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A LARGE-SCALEPHENOTYPICSCREENIDENTIFIES GENETICFACTORS LINKED TO GAME TO CYTE DEVELOPMENT IN PLASMODIUM FALCIPARUM

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Abstract:

Gametocyte development in *Plasmodium falciparum* is essential for malaria transmission from humans to mosquitoes and represents a critical bottleneck in the parasite life cycle. Despite its importance, the genetic and molecular mechanisms governing sexual commitment and gametocyte maturation remain incompletely understood. In the present study, a large-scale phenotypic screen was conducted to identify genes required for early and late stages of gametocytogenesis using a pooled library of approximately 600 isogenic piggyBac insertion mutants (Half-K library), representing nearly 7% of the *P. falciparum* genome. Gametocytogenesis was induced under in vitro culture conditions, and mutant representation was quantified at the onset of sexual commitment (day 0), early gametocyte development (day 3), and mature stage V gametocytes (day 14) using quantitative insertion-site sequencing (QIseq). Differential abundance analysis using DESeq2 enabled classification of mutants as gametocyte hypo-producers or hyper-producers at each developmental stage. Gene ontology enrichment analyses revealed that early gametocyte development is strongly associated with intracellular protein transport and organelle localization, whereas late-stage development is enriched for RNA metabolism, ribosomal modification, and endomembrane system organization. Notably, several transcription factors, RNA-binding proteins, and zinc-finger proteins were identified as essential for successful gametocyte progression. In parallel, comparison with a phenotypic screen under sub-lethal dihydroartemisinin (DHA) pressure demonstrated a significant association between increased gametocytogenesis and reduced DHA sensitivity, suggesting shared regulatory pathways linking sexual development and artemisinin response. Collectively, this study provides a comprehensive functional genomic framework for understanding gametocyte biology and identifies novel genetic targets with potential implications for transmission-blocking antimalarial strategies and resistance containment.

Keywords: *Plasmodium falciparum*; Gametocytogenesis; Sexual commitment; piggyBac mutagenesis; Quantitative insertion-site sequencing (QIseq); Functional genomics;

1. INTRODUCTION

Plasmodium falciparum is responsible for global malaria morbidity and mortality and is especially devastating in pregnant women and children in sub–Saharan Africa [1]. Transmission of the parasite is achieved through ingestion of mature sexual stages from an infected person by Anopheline mosquitoes. Once infected, the mosquito vectors go on to transmit the parasite to uninfected individuals during a blood meal. Human-to vector transmission represents a major bottleneck in the parasite life cycle and provides an opportunity to interrupt transmission. However, most antimalarials are ineffective in clearing the mature sexual stages (gametocytes), with some that are contradicted for use in certain patient populations [2, 3]. Better transmission blocking interventions driven by a deeper understanding of gametocyte biology is indeed crucial for malaria control and elimination. Game to cytogenesis begins with an epigenetic switch in asexually dividing parasites that commits the progeny to a non-replicative sexual fate [4, 5]. Post commitment, the gametocytes undergo five morphologically distinct stages (I-V) unique to P. falciparum, resulting in mature 'falci form' shaped gametocytes that are infectious to mosquitoes. This extensive 14-day period of development entails elaborate trans criptomic, proteomic, and metabolic changes essential for parasite transmission into a new host. The rate of commitment is influenced by various factors including inherent strain characteristics, host status, drug treatment and other environmental signals [6]. Unraveling the molecular mechanisms governing sexual commitment, the first step of game to cytogenesis and maturation, the last step of the intra erythro cytic sexual cycle is crucial for prioritizing potential high value targets for development of new therapies to block gametocyte development and prevent transmission. Even with recent progress made in the identification of genetic factors linked to game to cytogenesis, many processes vital for sexual stage development remain poorly understood. This chapter will pursue a large-scale functional genomic screen to identify the P. falciparum genes needed for gametocyte development using a large pool of piggy Bac mutants. The proof-of-concept necessary for a scalable phenotypic screen and elucidates the genotype-phenotype associations essential for stage V gametocyte development.

Here, we extend our forward genetic pB-QIseq approach to identify genes linked to the switch to sexual development, gametocyte conversion (early time point) and maturation (late time point). The mutants in large mixed pools are isogenic and contain a single random piggyBac transposon insertion in the parasite genome. A highly sensitive Illumina-based sequencing tool coupled with stringent computational criteria, Quantitative Insertion-site Sequencing (QIseq), is used to differentiate between mutants under phenotype selection by identifying and quantifying the piggyBac insertion sites [7]. QIseq can do so in large mixed mutant populations and even when mutants are present in very low numbers [8, 9]. Therefore, a genes' functional importance for sexual commitment and development is revealed by calculating its relative abundance at each time point. The gametocyte phenotypic screen enables identification of a set of genes involved in early and late game to cytogenesis, highlighting potential pathways and processes that can be targeted antimalarial drug discovery or vaccine development. More importantly, the methods established in this study, efficiently aid the functional annotation of many unknown proteins in the P. falciparum genome. Intriguingly, we also report an overlap of genes with increased game to cytogenesis and altered sensitivity to DHA through our parallel phenotypic screens, establishing a potential link between artemisinin resistance and game to cytogenesis.

2. MATERIALS AND METHODS

Parasite culture and maintenance

The parasites were cultured at 5% hematocrit (O+ erythrocytes) in complete medium containing 10% human AB serum (heat inactivated), 2.5% sodium bicarbonate (using 7.5% stock solution) in RPMI 1640 medium (KD Medical) supplemented with 50 µg/mL hypoxanthine and 25 mM HEPES. The culture flasks were grown in an incubator at 37°C and manually gassed with mixed gas (90% N2, 5% CO2 and 5% O2).

Development of a large-scale gametocyte screen

The Half-K piggyBac mutant library was generated [10]. A gametocyte screen was performed with 2 bio replicates of this mutant library. Briefly, the 1ml of the mixed pool population was thawed in non-vented T25 flasks (5 ml, 5% hematocrit, manual gassing, 37 °C) and after reaching 1-2% parasitemia the parasites were scaled up to 20-mL cultures in T75. Game to cytogenesis was induced by stressing the parasites with high parasitemia. Genomic DNA was harvested from the induced flasks using the Qiagen DNA extraction kit (QIAamp, cat no. 51104) and sequenced by QIseq as day 0. Gametocyte flasks were set with a starting parasitemia of 0.5%-0.8% and maintained with daily medium change. Early gametocyte enrichment on day 3 was accomplished by FACS using Pfs16, an early sexual- stage marker [11-14]. In brief, 200 ml of gametocyte cultures were first enriched using percoll gradient centrifugation [15]. A 100 µl aliquot of live, gametocyte enriched parasite-infected RBCs were incubated with polyclonal anti-P. falciparum Pfs16 (diluted 1:200) for 30 minutes on ice. Next, Alexa Fluor 488, goat anti-rabbit IgG (diluted 1:25) was added and allowed to incubate for 30 minutes on ice. To stain the nuclei, Hoechst dye was added to the samples for 20 mins at 370 C following which samples were washed 3 times with FACS buffer made from phosphate buffered saline supplemented with 1% bovine serum albumin (BSA). Flow cytometry was carried out in a high-speed BD FACS Aria IIu sorter, where compensation to exclude background fluorescence on the vertical and horizontal axes was done with samples single labelled with AF488, Hoechst and uninfected erythrocytes. Polyclonal rabbit anti-Pfs16 serum was obtained from MR4 BEI

resources (MRA-1276). Genomic DNA harvested from this population was sequenced by QIseq as day 3. Late gametocyte enrichment was achieved by treating cultures with 50 mM N-acetyl glucosamine (NAG) from days 4-9 to get rid of any asexual parasites [16]. When majority of the parasite population was stage V gametocytes on day 14, the mature stages were isolated by a Percoll- gradient centrifugation and washed thrice with incomplete medium [15]. Subsequently, genomic DNA was harvested from this population and sequenced by QIseq as day 14.

Phenotype identification

Previously described QIseq methodology was used to quantify insertion sites for each piggyBac mutant in the pool screened sample [7]. The DEseq2 from R was used to normalize original read counts per insertion-site and calculate fold changes for early (day 3/ day 0) and late (day 14 / day 0) time points. The mutants were ranked from lowest to highest to identify phenotypes of interest. Hits from the bottom quartile were categorized as hypo-producers and those from the top quartile were hyper-producers. Gene ontology (GO) enrichment All GO-enrichment analyses were performed by testing GO-terms mapped to the gametocyte phenotypic categories of interest (for example hypo-early, hyper-early, hypo-late, hyper-late, hypo-both, hyper-both and neutral) against a background of GO terms mapped to all other genes using our R package pfGO (v 1.1) [17]. The GO-term database was created from the latest curated P. falciparum ontology available at the time of analysis from Plasmo DB and enrichment was assessed via weighted Fisher/elim-hybrid p <=0.05 (v. 57). The fraction of genes represents the number of significant genes annotated to a given GO-term in each of the category divided by the total number of genes annotated to a given GO-term included in the analysis for all categories (background-set).

Comparison with DHA drug screen

In parallel, a phenotypic screen of the Half-K mutant library was performed using a sub lethal dose of DHA at an IC25 (4 nM) concentration for three asexual growth-cycles (144 hours) [8]. Genomic DNA was harvested from treated and untreated samples and sequenced by QIseq. Fold- change growth differences and phenotype classifications were determined using DEseq2 from R. Mutants in the bottom quartile (lower 25%) were categorized as sensitive and mutants in the top quartile were categorized as tolerant. These assigned DHA phenotypes were compared to the corresponding gametocyte phenotypes of the mutants in the Half-K mutant library.

3. RESULTS AND DISCUSSION

Phenotype identification

Previously described QIseq methodology was used to quantify insertion sites for each piggyBac mutant in the pool screened sample [7]. The DEseq2 from R was used to normalize original read counts per insertion-site and calculate fold changes for early (day 3/ day 0) and late (day 14 / day 0) time points. The mutants were ranked from lowest to highest to identify phenotypes of interest. Hits from the bottom quartile were categorized as hypo-producers and those from the top quartile were hyper-producers.

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All GO-enrichment analyses were performed by testing GO-terms mapped to the gametocyte phenotypic categories of interest (for example hypo-early, hyper-early, hypo-late, hyper-late, hypo-both, hyper-both and neutral) against a background of GO- terms mapped to all other genes using our R package pfGO (v 1.1) [17]. The GO-term database was created from the latest curated *P. falciparum* ontology available at the time of analysis from Plasmo DB and enrichment was assessed via weighted Fisher/elim-hybrid p <=0.05 (v. 57). The fraction of genes represents the number of significant genes annotated to a given GO-term in each of the category divided by the total number of genes annotated to a given GO-term included in the analysis for all categories (background-set).

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piggyBac mutant library. A large mixed mutant pool of 600 *piggyBac* isogenic mutants referred to as the Half-K library was used for the large-scale gametocyte screen. This collection of mutants represents ~7% of the parasite genome and contained insertions in the inter genic, exonic and un translated (UTR) regions of genes, (Fig 3.1 A). Gametocyte production was induced in two replicate cultures and genomic DNA was harvested post sexual induction on day 3 (early gametocyte time point) and day 14 (late gametocyte timepoint) to identify genes functionally important in early and late gametocyte development, respectively. Day 3 gametocytes were enriched by FACS selection of an early sexual- stage marker Pfs16 [11-14], while cultures for analysis of late gametocyte phenotypes done in parallel were enriched for mature gametocytes by percoll purification (see methods) (Fig 3.1 B) [15]. As described previously, relative sexual differentiation of each mutant was determined by fold-change differences as calculated at each of the time points, early (day 3) and late (day 14) versus the start of gametocyte culture (day 0) (Fig. 3.2 A). Mutants that performed poorly were ranked at the bottom of curve (lower quartile) and classified as hypo- producers while those mutants that had an increased fold change, present at the top of the curve (upper quartile) were classified as hyper- producers and unclassified mutants in between were categorized as neutral (Fig. 3.2).

Geneontology (GO) enrichment of gametocyte phenotypes

Gene ontology enrichment analysis was performed for gene sets classified as hypo- and hyper- producers of gametocytes during early and late development (Fig. 3.3). Gametocyte hypo- producers on day 3 were enriched for *intracellular protein transport* (GO:0006886) and *protein localization to organelle* (GO:0033365) (p <0.05). These included genes involved in vesicular trafficking and its regulation such as signal recognition particle subunit SRP72, putative (PF3D7_1136400), ADP-ribosylation factor, putative (PF3D7_1359800) and GTPase-activating protein, putative (PF3D7_1473100) [18-20]. The GO terms in hypo-producers for late gametocyte development were enriched for *tRNA modification (*GO:0006400), *rRNA modification* (GO:0000154), *pseudouridine synthesis* (GO:0001522) and *endomembrane system organization* (GO:0010256) (p <0.05), pointing towards the role of RNA metabolism in progression of gametocyte stages. These included tRNA modification genes such as tRNA pseudouridine synthase, putative (PF3D7_0516300), tRNA-2- methylation-N (6)- dimethylallyladenosine synthase (PF3D7_0622200) and rRNA modification genes like 60S ribosomal protein L7ae/L30e, putative (PF3D7_0419800), ribosomal RNA small subunit methyltransferase A1(PF3D7_1415800). In addition, two genes, trans membrane emp24 domain-containing protein, putative (PF3D7_1333300) and protein SEY1, putative (PF3D7_1416100) essential for the secretory pathway and organization of endoplasmic reticulum were gametocyte hypo-producers for late-stage development [21]. Meanwhile, early gametocyte hyper-producers (increased gametocytogenesis) were significantly enriched for *protein processing* (GO:0016485) at day 3. One of the genes associated with this GO term, signal peptidase complex catalytic subunit, PfSP21 (PF3D7_1331300) was found to be essential for intra-erythrocytic growth and involved in cell signaling [22, 23]. Late-stage hyper-producers were also enriched for *signal transduction (*GO:0007165) and others like *regulation of translation* (GO:0006417), *modulation by symbiont of host process* (GO:0044003), *ATP-dependent activity, acting on DNA* (GO:0008094) and *lipase activity* (GO:0016298) (p <0.05). Two putative DNA helicases PF3D7_0604600, PF3D7_081870, possibly serving as transcriptional regulators were gametocyte hyper- producers. Not surprisingly, lipid metabolism genes such as lysophospholipase putative (PF3D7_0937200) and lysophospholipase (LPL1) (PF3D7_1476700) were gametocyte hyper-producers in the screen. Previous studies have underlined the role of lysophosphatidylcholine (LysoPC) in regulating sexual conversion and the appearance of these genes as gametocyte hyper-producers suggest their role in regulating sexual commitment [24].

Link between artemisinin resistance (DHA) and gametocytogenesis

Many genetic factors in *P. falciparum* have been associated with resistance to artemisinin, the frontline antimalarial drug and recent evidence insinuates that resistance can favor mosquito transmission [25-27]. Therefore, we investigated the potential link between gametocyte development and altered sensitivity to artemisinin in a parallel screen using the same "Half-K" library. Based on a similar rationale, the phenotypic drug screen classified genes as sensitive or tolerant to DHA, an active derivate of artemisinin. The *piggyBac* mutants were tracked for asexual growth (3 cycles) under drug pressure (IC25) and QIseq was used to quantify the phenotypes [8]. We found a significant correlation between altered responses to DHA and changes in gametocyte conversion, as gametocyte hyper-producers at both early and late timepoints tended to have decreased sensitivity to DHA (Fig. 3.4 A). Of note were genes that have been previously

associated with artemisinin resistance such as protein Kelch13-interacting protein 7, KIC7 (PF3D7_0813000) [28, 29] and the Inosine-5'-monophosphate dehydrogenase, IMPDH (PF3D7_0920800) [30] supporting a link between the processes leading to altered sensitivity to DHA and gametocytogenesis (Fig. 3.4 B). Understanding sexual development and the molecular mechanisms governing it, is crucial for the development of novel antimalarial therapeutics. Gametocyte development in *P. falciparum* is elaborate and requires successful progression through different morphological stages until mature gametocytes infectious to mosquitoes are formed. Even though recent studies have identified the master regulator of gametocyte commitment, many genes involved in the early signaling mechanisms of sexual conversion and those essential for driving gametocyte maturation are yet to be discovered. In this study, we addressed this gap in sexual biology of the parasite using a forward genetic approach. We developed a high-throughput gametocyte screen with isogenic *piggyBac* mutants and identified genes that could be likely essential for gametocyte development. By using QIseq, we were able to screen a pool of 600 mutants simultaneously for identification of hits at two critical timepoints day 3 (early) and day 14 (late). Gametocyte hypo-producers were genes that performed poorly indicative of their role in gametocyte development, while gametocyte hyper- producers were genes that were linked to increased gametocytogenesis (Fig. 3.5).Not surprisingly, early gametocyte genes were enriched for protein transport and localization, processes that are essential during the early phases of sexual commitment. [31].

This category also included AP2 transcription factors that have been linked to *Plasmodium* sexual development like AP2-O2 (PF3D7_0516800), AP2-HC (PF3D7_1456000) and other putative factors like AP2 domain transcription factor, putative (PF3D7_1115500) [32, 33]. The rising role of zinc finger proteins as key regulators in gametocytogenesis have been highlighted by a few studies [34, 35]. In our study, we also identified two putative zinc finger proteins (PF3D7_1358600, PF3D7_1205500) as gametocyte hypo-producers. Noticeably, two putative RNA-binding proteins (PF3D7_0205700, PF3D7_0929200) emerged as gametocyte hypo- producers, reiterating the importance of transcriptional regulation in sexual development [36]. The methods established in this study enable identification of gametocyte hits for prioritization of drug and vaccine development and aid in the functional annotation of many *P. falciparum* genes. In our large-scale screen, a total of 110 conserved genes with unknown function were assigned a gametocyte phenotype, either as a hypo- or hyper-producer. These conserved processes offer avenues to extend our analysis to other widespread human malaria *Plasmodium* species like *P. vivax,* that lack an *in vitro* culture system. Recently, evidence showed that artemisinin treatment increases in sexual conversion rates *in vitro*, as well as in natural infections [27, 37, 38]. By comparing two parallel screens, selections in gametocyte development and selections in DHA drug pressure for the same “Half-K” library, we were able to point out a relationship between increased gametocytogenesis and decreased sensitivity to artemisinin. Factors that regulate sensitivity to artemisinin are correlating with factors that regulate gametocytogenesis. This link is significant because selective advantage for resistance transmission will fuel the spread of resistant genotypes in a population threatening the progress made in malaria control. These findings though compelling necessitate additional experiments to assess the impact of several factors such as parasite stage, time of exposure and drug dose which may influence the sexual conversion rates. development and altered sensitivity to artemisinin in a parallel screen using the same “Half-K” library. Based on a similar rationale, the phenotypic drug screen classified genes as sensitive or tolerant to DHA, an active derivate of artemisinin. The *piggyBac* mutants were tracked for asexual growth (3 cycles) under drug pressure (IC25) and QIseq was used to quantify the phenotypes [8]. We found a significant correlation between altered responses to DHA and changes in gametocyte conversion, as gametocyte hyper-producers at both early and late timepoints tended to have decreased sensitivity to DHA (Fig. 3.4 A). Of note were genes that have been previously associated with artemisinin resistance such as protein Kelch13-interacting protein 7, KIC7 (PF3D7_0813000) [28, 29] and the Inosine-5'-monophosphate dehydrogenase, IMPDH (PF3D7_0920800) [30] supporting a link between the processes leading to altered sensitivity to DHA and gametocytogenesis (Fig. 3.4 B). Understanding sexual development and the molecular mechanisms governing it, is crucial for the development of novel antimalarial therapeutics. Gametocyte development in *P. falciparum* is elaborate and requires successful progression through different morphological stages until mature gametocytes infectious to mosquitoes are formed. Even though recent studies have identified the master regulator of gametocyte commitment, many genes involved in the early signaling mechanisms of sexual conversion and those essential for driving gametocyte maturation are yet to be discovered. In this study, we addressed this gap in sexual biology of the parasite using a forward genetic approach. We developed a high-throughput gametocyte screen with isogenic *piggyBac* mutants and identified genes that could be likely essential for gametocyte development. By using QIseq, we were able to screen a pool of 600 mutants simultaneously for identification of hits at two critical timepoints day 3 (early) and day 14 (late). Gametocyte hypo-producers were genes that performed poorly indicative of their role in gametocyte development, while gametocyte hyper- producers were genes that were linked to increased gametocytogenesis (Fig. 3.5). Not surprisingly, early gametocyte genes were enriched for protein transport and

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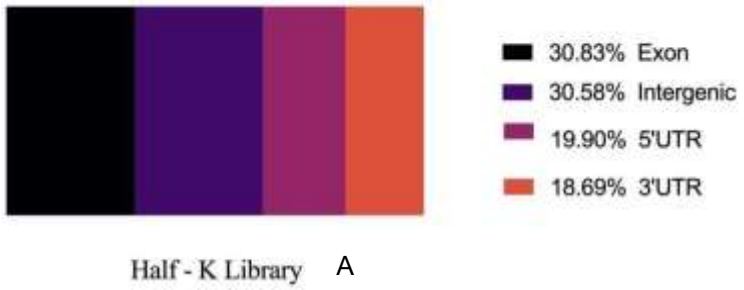

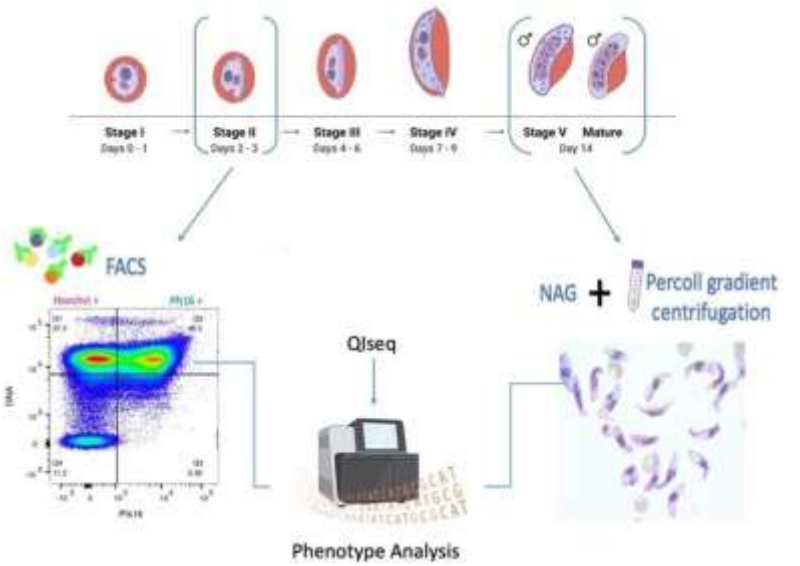

B

Figure 3.1 Approach for large-scale gametocyte screen.

Distribution of *piggyBac* insertions in the "Half-K" library. **B)** Methodology for enrichment of gametocyte populations. Early gametocytes were isolated with FACS selection for Pfs16, a parasitophorous vacuole membrane protein and one of the earliest known markers of sexual conversion. Briefly, gametocytes cultures at day 3 were first enriched by percoll (see methods) and incubated with anti-Pfs16 primary antibody and fluorescent secondary antibody followed by incubation with Hoechst dye. DNA from day 3 gametocytes (upper right quadrant) positive for both Hoechst and Pfs16 was harvested and sequenced. Stage V gametocytes were isolated using percoll purification as described in the methods.

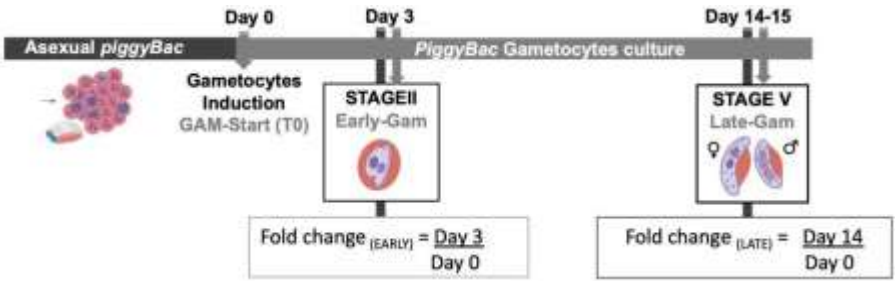

Figure 3.2 Gametocyte screen timeline for early and late development.

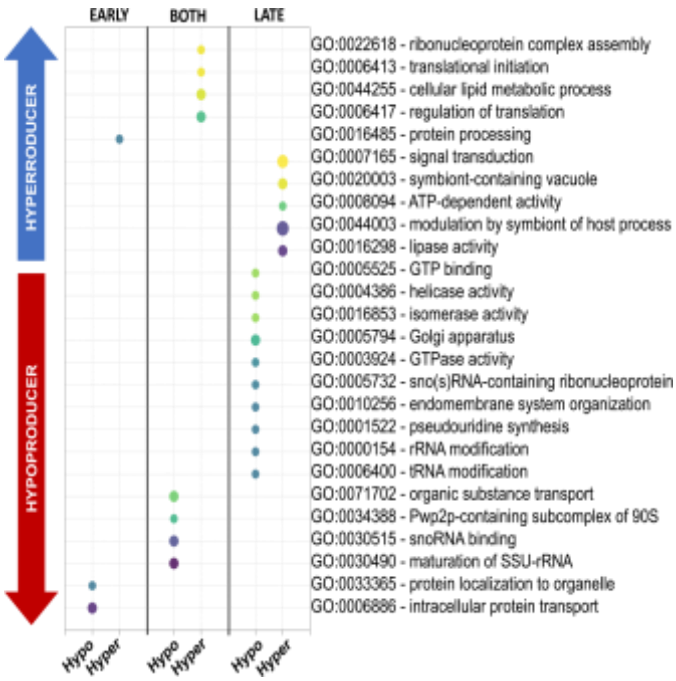

Figure 3.3 Gene ontology (GO) analysis for large-scale gametocyte screen.

Functional enrichment of significant gene ontology (GO) terms for gametocyte hypo- and hyper- producer *piggyBac* mutants vs all other mutants in the library (n=128). Circles represent the GO term, circle color represents, circle size represents the number of significant genes annotated to that category (see methods). The category 'Both' represents enrichment of terms from shared hypo- and hyper-producers between early and late developmental timepoints.

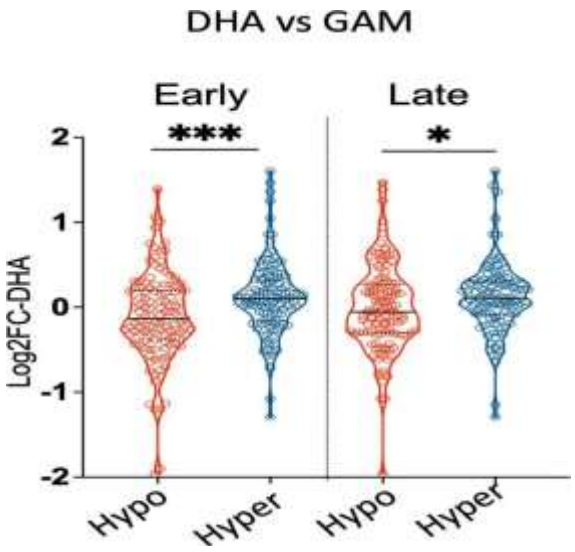

Figure 3.4 Link between gametocyte hyper-producers and sensitivity to DHA.

The violin plot compares the phenotypes for genes assigned in the large-scale gametocyte screen to phenotypes assigned in the drug screen. We observed a trend, where gametocyte hyper- producers tended to have decreased sensitivity to DHA, suggesting the presence of potential shared pathways that are essential for drug resistance and gametocytogenesis (Welch's t-test, $p < 0.05$).

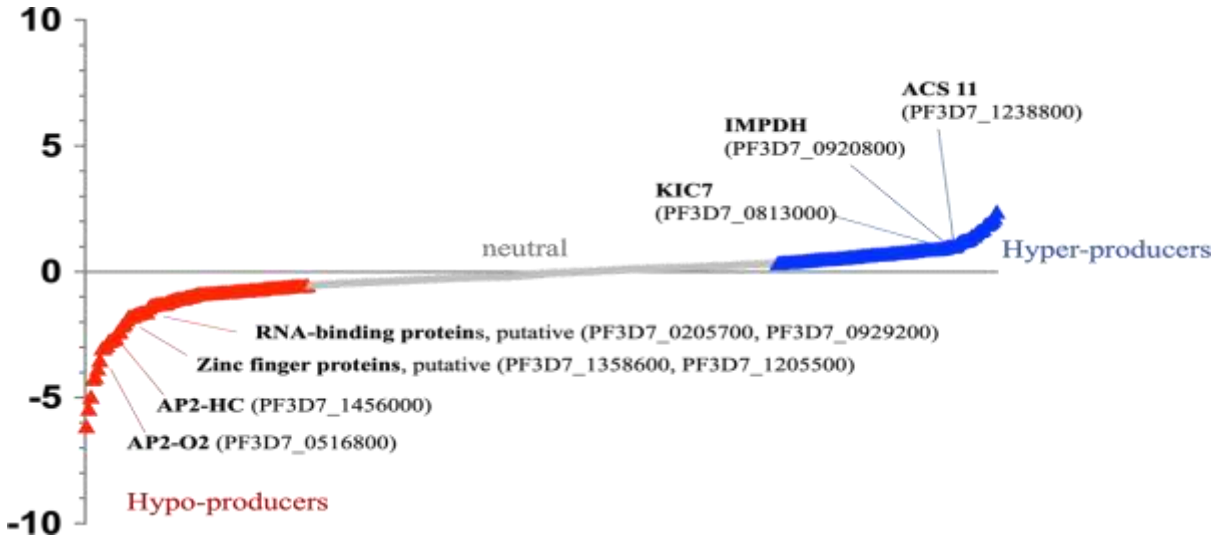

Figure 3.5 Likely-essential gametocyte genes identified in the phenotypic screen.

Relative differentiation of each *piggyBac* mutant in the library was determined ranking mutants from low to high. The genes in the bottom quartile (~102) indicated in red are inferred as likely- essential for gametocytogenesis. Hits from this category include genes that have been previously associated with sexual development such as AP2 transcriptional factors, zinc finger proteins and RNA-binding proteins. The top quartile (~102) indicated in blue are genes with a possible role in regulation of gametocytogenesis. Candidates highlighted in this category are genes related artemisinin resistance.

4. CONCLUSION

The present study provides a comprehensive functional genomic analysis of gametocyte development in *Plasmodium falciparum*, addressing a critical gap in understanding the genetic determinants that govern sexual commitment, early gametocyte differentiation, and maturation to infectious stage V gametocytes. By employing a large-scale phenotypic screen using a pooled library of approximately 600 isogenic piggyBac insertion mutants and quantitative insertion-site sequencing (QIseq), this research successfully identified a diverse set of genes that play essential regulatory and supportive roles across multiple stages of gametocytogenesis. The classification of mutants into gametocyte hypo-producers and hyper-producers at early (day 3) and late (day 14) developmental time points revealed stage-specific biological processes underlying sexual development. Early gametocyte development was predominantly associated with intracellular protein transport, vesicular trafficking, and organelle localization, highlighting the importance of cellular remodeling during the initial commitment to sexual fate. In contrast, late-stage gametocyte maturation was strongly enriched for RNA metabolism, ribosomal modification, and endomembrane system organization, underscoring the role of post-transcriptional regulation and translational control in the progression to mature, mosquito-infectious gametocytes. Importantly, the study identified numerous transcriptional regulators, including AP2 domain transcription factors, zinc-finger proteins, and RNA-binding proteins, reinforcing the concept that gametocytogenesis is tightly regulated at both transcriptional and post-transcriptional levels. In addition, a substantial number of conserved *Plasmodium* proteins of previously unknown function were assigned definitive gametocyte phenotypes, thereby contributing significantly to the functional annotation of the *P. falciparum* genome. A key novel finding of this research is the observed association between increased gametocyte production and reduced sensitivity to dihydroartemisinin (DHA). The overlap between gametocyte hyper-producers and mutants exhibiting tolerance to DHA suggests the existence of shared or interconnected pathways that modulate both sexual development and artemisinin response. This relationship has important epidemiological implications, as enhanced gametocytogenesis in drug-tolerant parasites may facilitate the transmission and spread of artemisinin-resistant genotypes, potentially undermining current malaria control efforts.

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