The ER chaperone PfGRP170 is essential for asexual development and is linked to stress response in malaria parasites.

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ABSTRACT

The vast majority of malaria mortality is attributed to one parasite species: *Plasmodium falciparum*. Asexual replication of the parasite within the Red Blood Cell (RBC) is responsible for the pathology of the disease. In *Plasmodium,* the endoplasmic reticulum (ER) is a central hub for protein folding and trafficking as well as stress response pathways. In other eukaryotes, ER chaperones assist with protein folding and unfolding, the crossing of biological membranes, ER stress, lipid metabolism, and protein trafficking. In this study we studied the role of an uncharacterized ER protein, PfGRP170, in regulating these key functions by generating conditional mutants. Our data show that PfGRP170 localizes to the ER and is essential for asexual growth, specifically required for proper development of schizonts. PfGRP170 is essential for surviving heat shock, suggesting a critical role in cellular stress response. The data demonstrate that PfGRP170 interacts with the *Plasmodium* orthologue of the ER chaperone, BiP. Finally, we found that knockdown of PfGRP170 leads to the activation of the *Plasmodium* eIF2□ kinase, PK4, suggesting a specific role for this protein in this parasite stress response pathway.

INTRODUCTION

Malaria is a deadly parasitic disease that causes over 212 million cases and nearly 430,000 deaths each year, primarily in children under the age of five[1]. The deadliest human malaria parasite, *P. falciparum*, infects individuals inhabiting subtropical and tropical regions. These are some of the most impoverished regions of the world, making diagnosis and treatment challenging. Moreover, the parasite has evolved resistance to all clinically available drugs, highlighting an important need for uncovering proteins that are essential to drive the biology of this parasite[2-6]. Malaria is associated with a wide array of clinical symptoms, such as fever, chills, nausea, renal failure, pulmonary distress, cerebral malaria, and cardiac complications. It is the asexual replication of the parasite within the Red Blood Cell (RBC) that is responsible for the pathology of the disease[7].

In *P. falciparum,* the endoplasmic reticulum (ER) is a uniquely complex, poorly understood organelle. In fact, recent data suggest that ER proteins play a major role in resistance to the frontline antimalarial, artemisinin[8-10]. It is in this organelle that a variety of essential cellular functions occur, including protein trafficking, cellular signaling, and activation of stress response pathways[11-17]. Compared with other organisms, the molecular mechanisms involved in these essential processes in *Plasmodium* remain poorly understood. Since many of the factors that drive these biological processes are not well characterized in *Plasmodium*, it is imperative to uncover proteins that regulate and maintain ER biology. One group of proteins likely governing many of these processes are ER chaperones[18-23].Very little is known about the roles that ER chaperones play in *Plasmodium*, many of them defined merely based on sequence homology to other organisms. The *Plasmodium* genome encodes a relatively reduced repertoire of predicted ER chaperones, but it is predicted to contain two members of the conserved ER HSP70 chaperone complex, GRP78 (or Binding immunoglobulin protein or BiP) and a putative HSP110, GRP170[24].

In this study, we generated conditional mutants for the putative ER chaperone, PfGRP170 (PF3D7_1344200). Using these conditional mutants, we localized PfGRP170 to the parasite ER, and show that unlike its homologs in other eukaryotes, PfGRP170 is essential for parasite survival in the clinically relevant RBC stage. Detailed life cycle analysis revealed that knockdown results in parasite death in early schizogony. The protein is required for surviving a brief heat shock, suggesting that PfGRP170 is essential during febrile episodes in the host. We show that despite a predicted transit peptide, PfGRP170 is not essential for protein trafficking to the apicoplast nor is it required for protein export into the host RBC. We demonstrate here that PfGRP170 interacts with the *Plasmodium* homolog of BiP suggesting a conserved HSP70 ER chaperone complex. Finally, we show that PfGRP170 knockdown leads to the activation of the only known ER stress response pathway in Plasmodium, the PK4 pathway[10,16].

RESULTS

PF3D7_1344200 is a putative GRP170 in P. falciparum

A blast search to identify ER localized Hsp70 proteins in *P. falciparum* revealed two proteins, HSP70-2 (PfGRP78/BiP) and a putative HSP110 (PF3D7_1344200). HSP110 proteins are considered large HSP70 chaperones, having sequence homology to both the nucleotide and substrate binding domains of other HSP70 members[25]. The increased size of HSP110 family members is the result of an extended α-helical domain at the C-terminus as well as an unstructured loop inserted in the substrate-binding domain[25,26] (Figure 1A). In other eukaryotic organisms, the ER localized HSP110 (referred to as GRP170) is a chaperone with four primary protein domains: a signal peptide, a nucleotide binding domain, a substrate binding domain, and an extended C-terminus[25,27]. A protein sequence alignment using the yeast GRP170 (Lhs1) was used to predict the boundaries of these domains in PF3D7_1344200 (PfGRP170) (Figure 1A and Supplemental Figure 1). Most of the sequence conservation between Lhs1 and PfGRP170 was found to be in the nucleotide binding domain (Supplemental Figure 1). PfGRP170 is well conserved across multiple *Plasmodium* species, including other human malaria-causing species (Supplemental Figure 2). This level of conservation decreases in another apicomplexan (*T. gondii*) and even more so in yeast and humans (Supplemental Figure 2).

Generation of PfGRP170-GFP-DDD conditional mutants

Conditional mutants for PfGRP170 were generated by tagging the endogenous PfGRP170 locus at the 3' end, using single homologous crossover, with a GFP reporter and the *E. coli* DHFR destabilization domain (DDD) (Figure 1B). In the presence of the small ligand Trimethoprim (TMP), the DDD is maintained in a folded state. However, if TMP is removed from the culture medium, the DDD unfolds and becomes unstable[28-32]. Intramolecular binding of the chaperone to the unfolded domain inhibits normal chaperone function (Figure 1B)[28,30,32]. Two independent transfections were carried out, and integrated parasites were selected via several rounds of drug cycling. PCR integration tests following drug selection indicated that the percentage of integrated parasites in both transfections were extremely low (Figure 1C). As a consequence, standard limiting dilution could not be used to clone out integrated parasites. To circumvent this issue, flow cytometry was used to enrich and sort extremely rare GFP

positive parasites. Despite low enrichments and sorting rates (GFP positive population =~1.13E-3), we successfully obtained two clones, termed 1B2 and 1B11, using flow sorting (Figure 1 C and D). Proper integration into the *pfgrp170* locus was confirmed by a Southern blot analysis (Figure 1E). Western blot analysis revealed that the PfGRP170-GFP-DDD protein was expressed at the expected size (Figure 1F). Immunofluorescence assays (IFA) and western blot analysis showed that the PfGRP170-GFP-DDD fusion protein was expressed and localized to the parasite ER at all stages of the asexual life cycle (Figure 1G and Supplemental Figure 3).

PfGRP170 is essential for asexual growth and surviving febrile episodes

To investigate the essentiality of PfGRP170, PfGRP170-GFP-DDD asynchronous parasites were cultured in the absence of TMP, and parasitemia was observed using flow cytometry. A growth defect was seen within 24 hours after the removal of TMP, resulting in parasite death (Figure 2A). Furthermore, the two clonal cells lines exhibited a dose-dependent growth response to TMP (Figure 2B). Consistent with data from other chaperones tagged with the DDD[28,30,32], TMP removal did not result in degradation of PfGRP170-DDD (Figure 2C). Conditional inhibition of *Plasmodium* proteins that does not involve their degradation, has also been observed for other non-chaperone proteins [33,34]. Moreover, knockdown of PfGRP170 did not affect its ER localization (Supplemental Figure 4).

Using a nucleic acid stain, acridine orange, we used flow cytometry to specifically observe each stage of the asexual life cycle (ring, trophozoite, and schizont) in PfGRP170-GFP-DDD parasites with and without knockdown (Figure 2D). The amounts of RNA and DNA increase over the asexual life cycle as the parasite transitions from a ring to trophozoite to a multi-nucleated schizont. We observed that upon TMP removal, mutant parasites are arrested in a relatively late developmental stage (Figure 2D). In order to identify the exact point in the asexual life cycle that the mutant parasites died, TMP was removed from tightly synchronized ring stage cultures and parasite growth and morphology was assessed over the 48-hour life cycle. We observed morphologically abnormal parasites late in the lifecycle, when control parasites had undergone schizogony (Figure 2E). Parasites undergoing knockdown of PfGRP170

ultimately failed to progress through schizogony and did not reinvade new RBCs (Figure 2E).

The cytoplasmic ortholog of PfGRP170, PfHSP110, was previously shown to be essential for surviving heat stress[32]. Therefore, we tested whether PfGRP170 mutants were sensitive to a brief heat shock. Asynchronous parasites were incubated in the absence of TMP for 6 hours at either 37°C or 40°C. Following the 6-hour incubation, TMP was added back to all cultures, which were then grown at 37°C for two growth cycles, while measuring parasitemia every 24 hours. The growth of parasites at 37°C was not significantly affected by the brief removal of TMP (Figure 2F). In contrast, incubating parasites at 40°C without TMP resulted in reduced parasite viability compared to parasites grown at 40°C with TMP (Figure 2F).

Apicoplast trafficking is not affected by PfGRP170 knockdown

Protein trafficking to the apicoplast is essential for parasite survival. Proteins targeted to the apicoplast contain an N-terminal transit peptide that is revealed upon signal peptide cleavage in the ER[15,35]. It remains unclear whether apicoplast targeted proteins go through the Golgi before reaching their final destination. It has been shown that disruption of ER to Golgi trafficking, using Brefeldin A (BFA), does not reduce apicoplast transport[15,36]. This same study also demonstrated that the addition of an ER retention sequence (SDEL), to a GFP with a transit peptide, did not reduce apicoplast trafficking or transit peptide cleavage[36]. However, a separate but similar analysis came to the opposite conclusion[37]. Thus, the identification, packaging, and transport of apicoplast-targeted proteins from the ER remain unanswered questions.

Two software analysis tools (Prediction of Apicoplast-Targeted Sequences (PATS) and PlasmoAP) predicted a strong apicoplast transit peptide for PfGRP170, despite our observation of a definite ER localization (Figure 1G and Figure 3A). We were therefore interested to find out whether PfGRP170 plays a role in apicoplast trafficking. We tested whether the putative PfGRP170 transit peptide (amino acids: 1 to 150) could be trafficked to the apicoplast by episomally expressing the predicted PfGRP170-transit peptide fused to a GFP reporter without an ER retention signal (Figure 3B). We

performed co-localization assays using ER, apicoplast, and Golgi markers and determined that the putative transit peptide localized to the ER due to colocalization with ER marker Plasmepsin V (Figure 3B).

To determine the role of PfGRP170 in trafficking proteins to the apicoplast, we removed TMP from PfGRP170-GFP-DDD parasites and examined the localization of the apicoplast-localized cpn60[30,38,39]. No defects in apicoplast localization of cpn60 were observed (Figure 3C). Additionally, incubation with the essential apicoplast metabolite IPP[40], failed to rescue or have any positive effect on TMP removal in PfGRP170-GFP-DDD parasites (Figure 3D).

Interactions of PfGRP170

Two independent approaches were taken to identify the interacting proteins of PfGRP170 (Figure 4A). The first was an anti-GFP Immunoprecipitation (IP) followed by mass spectroscopy. In the second approach we generated a cell line expressing PfGRP170 episomally tagged with an HA and promiscuous Biotin Ligase (BirA) that can tag and thus identify interacting partners via mass spectroscopy[41]. When exogenous biotin is added to the GRP170-BirA parasites, the BirA tagged protein will biotinylate interacting proteins or those that are in close proximity[41]. A western blot analysis confirmed expression of the PfGRP170-HA-BirA fusion protein (Supplementary Figure 5A). Colocalization IFA's confirmed that the PfGRP170-BirA protein localizes to the parasite ER (Supplementary Figure 5B). Additionally, western blot analysis demonstrates that proteins are biotinylated in the PfGRP170-BirA cell lines when biotin is added (Supplementary Figure 5C).

Mass spectroscopy data were obtained from two anti-GFP IP's of clones 1B2 and 1B11 and two biological replicates of PfGRP170-BirA IP experiments (Supplemental Table 1). In order to obtain a list of proteins specific to the ER and parasite secretory pathway, proteins identified by mass spectroscopy were filtered to include only those, which had a signal peptide or transmembrane domain. Mass spectroscopy data from anti-GFP IP of PfGRP170-GFP-DDD clones (1B2 and 1B11) and two PfGRP170-BirA IP's were compared, generating a high-confidence list of 28 proteins common to all four IP

experiments (Table 1). The proteins identified localized to a wide variety of subcellular localizations including the ER, parasitophorous vacuole, parasitophorous vacuole membrane, rhoptries, RBC, and parasite plasma membrane (Figure 4B and Table 1). Recently, a *piggyBac* based screen identified proteins in *P. falciparum*, which were predicted to be essential[42]. Using this analysis, we determined that 71.43% of the 28 potential interactors of PfGRP170 are predicted to be essential (Figure 4C).

PfGRP170 is not required for trafficking to the RBC

Several proteins in the PfGRP170 mass spectroscopy data localized to the RBC (Figure 4B and Table 1). In order for the parasite to grow, develop, and divide, it must drastically remodel the host RBC[12]. These modifications are accomplished through protein export into the RBC. In model eukaryotes, such as yeast and mammalian cells, molecular chaperones, and specifically those that are ER-localized, play central roles in protein trafficking[18,19]. We tested whether knockdown of PfGRP170 would prevent trafficking of several exported proteins (PfHSP70x, PfMAHRP1, and FIKK4.2). Knockdown of PfGRP170 did not affect the localization of these proteins to the host RBC (Supplemental Figure 6A-C).

PfGRP170 and BiP interact

The most abundant protein identified in our mass spectroscopy data was PfBiP (Figure 5A). This suggests that PfGRP170 may regulate its function as it does in other eukaryotes[27]. To confirm that PfGRP170 and PfBiP interact in *P. falciparum*, we performed an anti-GFP Co-IP, and probed the lysate for PfBiP. We observed that PfGRP170 and PfBiP interact and this interaction is not lost upon TMP removal (Figure 5B). As a control, we probed the GFP Co-IP lysates for a different ER protein, Plasmepsin V (PMV), and found that it did not pull down with PfGRP170 (Figure 5C).

To visualize the PfGRP170-PfBiP interaction within the cellular context of the infected RBC, we utilized a Proximity Ligation Assay (PLA)[43-45]. The PLA positive signal indicates that two proteins are within 40nm of each other, suggesting a close interaction within the cell. This approach has been used successfully in *Plasmodium* to

demonstrate interaction of exported proteins[46]. We performed this assay using anti-GFP and anti-BiP antibodies and observed a positive signal at all life cycle stages (Figure 5D). As a negative control we also probed with an antibody against the ER localized protease PMV and despite the co-localization of these two proteins in the ER, we did not see a positive PLA signal, suggesting distinct sub-organellar localizations (Figure 5E). Together, these results demonstrate that PfGRP170 and PfBiP interact during all stages of the asexual life cycle of *P. falciparum.*

PfGRP170 knockdown activates the PK4 stress response pathway

In addition to their function in the secretory pathway, molecular chaperones perform a vital role in the management of cellular stress. Typically, ER chaperones will attempt to fold substrates multiple times. If the protein cannot be folded, a process called ER associated degradation (ERAD) is activated[47]. This in turn leads to the transfer of the unfolded proteins out of the ER and into the cytosol where they are degraded via the proteasome. When the level of unfolded proteins in the ER increases above a certain threshold, several Unfolded Protein Response (UPR) signaling pathways are activated[17]. These pathways, many of which are regulated by ER chaperones, lead to transcriptional and translational changes which aid in reducing cell stress[17].

Plasmodium lack much of the ER machinery used to activate stress response pathways[16,48,49]. The only identified ER stress response pathway in *Plasmodium* is the PERK/PK4 pathway[10,16]. Signaling through this pathway has been shown to occur in the parasite following artemisinin treatment[10]. Under normal conditions, PK4 exists as a transmembrane monomeric protein in the ER. When the ER is stressed, PK4 oligomerizes and becomes active, phosphorylating the cytoplasmic translation initiation factor EIF2-alpha to halt translation and flux through the ER[10,16]. To determine whether this pathway was activated during knockdown of PfGRP170, PfGRP170-GFP-DDD parasites were tightly synchronized to the ring stage and grown without TMP for 24 hours, after which parasite lysate was collected and the phosphorylation state of EIF2-alpha determined by western blot. We observed that PfGRP170 knockdown resulted in the phosphorylation of EIF2-alpha, indicating that this pathway was activated (Figure 6A).

Since PfGRP170 knockdown resulted in EIF2-alpha phosphorylation, which has been shown to be required for resistance to artemisinin exposure, we tested if PfGRP170 plays a role in drug resistance. For this purpose, we utilized PfGRP170-BirA parasites, which have an extra copy of PfGRP170. Using the ring-stage survival assay, we compared the growth of the parental parasite line (3D7) with that of PfGRP170-BirA parasites after brief exposure to artemisinin. Our data show that overexpression of PfGRP170 did not result in artemisinin resistance (Supplementary Figure 7A-B).

Several *Plasmodium* kinases have been shown to phosphorylate EIF2-alpha in late developmental stages or in response to other cellular stress or artemisinin treatment[16,50-52]. We were therefore interested in identifying the specific kinase, which is responsible to the phosphorylation of EIF2-alpha during PfGRP170 knockdown. We incubated synchronized PfGRP170-GFP-DDD parasites without TMP for 24 hours, in the presence or absence of the PK4 inhibitor GSK2606414. Parasite lysates were used to determine the phosphorylation state of EIF2-alpha. We observed that in the presence of the PK4 inhibitor, EIF2-alpha phosphorylation was blocked, demonstrating that PfGRP170 knockdown specifically results in PK4 activation, that leads to phosphorylation of EF2-alpha (Figure 6B).

DISCUSSION

We present in this work the first characterization of PfGRP170 in the asexual life cycle of *P. falciparum*. We have generated conditional mutants that allow us to probe the role of this protein using the DDD conditional system[29-34]. Additionally, taking advantage of the GFP fused to PfGRP170, we were able to isolate an exceptionally rare clonal population using flow cytometry (Figure 1C-F). This technique could achieve what a traditional limiting dilution method could not. Moreover, this type of flow sorting can be further implemented not only for rare events but also to significantly cut down the time from transfection to a clonal cell population.

We demonstrate here that PfGRP170 is an ER resident protein that is essential for asexual growth in *P. falciparum* (Figure 1G and Figure 2A). Knockdown of PfGRP170 leads to a growth arrest of parasites late in development and their subsequent death (Figure 2D and E). In yeast and mammals, GRP170 functions in a complex with the ER

chaperone BiP, serving as the nucleotide exchange factor to regulate BiP activity[27,53]. Unlike *Plasmodium falciparum*, Yeast null for GRP170 are viable due to the upregulation of Sil1, another nucleotide exchange factor, that usually plays a role in the IRE1 stress response pathway[54]. *Plasmodium* genome does not encode Sil1 and IRE1, which goes in line with the observed essentiality of PfGRP170 during the blood stages. Additionally, research in mammalian systems suggests that GRP170 also has BiP-independent functions, such as binding unfolded substrates[26]. Our data show, via immunoprecipitation, mass spectroscopy, and proximity ligation assays (Figure 4, Figure 5, and Table 1), that PfGRP170 interacts with BiP in *P. falciparum* suggesting that it regulates BiP function.

Previously it was shown that apicoplast transit peptides are predicted to bind the ER chaperone BiP, and when these predicted binding sites were mutated, targeting to the apicoplast was disrupted[55]. Moreover, an Hsp70 inhibitor with an antimalarial activity was shown to inhibit apicoplast targeting[56,57]. These data, combined with the predicted transit peptide of PfGRP170, led us to investigate the role of this chaperone in apicoplast trafficking (Figure 3A). Interestingly, when the putative transit peptide was tagged with a GFP reporter and without an ER retention signal, the fusion protein was retained in the ER. Typically, proteins with a signal peptide that lack a trafficking signal or ER retention signal, are secreted to the parasitophorous vacuole[58-61]. One possibility is that this GFP fusion was unfolded and therefore retained in the ER[59]. Regardless, this reporter was not sent to the apicoplast indicating that it is not a functional apicoplast transit peptide (Figure 3B). In addition, PfGRP170 knockdown did not lead to any defects in trafficking to the apicoplast, nor could be rescued with the essential apicoplast metabolite IPP (Figure 3C and 3D).

Protein trafficking to the host RBC originates in the parasite ER and is essential for parasite viability, and therefore could potentially account for the observed death phenotype during PfGRP170 knockdown[11,12]. PfGRP170 was shown to associate with exported proteins in another study that identified proteins that bind to the antigenic variant surface protein, PfEMP1[62] and several exported proteins were found to interact with PfGRP170 in our study (Table 1 and Figure 4). However, our data show that there

is no significant difference in the trafficking of some exported proteins upon knockdown of PfGRP170, suggesting that protein export is not broadly affected (Supplemental Figure 5). The possibility that PfGRP170 activity is required for the trafficking of a subset of specific exported proteins remains to be tested.

ER chaperones are known in other eukaryotes to be vital to managing cellular stress[17,21]. Our data demonstrate that PfGRP170 is important for coping with a specific form of cellular stress, namely heat shock (Figure 2F). This finding highlights a potential critical role for PfGRP170 *in vivo*, as high febrile episodes are one of the main symptoms of clinical malaria and are considered an defense mechanism against parasites. GRP170 in mammalian systems have been shown to bind to the transmembrane proteins in the ER which are involved in the unfolded protein response (UPR), suggesting it may regulate these pathways[63,64]. *Plasmodium* genome does not encode many of the UPR orthologues, but a single ER stress pathway (PK4 signaling) has been previously described and was shown to be activated following artemisinin treatment[10,16]. Here, we demonstrate that PfGRP170 knockdown results in the activation of PK4 stress pathway (Figure 6), providing the first link between an endogenous ER resident protein and the activation of the PK4 pathway.

Finally, our data suggest that unlike its homologs in other eukaryotes, the essential function of PfGRP170 is not linked to its role in regulating BiP, since PfGRP170 binds to BiP upon removal of TMP and knockdown leads to upregulation of the stress response pathway (Figure 5B and Figure 6). Our data suggest a model where one (or more) of the substrates of PfGRP170 are essential for parasite survival within the red blood cell, and future work will determine which of the key substrates are solely dependent on PfGRP170 activity for their function. These data also suggest that given the divergence between mammalian and parasite GRP170 and their divergent biological roles, PfGRP170 could be a viable antimalarial drug target.

335 ***FIGURE LEGENDS***

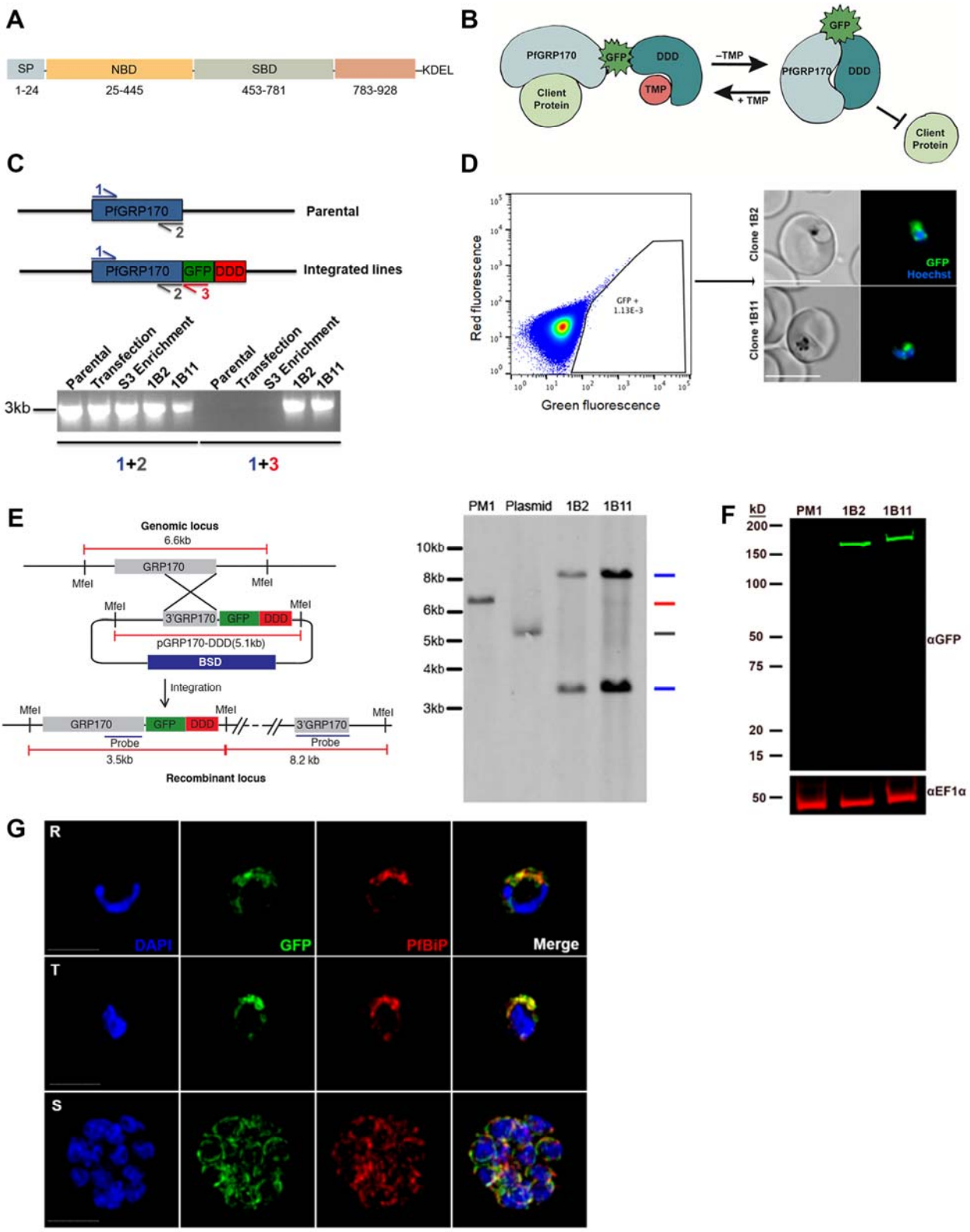

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Figure 1: Generation of PfGRP170-DDD Parasites

(A). Schematic detailing the putative domain boundaries of PfGRP170 (PF3D7_1344200) based on the yeast homolog, Lhs1: Signal Peptide (SP), Nucleotide Binding Domain (NBD), Substrate Binding Domain (SBD), Extended C-terminus region (783-928), and an ER retention signal (KDEL).

(B). Schematic diagram demonstrating the knockdown of PfGRP170. Knockdown of PfGRP170 is achieved by the removal of Trimethoprim (TMP), which results in the unfolding of the destabilization (DDD). The chaperone recognizes and binds the unfolded DDD and is inhibited from interacting with client proteins.

(C). **(Top)** Schematic diagram of the PfGRP170 locus in the parental line (PM1KO) and the modified locus where PfGRP170 is endogenously tagged with GFP and DDD. Primers used for integration test and control PCR are indicated by arrows. The relative positions of Primer 1 (blue) and Primer 2 (Gray) on the PfGRP170 locus are shown. These two primers will amplify PfGRP170 in parental and transfected parasites. Primer 3 (Red) recognizes the GFP sequence. Primers 1 and 3 were used to screen for proper integration into the PfGRP170 locus. **(Bottom)** PCR integration test and control PCRs on gDNA isolated from the PM1KO (parental), the original transfection of the PfGRP170-DDD plasmid after three rounds of Blasticidin (BSD) drug selection (Transfection), the PfGRP170-DDD transfected parasite lines after two rounds of enrichment for GFP positive cells (S3 enrichment), and PfGRP170-DDD clones 1B2 and 1B11 after MoFlo XDP flow sorting. The first 5 lanes are control PCRs using primers to amplify the PfGRP170 locus. The last 5 lanes are integration PCRs that only amplify if the GFP-DDD has been integrated into the genome.

(D). (Left) MoFlo XDP flow data demonstrating the percentage of GFP positive parasites in transfected PfGRP170-DDD parasites following three rounds of drug selection with Blasticidin (BSD) and two rounds of enrichment with an S3 cell sorter. Using the MoFlo, single GFP positive cells were cloned into a 96 well plate. Two clones, 1B2 and 1B11, were isolated using this method. **(Right)** 1B2 and 1B11 parasites, were observed using live fluorescence microscopy.

(E). Southern blot analysis of PfGRP170-DDD clones 1B2 and 1B11, PM1KO (parental control), and the PfGRP170-GDB plasmid is shown. Mfe1 restriction sites, the probe used to detect the DNA fragments, and the expected sizes are denoted in the schematic **(Left).** Expected sizes for PfGRP170-DDD clones (blue), parental DNA (red), and plasmid (gray) were observed **(Right).** Parental and plasmid bands were absent from the PfGRP170-DDD clonal cell lines.

(F). Western blot analysis of protein lysates from PM1KO (parental) and PfGRP170-DDD clonal cell lines 1B2 and 1B11 is shown. Lysates were probed with anti-GFP to visualize PfGRP170-DDD and anti-PfEF1α as a loading control.

(G). Asynchronous PfGRP170-DDD parasites were paraformaldehyde fixed and stained with anti-GFP, anti-PfGRP78 (BiP), and DAPI to visualize the nucleus. Images were taken as a Z-stack using super resolution microscopy and SIM processing was performed on the Z-stacks. Images are displayed as a maximum intensity projection. The scale bar is 2µM.

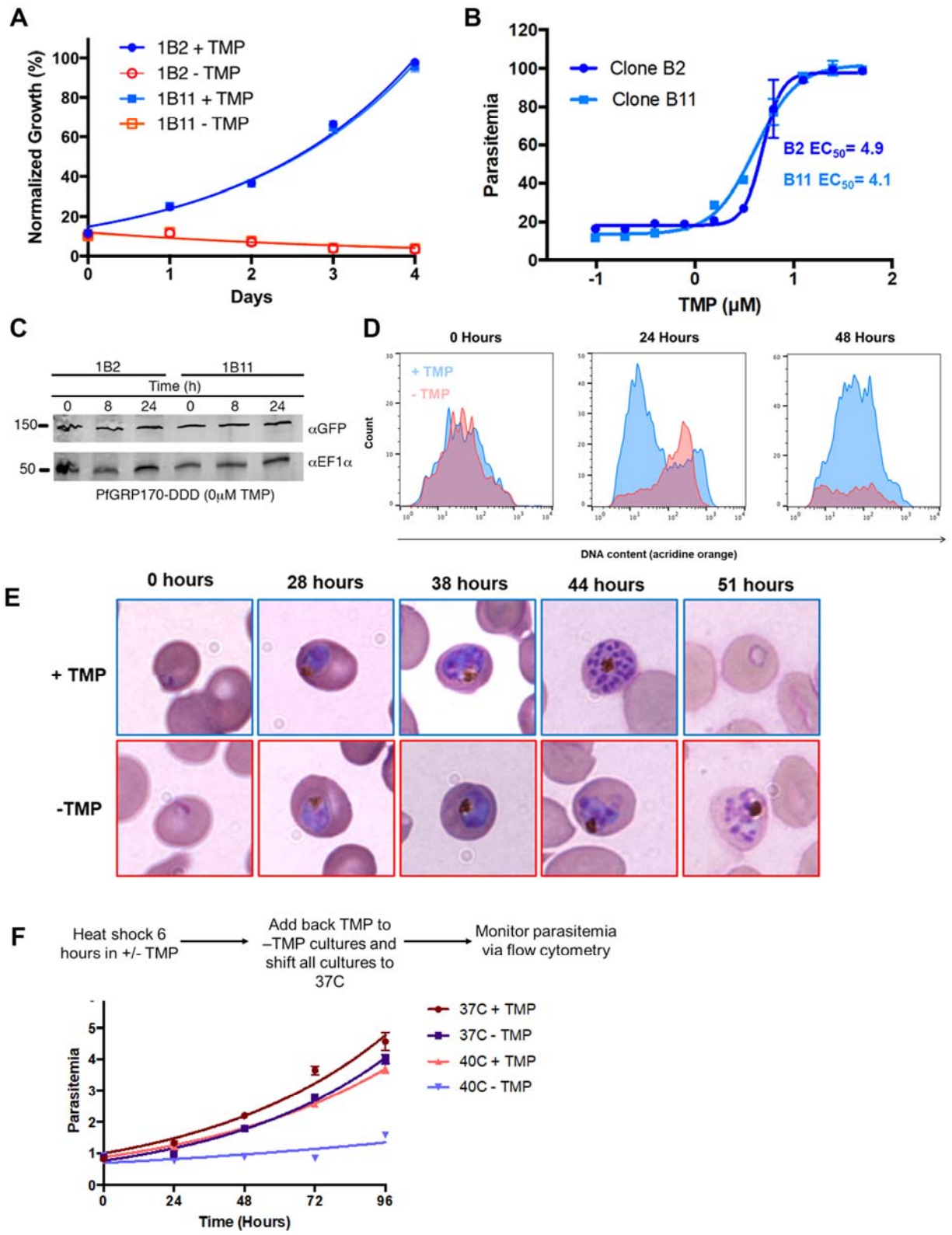


Figure 2: PfGRP170 is Essential and Required for Surviving a Heat Shock

(A). Parasitemia of asynchronous PfGRP170-DDD clonal cell lines 1B2 and 1B11, in the presence or absence of 20μM TMP, was observed using flow cytometry over 4 days. One hundred percent growth is defined as the highest parasitemia on the final day of the experiment. Data was fit to an exponential growth curve equation. Each data point is representative of the mean of 3 replicates ± S.E.M.

(B). Asynchronous PfGRP170-DDD clonal cell lines 1B2 and 1B11 were grown in a range of TMP concentrations for 48 hours. After 48 hours, parasitemia was observed using flow cytometry. Data was fit to a dose-response equation. Each data point is representative of the mean of 3 replicates ± S.E.M.

(C). Western blot analysis of PfGRP170-DDD lysates at 0, 8, and 24 hours following the removal of TMP is shown. Lysates were probed with anti-GFP to visualize PfGRP170-DDD and anti-PfEF1α as a loading control.

(D). Flow cytometric analysis of asynchronous PfGRP170-DDD parasites, incubated with (Blue) and without TMP (Red), and stained with acridine orange. Data at 0, 24, and 48 hours after the removal of TMP are shown.

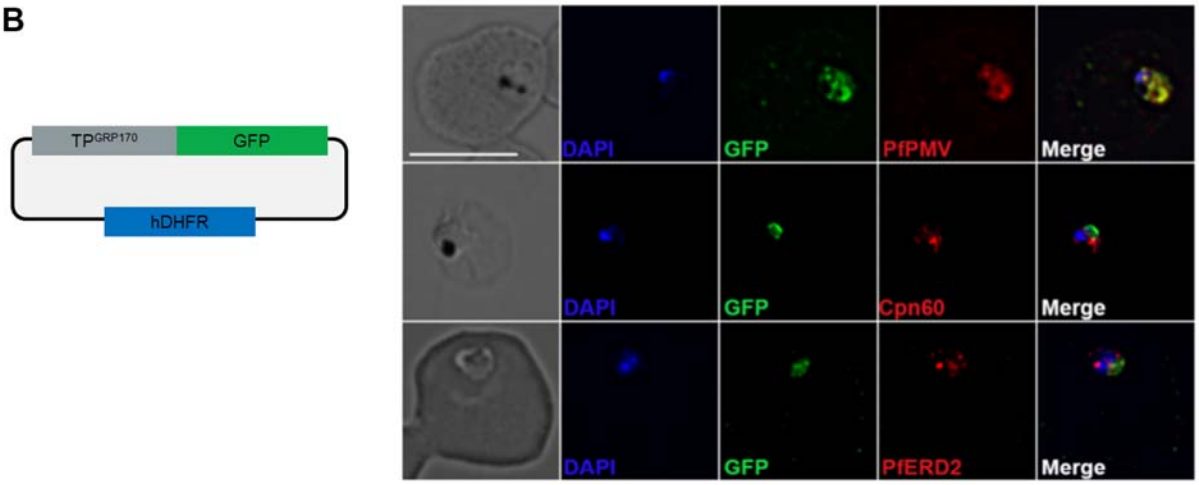
(E). TMP was removed from tightly synchronized PfGRP170-DDD ring stage parasites and their growth and development through the life cycle was monitored by Hema 3 stained thin blood smears. Representative images are shown from the parasite culture at the designated times.

(F). PfGRP170-DDD clones 1B2 and 1B11 were incubated with and without TMP for 6 hours at either 37°C or 40°C. Following the incubation, TMP was added back to all cultures and parasites were shifted back to 37°C. Parasitemia was then observed over 96 hours via flow cytometry. Data was fit to an exponential growth curve equation. Each data point shows the mean of 3 replicates ± S.E.M.

A

Protein	PATS Score	PlasmoAP Score
PfGRP170	Very Likely (0.943/1)	Very likely (5/5 tests positive)

B



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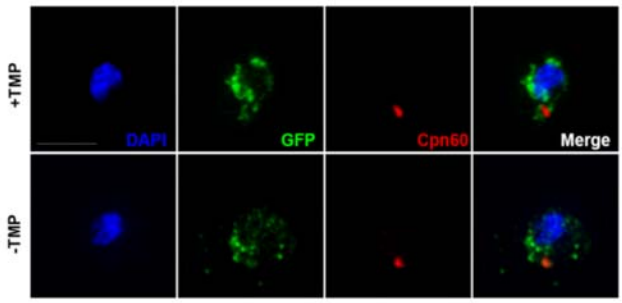


D

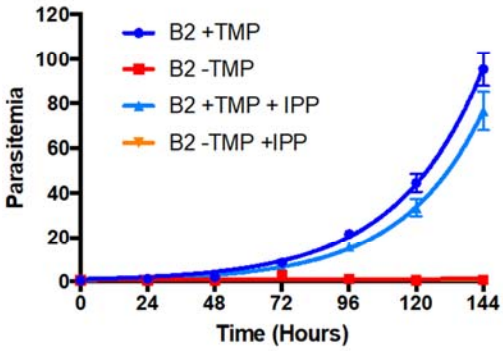


Figure 3: PfGRP170's putative plastid transit peptide localizes to the ER and knockdown of PfGRP170 does not affect Apicoplast Trafficking.

(A). Analysis of PfGRP170's protein sequence using two apicoplast transit peptide prediction programs: Prediction of Apicoplast-Targeted Sequences (PATS) and PlasmoAP.

(B). PfGRP170's putative apicoplast transit peptide was fused to GFP and transfected into 3D7 parasites. Parasites were fixed with acetone and stained with DAPI, anti-GFP (to label the PfGRP170 putative transit peptide) and either anti-PfPMV (ER), anti-PfERD2 (Golgi), or anti-Cpn60 (Apicoplast) to determine subcellular localization. The images were taken with Delta Vision II, deconvolved, and are displayed as a maximum intensity projection. The scale bar is 5µM.

(C). Synchronized ring stage PfGRP170 parasites were incubated for 24 hours with and without TMP. Following the incubation, the parasites were fixed with paraformaldehyde and stained with DAPI, anti-GFP (PfGRP170) and anti-Cpn60 (Apicoplast). Images were taken as a Z-stack using super resolution microscopy and SIM processing was performed on the Z-stacks. Images are displayed as a maximum intensity projection. The scale bar is 2µM.

(D). Asynchronous PfGRP170-DDD parasites were incubated with and without TMP and in the presence or absence of 200µM IPP. Parasitemia was monitored using flow cytometry for 144 hours. One hundred percent growth is defined as the highest parasitemia on the final day of the experiment. Data was fit to an exponential growth curve equation. Each data point is representative of the mean of 3 replicates ± S.E.M.

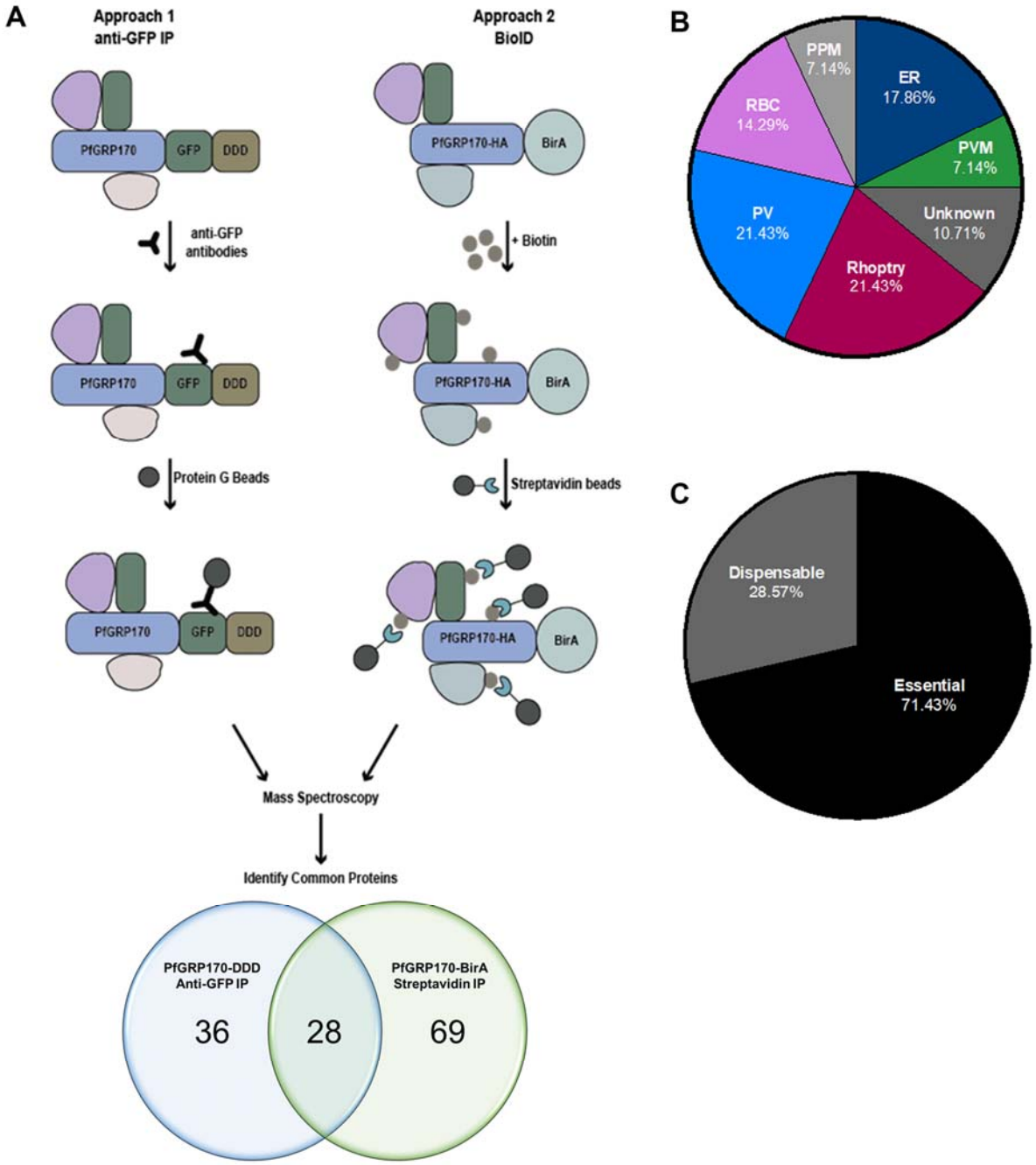


Figure 4: PfGRP170 interacts with Essential Proteins

(A). Schematic diagram illustrating the two independent methods were taken to identify potential interacting partners of PfGRP170: anti-GFP Immunoprecipitation (IP) using lysates from PfGRP170-DDD parasites and streptavidin IP of PfGRP170-BirA parasites

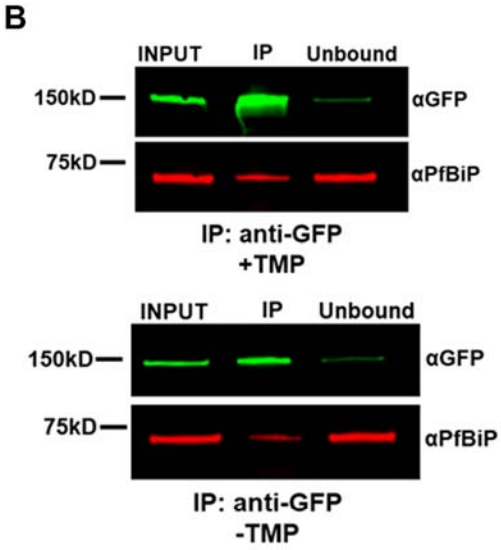
incubated with biotin for 24 hours followed by mass spectroscopy. The proteins identified from each IP were filtered to include only proteins that had a signal peptide and/or transmembrane domain using PlasmoDB. Proteins common between the anti-GFP and streptavidin IPs were cross-referenced to create a list of 28 common proteins.

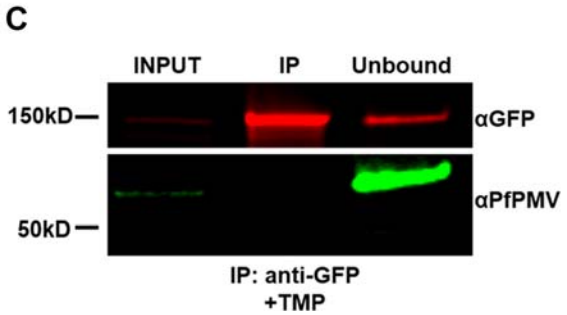
(B). The predicted subcellular localization of the 28 proteins identified using both approaches shown in (A).

(C). The percentage of interacting proteins that are predicted to be essential or non-essential for the asexual life cycle of *P. falciparum*[42].

A

Sample Type	# of unique Peptides for PfGRP170	# of unique peptides for BiP
Anti-GFP IP 1	132	83
Anti-GFP IP 2	141	80
BirA IP 1	55	27
BirA IP 2	53	30





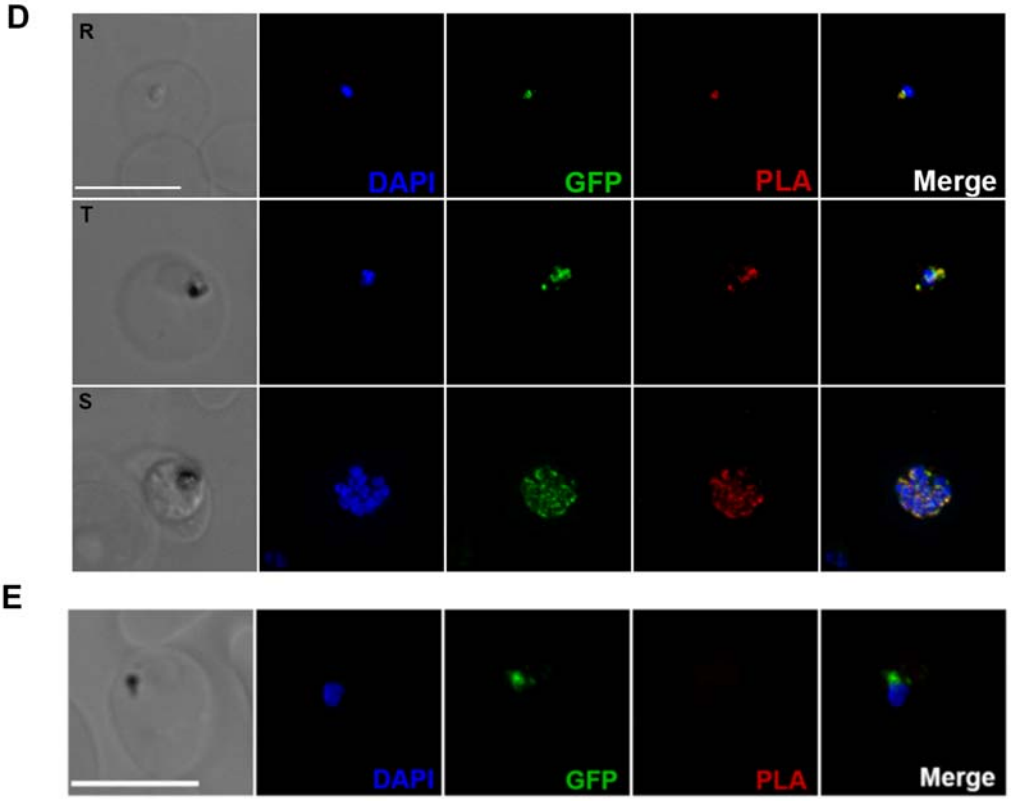


Figure 5: PfGRP170 Interacts with BiP

(A). Number of unique peptides identified for PfGRP170 and BiP by mass spectroscopy after anti-GFP immunoprecipitation (IP) of PfGRP170-DDD parasites and streptavidin IP of PfGRP170-BirA parasites (From Figure 4).

(B) Synchronized ring stage PfGRP170-DDD parasites were incubated with and without TMP for 24 hours. Following this incubation, an anti-GFP IP was performed and input, IP, and unbound fractions were analyzed using a western blot. The blot was probed using anti-GFP and anti-BiP.

(C). Western blot analysis of an anti-GFP IP performed on asynchronous PfGRP170-DDD parasites. Input, IP, and unbound fractions are shown. The blot was probed using anti-GFP and anti-PfPMV.

(D). *In vivo* interaction of PfGRP170 and BiP. PfGRP170-DDD parasites were paraformaldehyde fixed and stained with anti-GFP and anti-BiP. A Proximity Ligation Assay (PLA) was then performed. The scale bar is 5µM. A negative control using anti-GFP and anti-PfPMV is shown in **(E).**

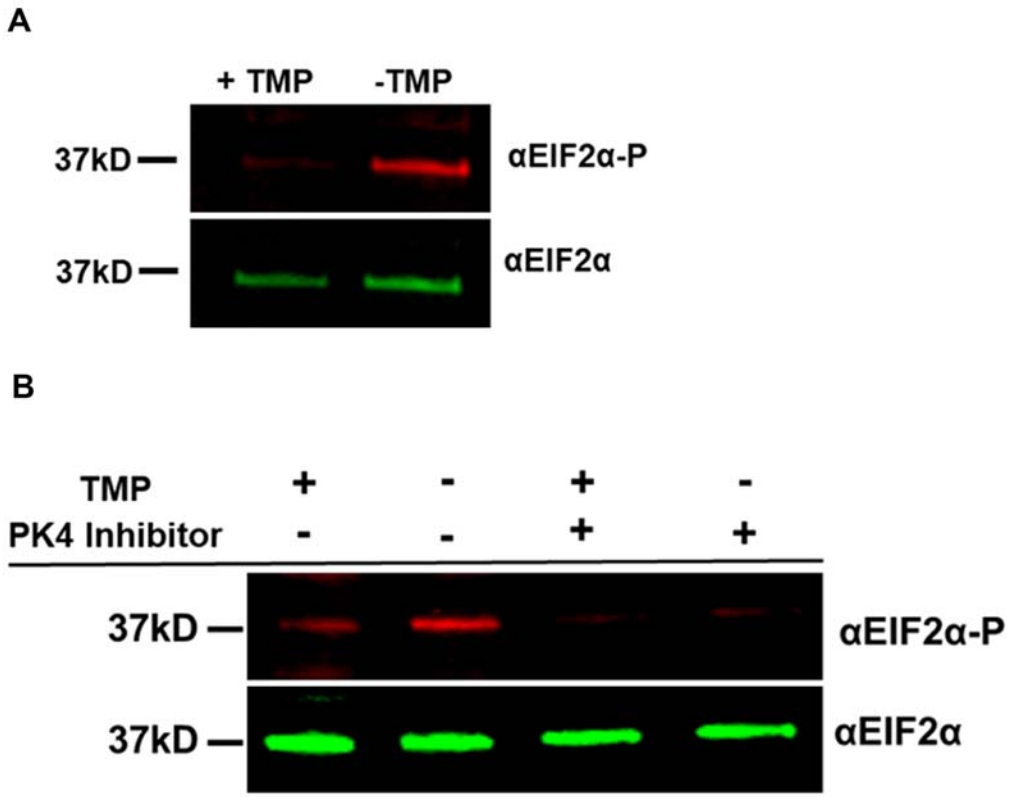


Figure 6: Activation of the PK4 Stress Pathway Following PfGRP170 Knockdown

(A). Synchronized ring stage PfGRP170-DDD parasites were incubated with and without TMP for 24 hours. Protein was isolated from these samples and analyzed via western blot, probing for anti-eIF2α and anti-Phospho-eIF2α.

(B). Synchronized ring stage PfGRP170-DDD parasites were incubated with and without TMP and in the presence and absence of 2μM PK4 inhibitor GSK2606414 for 24 hours. Protein was isolated from these samples and analyzed via western blot by probing for anti-eIF2a and anti-Phospho-eIF2α.

Table 1: List of high confidence interacting partners of PfGRP170

The table includes the 28 proteins identified using two independent mass spectroscopy approaches (See Figure 5), their subcellular localization, and whether they are

predicted to be essential using the a *piggyBac* mutagenesis screen recently performed in *P. falciparum*[42].

Gene ID	Gene Product	Subcellular Localization	Predicted to be Essential?
PF3D7_0917900	Heat shock protein 70	ER	Yes
PF3D7_1222300	Endoplasmin, putative	ER	Yes
PF3D7_0827900	Protein disulfide isomerase	ER	Yes
PF3D7_1108700	Heat shock protein J2	ER	Yes
PF3D7_1134100	Protein disulfide isomerase	ER	No
PF3D7_1364100	6-cysteine protein	PPM	No
PF3D7_0508000	6-cysteine protein	PPM	Yes
PF3D7_1116800	Heat shock protein 101	PV	Yes
PF3D7_0207600	Serine repeat antigen 5	PV	Yes
PF3D7_1335100	Merozoite surface protein 7	PV	Yes
PF3D7_1129100	Parasitophorous vacuolar protein 1	PV	No
PF3D7_1228600	Merozoite surface protein 9	PV	Yes
PF3D7_0207500	Serine repeat antigen 6	PV	Yes
PF3D7_0930300	Merozoite surface protein 1	PVM	Yes
PF3D7_1033200	Early transcribed membrane protein 10.2	PVM	No
PF3D7_0310400	Parasite-infected erythrocyte surface protein	RBC	Yes
PF3D7_0935800	Cytoadherence linked asexual protein 9	RBC	Yes
PF3D7_1038000	Antigen UB05	RBC	Yes
PF3D7_0501200	Parasite-infected erythrocyte surface protein	RBC	No
PF3D7_0929400	High molecular weight rhoptry protein 2	Rhoptry	Yes
PF3D7_1252100	Rhoptry neck protein 3	Rhoptry	Yes
PF3D7_1410400	Rhoptry-associated protein 1	Rhoptry	No
PF3D7_0707300	Rhoptry-associated membrane antigen	Rhoptry	No
PF3D7_0501600	Rhoptry-associated protein 2	Rhoptry	Yes
PF3D7_0905400	High molecular weight rhoptry protein 3	Rhoptry	Yes
PF3D7_0505700	Conserved Plasmodium membrane protein, unknown function	Unknown	No
PF3D7_1462300	GTP-binding protein, putative	Unknown	Yes
PF3D7_1352500	Thioredoxin-related protein, putative	Unknown	Yes

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METHODS

Primers and Plasmid construction

All primer sequences used in this study can be found in Supplemental Table 2.

Generation of pGDB-SDEL plasmid was done using the QuikChange II Site-Directed Mutagenesis Kit (Agilent Technologies) on the pGDB plasmid with primers P1 and P2 per the manufacturer's protocol[31].

Genomic DNA was isolated using the QIAamp DNA blood kit (Qiagen). gDNA used in this study was isolated from either 3D7 or Plasmepsin I knockout parasites (PM1KO)[31].

The pPfGRP170-DDD plasmid used to generate the PfGRP170-GFP-DDD mutants was made by amplifying via PCR an approximately 1-kb region homologous to the 3'end of the PfGRP170 gene (stop codon not included) using primers P3 and P4. The amplified product was inserted into pGDB-SDEL plasmid using restriction sites Xho1 and AvrII (New England Biolabs) and transformed into bacteria. The construct was sequenced prior to transfection.

The pGRP170-HA-BirA-KDEL plasmid was prepared by amplifying PfGRP170 (without the stop codon) from 3D7 gDNA using primers P5 and P6 and 3xHA-BirA from the pTYEOE-3XHA-BirA plasmid (From D. Goldberg) using primers P7 and P8. Both PCR products generated included homologous regions used for Sequence and Ligation Independent Cloning (SLIC)[65] .The primers to amplify the 3xHA-BirA included the

sequence of an ER retention signal (KDEL). These PCR products were fused together using SLIC as described previously and subsequently PCR amplified using primers P5 and P8[29]. The resulting product was then inserted into pCEN-DHFR[66] that was digested with Nhe1 and BglII (New England Biolabs) using SLIC and transformed into bacteria as described previously[29,30].

The pPfGRP170TP-GFP plasmid was prepared by amplifying the first 450 bp (includes the signal peptide and putative transit peptide sequence) of PfGRP170 from PM1 gDNA using primers P5 and P9. The GFP sequence used was amplified from pGDB using primers P10 and P11. The PfGRP170 transit peptide PCR was digested with Nhe1 and AatII (New England Biolabs) and the GFP PCR was digested with AatII and BglII (New England Biolabs). The two fragments were then ligated together (via the AatII digest site) using a T4 ligase (kit from New England Biolabs) and subsequently PCR amplified using primers P5 and P11. The resulting product was then digested with Nhe1 and BglII and inserted into pCEN-DHFR[66] that was digested with Nhe1 and BglII (New England Biolabs) using a T4 ligase and transformed into bacteria as described previously[29,30].

Cell Culture, transfections, and isolation of clonal cell lines

Parasites were grown in RPMI 1640 media supplemented with Albumax 1 (Gibco) and transfected as described previously[28-32].

To generate PfGRP170-GFP-DDD mutants, PM1KO parasites were transfected with the pPfGRP170-DDD plasmid in duplicate. PM1KO parasites contain the human dihydrofolate reductase (hDHFR) expression cassette which gives the parasites resistance to Trimethoprim (TMP)[31]. Drug selection and cycling were performed as described previously using 10µM TMP (Sigma) and 2.5µg/ml Blasticidin (Sigma)[30-32]. Following drug cycling, GFP positive cells were enriched using an S3 Cell Sorter (BioRad). Individual GFP positive cells from a single transfection were cloned into 96 well plates using a MoFlo XDP flow cytometer. After the EC_{50} of TMP was determined for clones 1B2 and 1B11, parasites were shifted into media containing 2.5µg/ml BSD and 20µM TMP to facilitate optimal growth.

The PfGRP170-BirA and PfGRP170TP-GFP parasites were generated by transfecting 3D7 parasites with plasmids pGRP170-HA-BirA-KDEL or pPfGRP170TP-GFP, respectively. Parasites expressing these episomal constructs were selected using 2.5nM WR99210.

Integration tests for PfGRP170-DDD mutants

Genomic DNA was isolated from parasites using the QIAamp DNA blood kit (Qiagen). Control primers to amplify the genome were P4 and P12 and primers used to amplify integrated DNA were P12 and P13.

Southern blot analysis was performed on DNA isolated from PfGRP170-GFP-DDD parasites (1B2 and 1B11) as described previously[29,30]. The assay was also performed on PM1KO parental DNA and the pGRP170-DDD plasmid as a control. DNA was isolated from parasites using the QIAamp DNA blood kit (Qiagen). 10µg of precipitated PM1KO DNA, 1B2, and 1B11 DNA and 10ng of pGRP170-DDD plasmid was digested overnight with Mfe1 (New England Biolabs). The biotinylated probe used was generated by PCR using biotinylated-16-UTP (Sigma) and primers P3 and P4. The biotinylated probe on the southern blot was detected using IRDye 800CW streptavidin-conjugated dye (LICOR Biosciences) and imaged using the Odyssey infrared imaging system (LICOR Biosciences).

Growth assays using flow cytometry

TMP was removed from asynchronous PfGRP170-DDD cultures for growth assays by washing the culture in equal volume of complete RPMI three times. The culture was then resuspended in complete RMPI media containing either 2.5µg/ml Blasticidin (Sigma) for knockdown parasites (Sigma) or 2.5µg/ml Blasticidin (Sigma) and 20µM TMP (Sigma) for the control. Parasitemia was monitored using a flow cytometer, either a CyAn ADP (Beckman Coulter) or CytoFLEX (Beckman Coulter) instrument, using either 1.5µg/ml acridine orange (Molecular Probes) as described previously[29] or similarly using 8µM Hoechst in filtered 1X phosphate-buffered saline (PBS). Flow cytometry data were analyzed using FlowJo software (Treestar Inc.). If the parasitemia was too high, parasites were subcultured during the experiment and the relative

parasitemia was then calculated by multiplying the calculated parasitemia by the dilution factor. Parasitemia was normalized by using the highest parasitemia as one hundred percent. Using Prism software (GraphPad Software Inc), the parasitemia data were fit to an exponential growth curve equation.

To determine the EC_{50} of TMP for PfGRP170-DDD cell lines, parasites were washed as described above and seeded into a 96 well plate with 2.5µg/ml Blasticidin and varying TMP concentrations. Parasitemia was measured after 48 hours using flow cytometry as described above. The parasitemia data were fit to a dose-response equation using Prism.

For the IPP rescue experiment, asynchronous PfGRP170-DDD parasites were washed as described above and resuspended in media either with 2.5µg/ml Blasticidin or 2.5µg/ml Blasticidin and 20µM TMP with or without 200µM Isopentenyl pyrophosphate (Isoprenoids LC). Parasitemia were monitored using flow cytometry as described above and the data were fit to an exponential growth curve equation using Prism.

For the heat shock experiment, asynchronous PfGRP170-DDD parasites were washed as described above and resuspended in media either with 2.5µg/ml Blasticidin or 2.5µg/ml Blasticidin and 20µM TMP. Parasites were then incubated at either 37°C or 40°C for 6 hours. After 6 hours, 20µM TMP was added to cultures that were incubated without it and all parasites were shifted back to 37ºC. Parasitemia was monitored using flow cytometry as described above and the data were fit to an exponential growth curve equation (GraphPad Software Inc).

Synchronized growth assay

PfGRP170-DDD Parasites were synchronized as described previously by sorbitol (VWR), followed by percoll (Genesee Scientific) the next day and then sorbitol four hours later to obtain 0-4 hour rings[30,31]. Parasites were washed as described above to remove TMP from the media and incubated in media either with 2.5µg/ml Blasticidin or 2.5µg/ml Blasticidin and 20µM TMP. Thin blood smears using the Hema 3 Staining Kit (PROTOCOL/Fisher) were prepared every few hours to monitor parasite growth and

morphology. Slides were imaged using a Nikon Eclipse E400 microscope with a Nikon DS-L1-5M imaging camera.

Western blot

Western blotting was performed as described previously[30]. Parasite pellets were isolated using cold 0.04% Saponin (Sigma) in 1X PBS for 10 minutes as described previously[30,31]. Antibodies used for this study were: mouse anti-GFP JL-8 (Clontech, 1:3000), rabbit anti-PfEF1α (from D. Goldberg, 1:2,000), mouse anti-plasmepsin V (from D. Goldberg, 1:400), rabbit anti-PfBiP MRA-1246 (BEI resources, 1:500), rabbit anti-GFP A-6455 (Invitrogen, 1:2,000), mouse anti-eIF2α L57A5 (Cell Signaling, 1:1,000), rabbit anti- Phospho-eIF2α 119A11 (Cell Signaling, 1:1,000), rat anti-HA (Roche 3F10, 1:3000), and mouse anti-Ub P4D1 (Santa Cruz Biotechnology, 1:1,000). Secondary antibodies used were IRDye 680CW goat anti-rabbit IgG and IRDye 800CW goat anti-mouse IgG (LICOR Biosciences, 1:20,000). The western blots were imaged using the Odyssey infrared imaging system. Polyacrylamide gels used in this study were either prepared using 10% EZ-Run protein gel solution (Fisher) or precast gradient gels (4-20%, from Biorad).

For testing PK4 activity, synchronized ring stage PfGRP170-DDD parasites were incubated in media with either 2.5µg/ml Blasticidin or 2.5µg/ml Blasticidin and 20µM TMP in the presence or absence of a PK4 inhibitor GSK2606414 (Millipore Sigma) at 2µM for 24 hours. After 24 hours, the parasites were lysed for western blot analysis using 0.04% saponin in 1X PBS as described above.

Live Fluorescence Microscopy

To visualize PfGRP170-DDD live parasites, 100µL of parasite culture was pelleted. The supernatant was removed, and the parasites were resuspended in 100µL medium with 2.5µg/ml Blasticidin and 20µM TMP and 5µM Hoechst. The parasites were incubated at 37°C for 20 minutes. The parasites were then pelleted again and 90% of the medium was removed. Parasites were resuspended in the remaining medium and 8µL of this culture was placed on a glass slide and covered with a coverslip. The edges were sealed with nail polish and the cells were imaged using a DeltaVision II Microscope.

Immunofluorescence trafficking assays and imaging processing

Immunofluorescence assays (IFA) were performed as described previously using a combination of 4% Paraformaldehyde and 0.015% glutaraldehyde for fixation and permeabilization using 0.1% Triton-X100[29,30] or by smearing cells on a slide and fixing them with acetone. For apicoplast and red blood cell trafficking assays, cells were synchronized and TMP was removed as described above. Cells were then fixed as described above, 24 hours after the removal of TMP.

Primary antibodies used for IFAs in this study were: rabbit anti-GFP A-6455 (Invitrogen, 1:200), rat anti-PfBiP MRA-1247 (BEI resources, 1:125), rabbit anti-PfBiP MRA-1246 (BEI resources (1:100), mouse anti-plasmepsin V (From D. Goldberg, 1:1), mouse anti-GFP clones 7.1 and 13.1 (Roche 11814460001, 1:500), rabbit anti-Cpn60 (From. B. Striepen, 1:1,000), rabbit anti-PfERD2 (MR4, 1:2,000), rabbit anti-HA 9110 (Abcam, 1:200), rabbit anti-PfMAHRP1C (From. Hans-Peter Beck, 1:500), mouse anti-PfFIKK4.2 (From David Cavanagh/EMRR, 1:1,000), and rabbit anti-PfHSP70X (From Jude Przyborski, 1:500). Secondary antibodies used in this study are Alexa Fluor goat anti-rabbit 488, Alexa Fluor goat anti-rabbit 546, Alexa Fluor goat anti-mouse 488, Alexa Fluor goat anti-mouse 546, and Alexa Fluor goat anti-rat 546 (Life Technologies, 1:100). The mouse anti-PfFIKK4.2, rabbit anti-PfHSP70X, and anti-PfMAHRP1C require acetone fixation.

All fixed cells were mounted using ProLong Diamond with DAPI (Invitrogen) and imaged using the DeltaVision II microscope system or Zeiss ELYRA S1 (SR-SIM) Super Resolution Microscope using a 100X objective. Images taken using the DeltaVision II were collected as a Z-stack and were deconvolved using the DeltaVision II software (SoftWorx). The deconvolved Z-stacks were then displayed as a maximum intensity projection using SoftWorx. Images taken using the Super Resolution Microscope were taken as a Z-stack. The Z-stacks were analyzed using Zen Software (Zeiss, version from 2011) for SIM processing and obtaining the maximum intensity projection. Any adjustments made to the brightness and/or contrast of the images were made using either Softworx, Zen Software, or Adobe Photoshop and were done for display purposes only. Any quantification performed for microscopy images was done using ImageJ

software as described previously[29]. The quantification data were analyzed using Prism (GraphPad Software, Inc.).

Co-immunoprecipitation assays and Mass Spectroscopy

Parasites pellets were isolated from 48 mL of asynchronous culture at high parasitemia (10% or higher) using cold 0.04% saponin in 1X PBS as described above. Parasite pellets were lysed by resuspending the pellet in 150µL of Extraction Buffer (40mM Tris HCL pH 7.6, 150mM KCL, and 1mM EDTA) with 0.5% NP-40 (VWR) and 1X HALT protease inhibitor (Thermo). The resuspended parasites were then incubated on ice for 15 minutes and then sonicated three times (10% amplitude, 5 second pulses). In between each sonication, the lysate was placed on ice for 1 minute. The lysate was then centrifuged at 21,100g for 15 minutes at 4ºC. The supernatant was collected in a fresh tube and placed on ice. The remaining pellet was subjected to a second lysis step using 150µL of Extraction buffer as above without NP-40. The lysate was sonicated and centrifuged as above (no 15-minute incubation on ice). The supernatant was collected and combined with the lysate from the first lysis step (INPUT sample). 20µL of the input sample was collected into a fresh tube and stored in the -80ºC. The remaining input sample was combined with 2µL of rabbit anti-GFP monoclonal G10362 (Thermo) and incubated rocking for two hours at 4°C.

After the two-hour incubation, the lysate with antibody was added to 50µL of prepared protein G Dynabeads (Invitrogen). Dynabeads were prepared by washing 50µL of beads three times with 100µL of IgG binding buffer (20mM Tris HCL pH 7.6, 150mM KCL, 1mM EDTA, and 0.1% NP-40). The IgG binding buffer was removed from the beads each time using a magnetic rack (Life technologies). The beads, antibody, and lysate were incubated rocking for two hours at 4°C. After the two-hour incubation, the unbound fraction of protein was collected using the magnetic rack into a fresh tube and stored at -80°C until needed for western blot analysis. The beads were then washed two times in 300µL of IgG binding buffer with 1X HALT and one time in IgG binding buffer with 1X HALT without NP-40. Each wash was done for 10 minutes rocking at 4°C.

For Co-IP's to show PfGRP170-DDD/BiP interaction 0-4 hour ring stage parasites were obtained and TMP was removed as described under the synchronized growth assay

section. Parasites were lysed and an anti-GFP IP was performed as described above, approximately 24 hours after the removal of TMP. Protein was eluted off the beads for western blot using 1X Protein Loading Dye (LICOR) with 2.5% beta-Mercaptoethanol (Fisher) and boiled for 5 minutes. This was followed by a centrifugation at 16,200 g for 5 minutes. The eluted proteins are collected by placing the tube on a magnetic rack. The isolated proteins on magnetic beads were digested with trypsin and analyzed at the Emory University Integrated Proteomics Core using a Fusion Orbitrap Mass Spectrometer.

PfGRP170-BirA biotinylation and mass spectrometry

To confirm that proteins were biotinylated when biotin was added to the PfGRP170-BirA parasites, parasites were incubated 24 hours in media containing 2.5nM WR + 150µg of biotin (Sigma). Parasites were isolated using 0.04% saponin in 1X PBS and the lysate was ran on a western blot as described above. Secondary antibodies used were IRDye 680CW goat anti-rabbit IgG and IRDye 800CW Streptavidin (LICOR). 3D7 parasites incubated with media containing 150µg of biotin for 24 hours was used as a control.

For PfGRP170-BirA streptavidin IP's, cultures were incubated for 24 hours in media containing 2.5nM WR + 150µg of biotin (Sigma). 48 mL of asynchronous culture at high parasitemia (10% or higher) were harvested for IP as described above with the following modifications. Streptavidin MagneSphere Paramagnetic Particle beads (Promega) were used to isolated biotinylated proteins. To prepare the Streptavidin beads for IP, beads were washed three times in 1 mL of 1X PBS. Incubations of lysate with the magnetic beads were performed at room temperature for 30 minutes. After the unbound fraction was removed, beads were washed twice in 8M Urea (150mM NaCL, 50mM Tris HCL pH 7.4) and once in 1X PBS. The biotinylated proteins on magnetic beads were digested with trypsin and analyzed at the Emory University Integrated Proteomics Core using a Fusion Orbitrap Mass Spectrometer.

Proximity Ligation Assays

Asynchronous PfGRP170-DDD parasites were fixed as described above, approximately 24 hours after the removal of TMP. The proximity ligation assay was performed using the Duolink PLA Fluorescence kit (Sigma) per the manufacturers protocol.

Ring Stage Survival Assay

The ring-stage survival assay method was performed on 3D7 (control) and PfGRP170-BirA parasites as described previously**,** with a slight adjustment[67]. Cultures were synchronized using 5% sorbitol (Sigma-Aldrich, St. Louis, MO, USA), pre-warmed to 37°C, to obtain the highest proportion of rings, $\geq$50%. The cultures were placed back under previously described conditions for 24 hours and followed-up the next morning. Thin blood smears were methanol fixed and stained with 10% Giemsa for 15 minutes and evaluated for mature schizonts with visible nuclei (10-12). The parasites were independently suspended in PRMI-1640 supplemented with 15U/ml of sodium heparin (Sigma-Aldrich, St. Louis, MO, USA) to disrupt spontaneous rosettes formation for 15 minutes at 37 °C. After incubation, each parasite culture was layered onto a 75/25% percoll (GE Healthcare Life Sciences, Pittsburgh, PA, USA) gradient, and centrifuged at 3000rpm for 15 minutes. The intermediate phases containing the mature schizonts of each culture, were independently collected, gently washed in RPMI and transferred into two new T25 flasks with fresh cRPMI and erythrocytes for 3-hour incubation at previously described conditions. Thin blood smears were prepared as previously described, to ensure >10% schizonts count.

At the 3-hour mark, the parasites were taken-out of incubation and treated with 5% sorbitol to remove the remaining mature schizonts, which had not invaded erythrocytes yet. Parasitemia was adjusted to 1% at 2% hematocrit by adding uninfected erythrocytes and cPRMI, after the evaluation of quick stained Giemsa smears. The parasites were exposed to 700nM DHA or 1% dimethyl sulfoxide (DMSO) for 6 hours. After the 6-hour incubation period, the parasites were washed to remove the drug or DMSO and re-suspended in 1ml of cRPMI. The parasites were then transferred into two new well in the 48-well culture plate, incubated at 37 °C under a 90 % N_2, 5 % CO_2, and 5 % O_2 gas mixture for 66 hours, after which thin blood smears were prepared,

methanol fixed, stained with 10% Giemsa for 15 minutes and read by three operators. Growth rate and percent survival was calculated by counting the number of parasitized cells in an estimated 2000 erythrocytes**.**

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