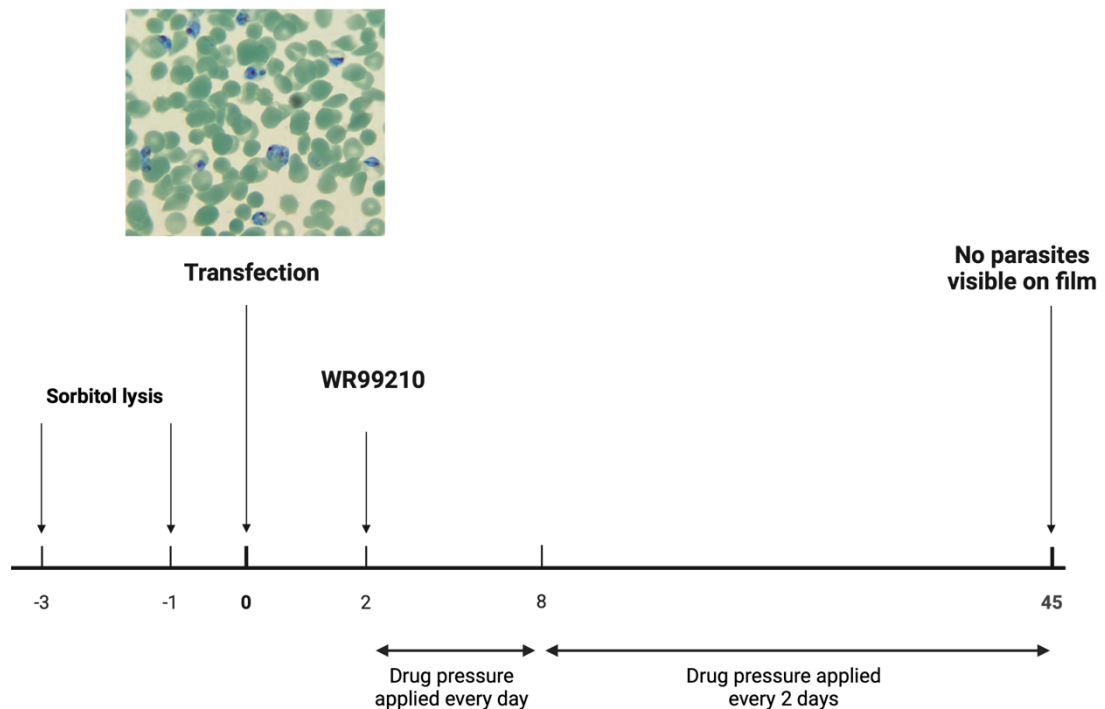


Figure 5.5. Electroporation of uninfected RBCs with pARL-DMT-DiCre and subsequent transfection of *P. falciparum*. Fresh human RBCs were transfected with seventy-five micrograms of each of the three pARL-DMT-DiCre plasmids and subsequently infected with highly synchronous late-stage *P. falciparum*. Parasites were synchronised 30 and 78 h prior to transfection and added to transfected RBCs. Drug pressure was applied 48 h after transfection with WR99210 (2.5 nM) to select for parasites containing the plasmid. Media was changed every 24 h for a further 6 d and then every 48 h for 37 d.

5.2.2. Effects of chelators and DMT inhibitors on parasite growth

To examine the effects of modulating iron homeostasis on the blood stage malaria parasite and to provide a wild-type comparison for subsequent and future experiments that feature transgenic parasites, *P. falciparum* 3D7 was treated with the divalent metal chelators deferoxamine (DFO) and bathophenanthrolinedisulfonic acid (BPS). Highly synchronous ring-stage parasites, at 0.5% parasitaemia, were cultured in one of ten concentrations of each chelator for 48 h and relative parasitaemia was determined by

measuring fluorescence after treatment with the fluorescent DNA stain PicoGreen. Three replicate experiments were undertaken for each chelator (Figure 5.6) and the mean IC₅₀ values – the concentration at which was reduced by 50% of the untreated control (% NDC) – for BPS and DFO were 19.8 μ M and 17.2 μ M respectively (Table 5.1). Furthermore, a single dose-response assay for iron was performed (Figure 5.7). At the highest concentration tested (1 mM), 63.8% of the untreated control was recorded. It is noteworthy that concentrations that exceed 1 mM are tough to test as FeSO₄ starts becoming insoluble in RPMI at this concentration.

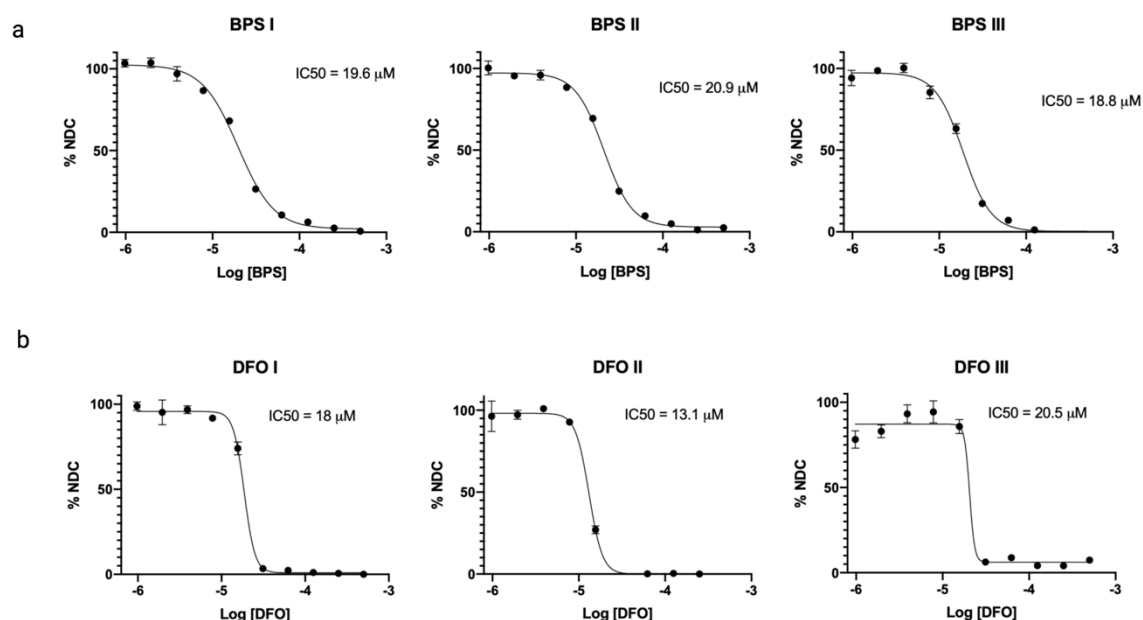


Figure 5.6. Dose responses to iron chelators. Highly synchronous ring-stage *P.*

falciparum were exposed to ten concentrations of the iron chelators **(a)**

bathophenanthrolinedisulfonic acid (BPS) and **(b)** desferrioxamine (DFO) for 48 h. Cultures were treated with the fluorescent stain PicoGreen and fluorescence was measured.

Individual data points represent the mean ($\pm$ SEM) relative fluorescence of five technical replicates for each concentration as a percentage of an untreated control. To each set of means was fitted a sigmoidal, four parameter logistic (4PL) curve, thus enabling the IC₅₀ to be estimated.

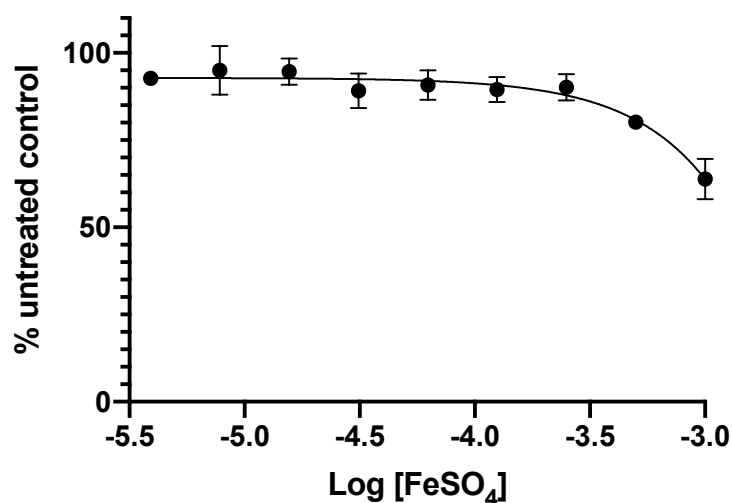


Figure 5.7. Dose response to iron. Highly synchronous ring-stage *P. falciparum* were exposed to nine concentrations of iron and an equal concentration of ascorbate for 48 h. Cultures were treated with the fluorescent stain PicoGreen and fluorescence was measured. Individual data points represent the mean ($\pm$ SEM) relative fluorescence of five technical replicates for each concentration as a percentage of an untreated control.

Isothiourea and pyrimidinone are validated inhibitors of human DMT1/NRAMP2 [255]. Accordingly, we decided to undertake dose-response assays with them in *P. falciparum* 3D7 to generate baseline IC₅₀ values to enable comparison among PfDMT mutant parasites and a wild-type. The assays were performed in exactly the same way as for the two chelators and gave IC₅₀ values for isothiourea and pyrimidinone of 8.22 μ M and 29.4 μ M (mean; n = 1), respectively (Figure 5.8).

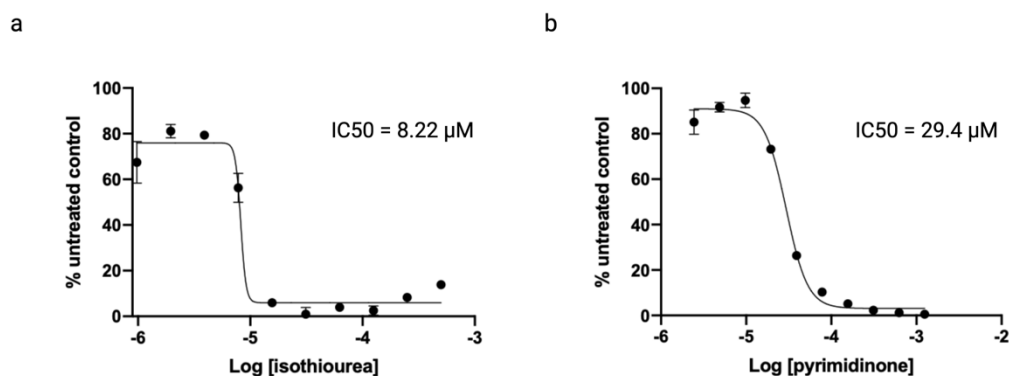


Figure 5.8. Dose responses to the DMT inhibitors isothiourea and pyrimidinone. Highly synchronous ring-stage *P. falciparum* were exposed to ten concentrations of the DMT inhibitors **(a)** isothiourea and **(b)** pyrimidinone for 48 h. Cultures were treated with the fluorescent stain PicoGreen and fluorescence was measured. Individual data points represent the mean ($\pm$ SEM) relative fluorescence of five technical replicates for each concentration as a percentage of an untreated control. To each set of means was fitted a sigmoidal, four parameter logistic (4PL) curve, thus enabling the IC₅₀ to be estimated.

Table 5.1. Summary of chelator and DMT inhibitor IC₅₀ values

Chelator	IC ₅₀ (μM)
Desferroxamine (DFO)	17.2*
Bathophenanthrolinedisulfonic acid (BPS)	19.8*
FeSO ₄	>1000**
DMT inhibitor	IC ₅₀ (μM)
Isothiourea	8.22**
Pyrimidinone	29.4**

*Calculated from the means of three independent experiments

**Calculated from a single experiment

5.2.3. Effects of arsenic trioxide on artemisinin potency

To explore whether artemisinin derivatives and arsenicals exhibit a synergistic relationship when used in combination against *P. falciparum*, dose-response assays were undertaken with artesunate (ART) and arsenic trioxide (ATO) to establish IC₅₀ concentrations. Highly synchronous ring stage *P. falciparum* were cultured in the presence of eight concentrations of each drug for 48 h in exactly the same way as assays described in the previous section. The calculated IC₅₀s were 621 nM and 7.12 nM for ATO and ART respectively (Figure 5.9).

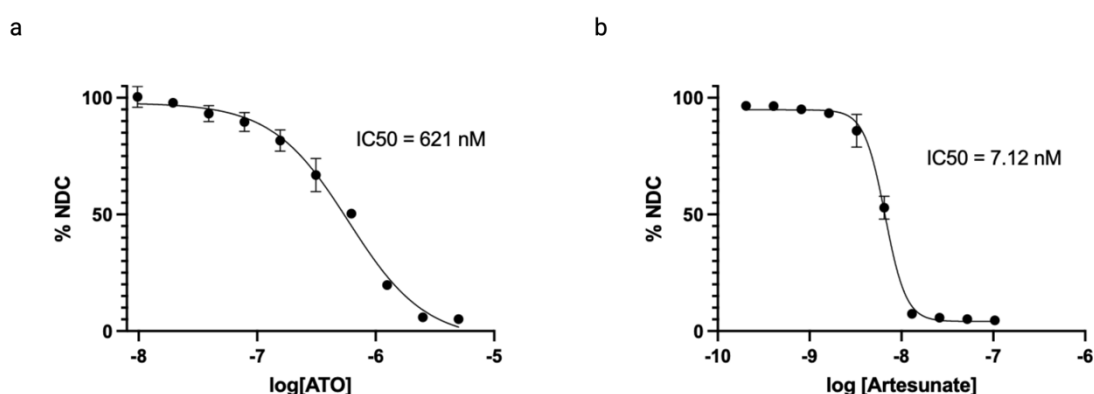


Figure 5.9. Artesunate (ART) and arsenic trioxide (ATO) dose responses. Highly synchronous ring-stage *P. falciparum* were exposed to nine concentrations of either artesunate (a) or arsenic trioxide (b) for 48 h. Cultures were treated with the fluorescent stain PicoGreen and fluorescence was measured. Individual data points represent the mean ($\pm$ SEM) relative fluorescence for each concentration as a percentage of an untreated control from three independent experiments. To each set of means was fitted a sigmoidal, four parameter logistic (4PL) curve, thus enabling the IC₅₀ to be estimated.

To assess the presence of a synergistic relationship, IC₅₀s were calculated for each drug when also cultured in a sub-lethal concentration of the other (Figure 5.10). Combination indices (CI) indicate whether such a relationship exists at the drug

concentrations tested. A CI of one indicates an additive relationship between the drugs at those concentrations, whereas CIs greater than or less than one, indicate antagonism or synergism respectively. For five of the six combinations tested, the calculated CI was greater than one, thus suggesting slight antagonism. The IC₅₀ of artesunate was seemingly unaffected by treatment with 10 nM ATO.

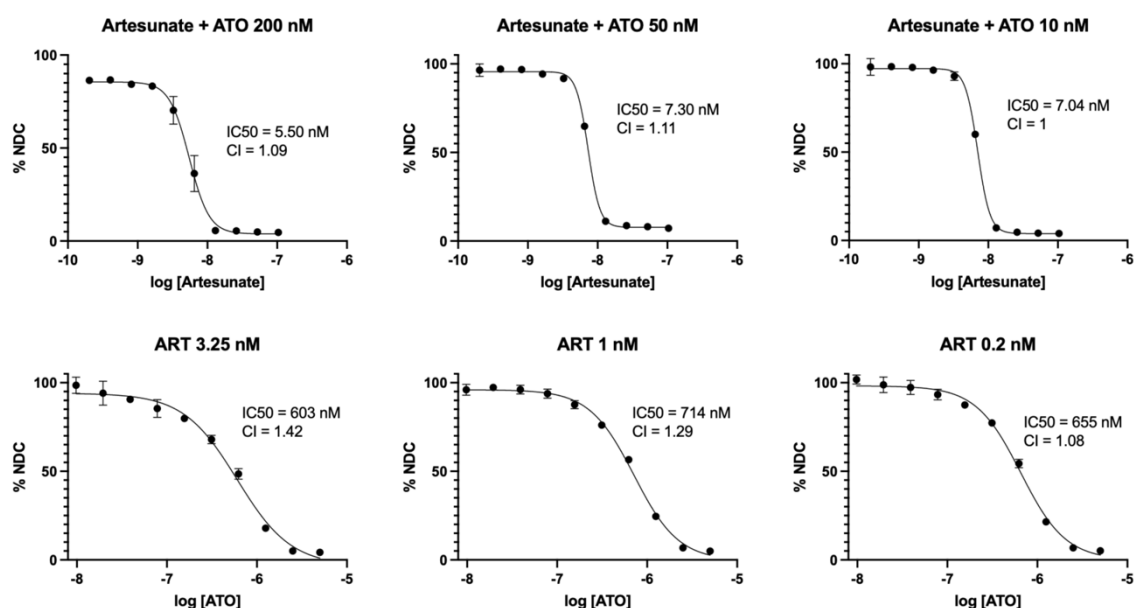


Figure 5.10. Artesunate-arsenic trioxide combinations. Highly synchronous ring-stage *P. falciparum* were exposed to nine concentrations of either artesunate or arsenic trioxide in combination with a fixed, sub-lethal dose of the other for 48 h. Cultures were treated with the fluorescent stain PicoGreen and fluorescence was measured. Individual data points represent the mean ($\pm$ SEM) relative fluorescence for each concentration as a percentage of an untreated control from three technical replicates. To each set of means was fitted a sigmoidal, four parameter logistic (4PL) curve, thus enabling the IC₅₀ to be estimated. For combination indices - synergy < 1 < antagonism.

5.3. Discussion

The malaria parasite has an absolute requirement for iron throughout its lifecycle and therefore must have evolved mechanisms through which host iron is accessed [120]. Of the putative iron transporters that have been identified, the NRAMP orthologue PfDMT appears an interesting candidate to fill that role as its orthologues play similar roles in other organisms [256–259]. Notably, in mammals NRAMP2 facilitates the uptake of dietary iron by enterocytes [260].

Accordingly, work presented in this chapter aimed to generate transgenic parasite lines that would aid in the study of this largely yet-to-be-characterised transporter. Using the CRISPR-Cas9 system, we aimed to produce mutants that would express a C-terminally truncated (and therefore likely non-functional) PfDMT, as well as a mutant that featured a single amino acid change in PfDMT (S433M). It has been demonstrated in the *Deinococcus radiodurans* DMT orthologue that when the corresponding and highly conserved methionine residue is mutated to an alanine the protein becomes capable of transporting calcium and magnesium rather than softer divalent metals such as iron and cobalt [251]. Successful generation of this mutated line would therefore enable similar studies in *P. falciparum*. Furthermore, the same approach could be employed to inspect a vast array of residues to better understand their role in function of the protein. In other organisms NRAMP proteins are known to show poor selectivity for specific divalent transition metal ions and thus have been shown to transport a wide variety of metals in addition to iron, including manganese and cadmium [261,262]. It has been shown in an NRAMP of the plant model *A. thaliana* that several mutations resulted in a reduced transport capacity of zinc, cadmium and manganese [263].

Studies that screen for mutations that affect the substrate specificity of a *Plasmodium* transporter naturally lend themselves to being undertaken in a heterologous system

such as yeast, as it's a far easier organism and is far more genetically tractable. With that considered it is noteworthy that PfDMT1 is yet to be functionally expressed in yeast (or *X. laevis* oocytes) despite great efforts from our group and collaborators.

In addition to the mutants that were to be generated using CRISPR-Cas9, we also aimed to generate conditional mutants using selection-linked integration [177]. This set of three mutants were to feature the same mutations as for the aforementioned set but were to be inducible using the DiCre system [264,265]. DiCre and other inducible systems enable exogenous control of the expression of mutated genes and thus allow the study of their effects on specific life cycle stages [266]. The mutant lines we aimed to generate were to be in a DiCre-expressing NF54 line, which readily produces the sexual blood stages of *P. falciparum* in *in vitro* culture and would therefore have the potential to be taken through the mosquito and liver life cycle stages and PfDMT function could be characterised in these stages [173]. Future work to characterise the function of PfDMT should focus on using the reverse genetic approaches described in this chapter to generate conditional mutants featuring different amino acid residue substitutions to examine the resultant effect on responses to various transition metals, including iron. This can be achieved by modulating iron conditions through supplementation of ferrous iron or its depletion with chelators – as shown here.

PfDMT was shown to be refractory to knockout by transposon mutagenesis and this result was recently corroborated by eight failed attempts to conditionally knockdown the gene using the glmS ribozyme system [222,246]. With that considered it is very highly unlikely that our attempts to produce a line with a C-terminal truncation would have yielded any viable parasites. Interestingly the work of Wichers et al. suggests that PfDMT localises to the food vacuole in the asexual blood stages of *P. falciparum*, which contradicts the unpublished work of David Sullivan's group which suggests a plasma membrane locale [126,246]. Both locations could enable PfDMT to facilitate iron influx

into the cytosol of the parasite and seem plausible, with the former being from the parasitophorous vacuole and the latter from the vacuole.

We showed the anti-cancer drug arsenic trioxide to have a sub-micromolar IC₅₀ in the asexual blood stages of *P. falciparum* and our IC₅₀ estimation for artesunate aligns with what has been reported elsewhere [267]. Our combination index data suggest that, at the concentrations tested, ATO and artesunate do not synergise in the *P. falciparum* asexual blood stages. It is noteworthy that these indices were calculated from single experiments done in a single strain of the parasite. If a potential synergistic relationship is in fact mediated by ATO inducing increased iron-mediated activation of artesunate, future dose-response assays using kelch13 mutants, whose ring-stages have a reduced sensitivity to artemisinins, could be undertaken [65].

6. Concluding remarks

The malaria parasite *Plasmodium falciparum* is the most important parasite of mankind in terms of both deaths and socio-economic impact and accordingly remains a major public health threat [1,2,268]. Despite major reductions in both reported cases and deaths in the latter half of the twentieth century and first two decades of the twenty-first century, progress seems to have stalled, and it is expected that the ongoing coronavirus pandemic will only impede further progress [27,269,270]. Globalisation and the increased travel and migration that accompanies it is increasing the transmission of various infectious diseases including malaria. Drug resistance has been reported for virtually all major antimalarial drug classes, thus highlighting the exigent need for new drugs and new drug combinations. Whilst many would argue that “true” resistance to the artemisinin derivatives has not yet emerged, such an emergence would undoubtedly be catastrophic for control efforts and accordingly it should be of the utmost importance to develop new partner drugs, with novel modes of action to be used as part of artemisinin-based combination therapies [71,271].

As discussed in previous chapters, malarial transporters represent an area of great therapeutic potential. As a protein class they are well-exploited targets, representing 13% of proteins targeted by FDA-approved oral drugs [272]. Of the antimalarial drugs, the artemisinin derivatives target the essential calcium pump PfATP6, as well as several other proteins, including perhaps PfCRT and PfMDR1 [60,61,63]. Inhibitors of the sodium pump PfATP4 have been studied in clinical trials [273]. Of the putative transition metal transporters that were focused upon in this thesis, PfDMT, PfCRT and PfCTR1 have been shown to be essential to the asexual blood stages of *P. falciparum* and *P. berghei* and thus all represent highly attractive targets.

PfDMT is an orthologue of the human NRAMPs, which are both transporters of iron [274]. NRAMP2 (also known as DMT1) is responsible for the uptake of dietary iron from the lumen of the small intestine into the enterocyte, with ferroportin facilitating its release into the blood [118,119]. NRAMP1 mediates the transport of iron from senescent RBCs that are broken down in the phagolysosome of the macrophage into the cytosol, from where it is either stored in ferritin or released back into the bloodstream via ferroportin [275]. The hormone hepcidin plays a key role in iron homeostasis, by modulating the expression of ferroportin. Mutations in the HFE (human homeostatic iron regulator protein) gene, which controls hepcidin expression, cause iron overload, or haemochromatosis [275]. As discussed for *Plasmodium*, iron levels must be tightly regulated to avoid its toxic effects. Humans, and other mammals, have no means to excrete iron (other than through menstruation), and thus a defect leading to excessive uptake will be damaging. It is commonly treated using iron chelators such as DFO [276]. Conversely, iron deficiency is caused by reduced or impaired uptake of iron and leads to anaemia. Specific causes of this include gastrointestinal helminth infection, lack of dietary iron, excessive menstrual bleeding, or malabsorption. As discussed in the previous chapter, while the effect of iron deficiency on malaria is yet to be completely understood, it seems that iron deficiency may impair parasite growth in the host – presumably this is simply due to the parasite not being able to acquire enough iron to satisfy its own requirements. Targeting iron acquisition pathways in the parasite therefore represents a theoretically effective chemotherapeutic strategy.

Future work on PfDMT should aim to further validate it as a drug target. With the absence of a solved crystal structure, the structure predicted by artificial intelligence-driven platform AlphaFold, could substitute in molecular docking prediction studies [277]. It is noteworthy that any potential drug would have to selectively inhibit PfDMT over its human orthologue NRAMP2, to avoid the effects of potentially inhibiting dietary iron uptake by the host. To examine the selectivity of potential lead compounds, one could

replace the entire PfDMT coding region with that of NRAMP2 (with appropriate codon optimisation) in the *P. falciparum* genome and compare the drug IC₅₀ value derived in the resultant line with that of an appropriate control. In addition to orthologue replacement with the human protein, the same could be done with PfDMT orthologues from across the *Plasmodium* genus to examine potential differences in drug susceptibility between species. This approach has been used in *P. knowlesi* to show species-specific differences in potency of PfATP4 inhibitors [278].

The putative copper transporter PfCTR1 is likely essential to the *P. falciparum* blood stages and presumably (based on substrate specificity of orthologues) facilitates copper uptake. Mutations in the human copper transporter hCTR1 result in an autosomal recessive condition in infants characterised by copper deficiency in the central nervous system [279]. In addition, both Menkes and Wilson diseases are caused by impaired copper uptake due to mutations in human copper transporters [280]. As discussed in the opening chapter, both PfCTR1 and PfCTR2 seem to be expressed in the dormant liver stages of *P. cynomolgi* and are thus attractive radical cure targets. It is noteworthy that despite being identified as thus, future study of these proteins in the enigmatic dormant liver stages will likely prove challenging. With that being said, unlike *P. vivax* and *P. ovale*, *P. cynomolgi* is genetically tractable and culturable and could perhaps enable future work [281,282].

Whilst not essential to the malaria blood stages, PfCDF likely provides an important zinc tolerance mechanism to the sexual stages of the parasite whilst in the mosquito vector. Future work should aim confirm this hypothesis through generating transgenic parasites that do not express functional PfCDF and examining the effect of altered zinc concentration on their relative progression through the vector stages of the life cycle.

To study the function of any of the putative transporters described in this thesis, the generation of transgenic parasite lines that either feature a non-functional transporter, or one mutated in such a way as to alter substrate specificity opens the door to a multitude of options. Comparative elemental analysis of transgenic parasites versus wildtype using ICP-MS (induction coupled plasma mass spectrometry) would allow relative transition metal concentrations to be compared [283,284]. ICP-MS combined with a genome-wide knockout screen has enabled the identification of novel transition metal regulator proteins in other systems such as HeLa cell and yeast [284,285]. Such an approach in *Plasmodium* would likely add to our current repertoire of proteins involved in transition metal homeostasis. In addition, a transcriptomic screen that compared parasites cultured in differing concentrations of iron, copper, or zinc could also aid in identifying novel proteins – as has been done in other organisms [286–288].

Whilst many of the key players have likely been identified, we still have an incomplete picture of transition metal homeostasis in the malaria parasite. Clearly transporters play a key role, but with all but one lacking functional characterisation, there is a clear trajectory for immediate future study. In particular, this will focus on attractive drug targets such as the essential PfDMT, but also aim to paint a clearer picture of the system as a whole.

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Publications & Presentations

Publications

- Cafry, J. et al. (2023) The QuantuMDx Q-POC SARS-CoV-2 RT-PCR assay for rapid detection of COVID-19 at point-of-care: preliminary evaluation of a novel technology. Scientific Reports. Accepted for publication
- Kay, G. A. et al. (2022) SARS-CoV-2 enzyme-linked immunosorbent assays as proxies for plaque reduction neutralisation tests. Scientific Reports. 12, 3351
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Presentations

- The *Plasmodium falciparum* cation diffusion facilitator confers zinc tolerance to a zinc-sensitive yeast line – British Society for Parasitology conference in York (2022) - poster
- Characterisation of a cation diffusion facilitator from the malaria parasite *Plasmodium falciparum* – British Society for Parasitology conference (2021) – online poster
- Transition metal ion transport in the malaria parasite *Plasmodium falciparum* – St George's, UoL annual research day (2019) - poster