The Role of Erythrocytic miRNA in the lifecycle of *Plasmodium falciparum*

by

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Dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the University Program in Genetics and Genomics in the Graduate School of Duke University

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ABSTRACT

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Abstract

Malaria, caused by the apicomplexan parasite *Plasmodium*, is a disease which affects up to 500 million people each year. Historically, malaria infection has been combated both through the control of its vector, the *Anopheles* mosquito, and use of a variety of drugs, such as quinine (1800s) and chloroquine (1900s). However, with the evolution of resistance to the majority of available anti-malarial drugs, current approaches have settled upon combinatorial therapies. The most effective of these currently are ACTs (Artemisinin Combination Therapies – Artemisinin derivatives combined with a number of other drugs). However reports of Artemisinin resistance are continuing to emerge, suggesting that new approaches and increased understanding of the *Plasmodium* parasite is required.

Beginning with the complete sequencing of *Plasmodium falciparum* genome and continuing with comprehensive profiling of both the parasite’s proteome and transcriptome, various genomic approaches applied in the study of malaria have led to significant new insights into the underlying biology of this parasite. While these new findings have greatly increased our understanding of genetic regulation within the malaria parasite, they largely have not yet translated into new therapeutic approaches. For this reason, considerable attention has been paid to the study of human genetic disorders which convey resistance to malaria, in the hopes that elucidating the mechanisms behind these resistances might lead to increased understanding of the parasite’s biology and thus novel therapeutic approaches.

Sickle cell (HbS) erythrocytes are well known to resist malaria infection. However, the molecular basis of this resistance, long been recognized as multifactorial, contains
elements which remain poorly understood. Here we show that the dysregulated erythrocytic microRNA composition, present in both HbAS and HbSS erythrocytes, is a significant determinant of resistance against the malaria parasite *Plasmodium falciparum*. During the intraerythrocytic lifecycle of *P. falciparum*, a subset of erythrocyte microRNAs translocate into the parasite. Two microRNAs, miR-451 and let-7i, were highly enriched in HbAS and HbSS erythrocytes and these miRNAs, along with miR-223, negatively regulated parasite growth. Surprisingly, we found that miR-451 and let-7i integrated into essential parasite mRNAs and, via impaired ribosomal loading, resulted in translational inhibition of the target mRNA. Hence, sickle cell erythrocytes exhibit cell-intrinsic resistance to malaria in part through an atypical microRNA activity which may present a novel host defense strategy against complex eukaryotic pathogens. In addition, the formation of these chimeric transcripts even in normal host erythrocytes illustrates a unique parasitic post-transcriptional adaptation to the host-cell environment.
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1. Introduction

1.1 Malaria

1.1.1. Human malaria diseases

Malaria is an extremely widespread parasitic disease caused primarily by the apicomplexan parasite *Plasmodium*. It is the most common human parasitic disease, infecting approximately 300 million people each year and causing up to 1 million deaths (W.H.O., 2011). Malaria is largely confined to the developing world and the tropics (Figure 1), due to the environmental conditions favored by its primary vector, the *Anopheles* mosquito and consequently the extent of urbanization within potentially affected regions.

![Figure 1: Global Distribution of Plasmodium infection. (Hay 2009)](image)

Malaria has been controlled in many countries through the combined use of public health infrastructure and medical treatment. However, due to the lack of effective vaccines and the emergence of drug-resistant malaria strains, malaria still presents a huge challenge for many tropical and subtropical regions (sub-Saharan Africa in particular).
More than two billion people live within these malaria endemic regions and thus are at risk for malaria infection (Snow et al, 2005). In endemic regions, malaria also exacts a significant economic toll. It has been estimated that malaria causes US $12 billion a year in lost productivity in Africa alone (Snow et al, 2005). In many endemic countries, malaria is responsible for economic stagnation, dramatically lowering the annual economic growth in some regions (Orem et al, 2012). While isolated efforts to curb malaria with combinations of vector control, education, and drugs have proven somewhat successful in the past half-century, large areas of the globe remain exposed to malaria (Figure 2).

![Figure 2: Changes in the areas exposed to malaria infection at three timepoints (1946, 1966 and 1994). (Sachs and Malaney, 2002)](image)

Human malaria is caused by the infection of five closely related parasitic organisms: *Plasmodium falciparum, P. vivax, P. ovale, and P. malariae,* and most recently *P. knowlesi* (Oddoux et al, 2011). *Plasmodium falciparum* (*P. falciparum*) is the most common form in Africa, South America and South-East Asia, while *P. vivax* is the
most common form in the Middle East and South Asia (Guerra et al, 2010). From a public health perspective, the most important *Plasmodium* species, due to both their widespread distributions and potential for more severe malaria complications, are *P. falciparum* and, to a lesser degree, *P. vivax* (Hay et al, 2009). However, given that *P. falciparum* causes >90% of all cases of severe malaria, this dissertation will primarily focus upon *P. falciparum*.

1.1.2 The Lifecycle of *Plasmodium falciparum*

*Plasmodium* parasites have a bi-organismal lifecycle, which involves stages of development within both humans and *Anopheles* mosquitoes. *P. falciparum* is a unicellular parasite of the apicomplexan family, which also contains *Toxoplasma* (Foth and McFadden, 2003) and several other disease-causing parasites. In terms of cellular morphology, *P. falciparum* is similar in structure to most eukaryotic cells. However, as is common to all apicomplexan parasites, *P. falciparum* also contain an apicoplast which possesses an additional genome. The complete *P. falciparum* lifecycle encompasses three major developmental stages: the mosquito (Sporogonic), liver (Exo-erythrocytic), and blood (Erythrocytic) stages (C.D.C., 2010).
As shown in Figure 3, when an infected mosquito takes a blood meal, the parasite sporozoites from the salivary glands of the mosquito enter the blood stream. Once there, those sporozoites infect human hepatocytes, and once there undergo several rounds of asexual replication to form merozites (Tarun et al, 2008). In several strains of *Plasmodium* (such as *P. vivax* and *P. ovale*), but not *P. falciparum*, infection of the hepatic cells establishes a reservoir of infection, which is capable of reestablishing the erythrocytic infection (Tarun et al, 2007).

Later, the parasite ruptures the infected liver cells and the new merozoites enter the blood stream, which are then capable of infecting mature erythrocytes. After invasion by the merozoite, the 48-hour erythrocytic life cycle of *P. falciparum* then proceeds through...
three stages of development, from ring-stage (so called due to its morphology),
transitioning to trophozoites, which are metabolically quite active and are the stage where
most of the erythrocyte cytosolic material is taken up by the parasite, and finally to
schizonts, from which new merozoites bud off following several rounds of nuclear
division in the absence of cytokinesis (Boyle et al, 2010). The new merozoites proceed
to infect neighboring erythrocytes, thus perpetuating the asexual erythrocytic cycle.

In addition to the asexual stage of erythrocyte development described above, there
is an alternative pathway of development during the erythrocytic stage in which
merozoites can develop into microgametocytes and megagametocytes, known as the
sexual cycle of erythrocytic development. It is not clear how this process is controlled,
but evidence has shown that several cell signaling pathways, including PKA signaling,
are involved (Trager and Gill, 1989). Gametocytes are a critical component of
continuing parasite development through subsequent infection cycles. The gametocytes
formed during the sexual stage do not divide within the blood stream and do not complete
maturation until they enter the mosquito midgut, where they fuse to form a gamete, which
begins the mosquito stages of infection. This stage is necessary to infect additional
humans in a new round of the parasite lifecycle, as asexual stage parasites do not
replicate within the mosquito (C.D.C., 2010).

Given that most of the pathology related to malaria results from high parasite
burden during the erythrocytic stage of development, and in particular due to the asexual
stage, I will primarily focus on this stage of development. In terms of host cell
preference during the erythrocytic cycle, there are some differences between the various
Plasmodium species. P. falciparum exhibits no preference in erythroid host-cell types. In
contrast, *P. vivax, P. knowlesi* and *P. ovale* have a demonstrable preference to infect host reticulocytes. Due to the relative scarcity of circulating reticulocytes, this in part explains why these parasites cause significantly lower parasitemia and therefore reduced severity relative to *P. falciparum* (Tarun et al, 2007). While *P. malariae* prefers mature senescent erythrocytes, it has a 72 hour erythrocytic lifecycle, instead of 48 hours for the other *Plasmodium* species (Daneshvar et al, 2009), which helps to explains its decreased severity.

The initial stages of infection are generally not life-threatening. The disease, in its uncomplicated state, is characterized by cycles of severe fever every 48 hours, coinciding with the rupture of infected erythrocytes during the erythrocytic life cycle. The primary risk to humans comes not from the initial infection, but from complications which can arise as the infection progresses and the parasitemia increases (Snow et al, 1999). Complications from malaria infection include cerebral malaria which can cause a coma, severe anemia and organ failure due to lactic acidosis, hypoglycemia and hypotension.

### 1.1.3 Genetic Regulation within the Erythrocytic stage of *Plasmodium falciparum*

The genome of *P. falciparum* was initially sequenced in 2002 (Gardner et al, 2002), followed more recently by genome sequencing of three additional *Plasmodium* species, *P. vivax, P. yollei, and P. burghei* (Carlton et al, 2008, Pain et al, 2008, Carlton et al, 2002). The 22.8 Mb genome of *P. falciparum* consists of 14 linear chromosomes, the linear mitochondrial genome and the apicoplast’s circular plastid-like genome (Bozdech et al, 2003). At the time the genome was sequenced, genomic alignments of the malaria parasite estimated that more than half of the ~5500 predicted open reading frames
(ORFs) lacked sequence similarity to any other known organism (Gardner et al, 2002, Florens et al 2002). Given the lack of sequence homology, annotation remains a challenge, with ~50% of the plasmodium transcriptome still being unannotated (Florens et al, 2002). More recently, the transcriptome of the erythrocytic stage (IDC) of *P. falciparum* has been profiled for three distinct laboratory strains across the erythrocytic life cycle (Bozdech et al, 2003, Llinas et al, 2006). These profiles indicate that there is significant temporal regulation in the transcription of genes within the erythrocyte stage of *P. falciparum*. Differences in transcriptional profile have also been assessed for clinical field isolates of *P. falciparum* (Mackinnon et al, 2009), and gene expression data has demonstrated dramatic alterations in steady-state gene expression in response to various drugs (Gunasekera et al, 2007, Hu et al, 2010).

Transcriptional regulation within malaria parasites has been the subject of intense investigation due to its potential as a therapeutic target. Examples of transcriptional regulation include the elucidation of epigenetic control of transcription (Cui et al, 2008, Cui et al, 2007), monoallelic expression of *var* gene in antigen variation (Chookajorn et al, 2008, Scherf et al, 2008), a critical component in immune system evasion, and in depth transcriptomic comparisons between the asexual and sexual parasite stages (Hayward et al, 2000).

Nonetheless, given that *P. falciparum* has fewer than 6000 genes in its genome (Otto et al, 2010, Lasonder et al, 2002), transcriptional regulation alone may be insufficient to account for the parasite’s complexity. Therefore, extensive post-transcriptional regulation, alternative splicing and other forms of regulation of *Plasmodium* transcripts may be important to generate further diversity of transcripts in
order to generate its biological complexity. Furthermore, the relative paucity of transcription factors (Painter et al, 2010, Templeton et al, 2004) coupled with the high number of putative RNA-binding proteins (Hughes et al, 2010), suggests that post-transcriptional regulation plays a major role in parasite gene regulation.

1.1.4 Post-Transcriptional Regulation in *P. falciparum*

When the initial genomic and proteomic studies of *P. falciparum* were completed, a theory of “just-in-time” transcription was proposed, in which genes were translated soon after transcription, and thus most genetic regulation within the parasite was transcriptional in nature (Le Roch et al, 2003). However, with the numerous advances in genomic technology, many of which have been applied to the study of *P. falciparum*, significant discrepancies have since been noted between the steady-state transcriptome (Bozdech et al, 2003, Otto et al, 2010) and proteome (Florens et al, 2002, Lasonder et al 2002, Lasonder et al, 2008), a fact which highlights post-transcriptional and translational regulation as potentially key regulatory steps in *Plasmodium* genetic regulation.

Indeed, recent investigations into gene expression have revealed the importance of mRNA stability (Shock et al, 2007), gene splicing (Saenz et al, 2008) and gene silencing (Deitsch et al, 2001, Duraisingham et al, 2005, Le Roch et al, 2003) in various *Plasmodium* species. Post-transcriptional regulation has also been implicated in systemic parasite gene regulation, where a global analysis of RNA decay in *P. falciparum* indicates a dramatic increase in the half-life of RNA messages during the asexual IDC. The average mRNA half-life is approximately 9.5 minutes during the ring stage, extending to an average of 65 minutes during the late schizont stage of development (Shock et al, 2007). Extensive
post-transcriptional and post-translational modifications have also been noted in during the erythrocytic stage of development (Foth et al, 2008). RNA binding proteins have also been implicated in cell-fate choice between the asexual and sexual stage of development, as knockdown of PbPuf2, a *P. burghei* homolog of the pumilio family of RNA-binding proteins, lead to increased gametocytogenesis and is required for sporozoite invasion of hepatocytes (Gomes-Santos et al, 2011).

Translational repression, in tandem with transcriptional responses, has been implicated in both var gene switching and PfEMP1 expression levels in *P. falciparum* (Mok et al, 2008). Translational regulation has also been shown to be critical for both rapid parasitic responses to cell stress and drug exposure (Holcik et al, 2005, Kreidenweiss et al, 2008) and cell fate determination between sexual and asexual forms (Mair et al, 2006). In *P. berghei*, translational repression is essential for sexual stage development; several mRNAs (e.g. p28) require proper partitioning into cytoplasmic messenger ribonucleoprotein (mRNPs) complexes to prevent their translation until after fertilization (Mair et al, 2006). The maintenance and assembly of such complexes depends on the highly evolutionarily conserved DEAD-box RNA helicase DOZI (Mair et al, 2006). The presence, and sequence features within, both the 5’ and 3’ UTRs seems to be important for this translational repression. Recent evidence has pointed to the importance of the transmembrane isoform of MAEBL for the invasion of *Anopheles* salivary glands (Saenz et al, 2008). Taken together, these examples all highlight the importance and complexity of post-transcriptional, and in particular translational, genetic regulation in *Plasmodium*. 
While we have substantially enhanced our understanding of the parasite in the last decade, these discoveries have not yet translated into new avenues of treatment. Compounding this paucity of new drugs is a worldwide increase in *P. falciparum* strains resistant to the mainstays of anti-malarial treatment (Ridley, 2002).

### 1.1.5 Drug-Resistance in Treatment of *P. falciparum*

Many drugs have been devised and/or naturally discovered to combat the spread of malaria. Throughout the middle/late 20th century, malaria was treated through the use of several different compounds, most notably chloroquine. Beginning in the 1960s however, there began to be reports of *P. falciparum* strains which were resistant to chloroquine (Sidhu et al, 2002, Suwanarusk et al, 2007). This resistance is now largely believed to be caused by the PfCRT gene (*P. falciparum* chloroquine resistance transporter), which expels chloroquine from the parasite food vacuole as soon as it enters (Sidhu et al, 2002).

Chloroquine would prove to be only the first major example of evolution of resistance to a major class of therapeutics, as resistance has been observed to nearly all classes of drugs currently available. It is this evolution of resistance over a wide geographical range which makes *P. falciparum* the most dangerous *Plasmodium* parasite. In contrast, *P. vivax* is also extremely widespread, but it has not yet developed extensive resistance to chloroquine (Suwanarusk et al, 2007), and thus is not regarded nearly the threat to global health.

Due to the rapid loss of efficacy for many individual therapies, modern malaria treatment takes advantage of combinations of therapies to diminish the potential for
Drug resistance is obviously not a problem unique to *P. falciparum* malaria. In several diseases where drug resistance has proved problematic, one means to potentially subvert this resistance is to study the underlying mechanisms of naturally occurring genetic variants which convey resistance. Given the long-established nature of these resistances, they may elucidate therapeutic directions which are more resistant to the rapid evolution of disease resistance. There are several genetic diseases which convey resistance to malaria, the longest recognized of which is sickle cell anemia. However, while the fact that the sickle cell allele conveys resistance to malaria has been recognized since the 1970s (Friedman et al, 1978, Pasvol et al, 1978), the underlying cause of sickle cell’s resistance to malaria remains largely unexplained, reducing any potential therapeutic benefit.
1.2 Sickle Cell Anemia

1.2.1 Molecular basis of Sickle Cell Anemia

Sickle cell anemia is generally considered as an erythrocytic disease. Human erythrocytes, or red blood cells, are end-products of a highly regulated differentiation process that involves the gradual loss of cellular organelles including the extrusion of the nucleus, a decline in nucleic acid content, and a step-wise acquisition of erythrocyte characteristics, such as the production of hemoglobin (Hoffman et al, 2004).

Sickle cell disease (SCD) was the first single-gene disorder to be characterized at the molecular level, caused by an A->G nucleotide substitution in the sixth codon of the β-globin gene which causes a valine to glutamic acid substitution, resulting in sickle hemoglobin (HbS) (Pauling et al, 1949). This mutation reduces the protein solubility and enhances the occurrence of polymerization during deoxygenation, resulting in “sickling” and poor deformability of erythrocytes, which can often occlude microvascular circulation (Embury et al, 1994). SCD is characterized clinically by hemolytic anemia, episodic painful events, chronic organ deterioration and various other acute complications. These clinical manifestations are attributable to HbS polymerization and its downstream effects (Hillery et al, 2004, Stuart and Nagel 2004). Although erythrocyte sickling is often thought to be responsible for malaria resistance, the fact that similar malaria resistance is observed in other genetic erythrocyte diseases which lack erythrocyte sickling, such as HbC (Fairhurst et al, 2005), pyruvate kinase deficiency (Ayi et al, 2008) and G6PD deficiency (Bernstein et al, 1980, Cappellini et al, 2008) suggests that sickling alone does not adequately explain malaria resistance in these diseases.
1.2.2. Malaria resistance in Sickle Cell Diseases and other Erythrocyte Diseases

Although SCD causes many health problems, its frequency remains quite high amongst people living in malaria-endemic regions (Figure 4). Many epidemiological studies suggest that the high frequency of HbS alleles among people living in malaria-endemic regions reflects the positive selective pressure imposed by the malaria parasite *P. falciparum* (Allison, 1954, Livincstone 1971, Aidoo et al, 2002).

![Figure 4: The concordance between the allele frequency of the HbS allele and the presence of *P. falciparum* malaria (adapted from Aidoo et al, 2002)](image)

When infected with *P. falciparum*, fewer individuals with the sickle cell trait (HbAS) die of the infection compared to normal (i.e., HbAA) individuals (Aidoo et al, 2002). This malaria resistance helps to explain both the relative survival advantage and the increased frequency of HbS in malaria endemic regions. Genome-wide association studies have shown evidence for several distinct mechanisms of resistance to *P. falciparum* (Gong et al, 2012). The underlying causes of malaria resistance in SCD and other erythrocyte diseases remain the subject of considerable debate, given that malaria resistance in SCD appears to be due both to factors outside of SCD erythrocytes (cell-extrinsic factors), such as enhanced anti-malaria immunity and clearance (Marsh et al,

Parasitized mutant polymorphic erythrocytes have been shown to be subject to enhanced phagocytosis by monocytes (Ayi et al, 2004), suggesting that *P. falciparum* is more rapidly cleared by the immune system. Further, the Hemoglobin C allele was shown to provide resistance to *P. falciparum* by altering the cell surface properties of infected erythrocytes, resulting in abnormal display of the cytoadheren ligand PfEMP-1 and leading to decreased cell adhesion, impaired rosetting, and reduced haemagglutination (Fairhurst et al, 2005). In addition to these cell-extrinsic factors, *P. falciparum* grows poorly within homozygous sickle (HbSS) erythrocytes (Friedman et al, 1978, Pasvol et al, 1978), indicating that cell-intrinsic erythrocytic factors also contribute to overall resistance at the organism and population levels.

The establishment of an *in vitro* culture system of *P. falciparum* has facilitated more detailed investigations of the mechanism by which cell-intrinsic erythrocyte differences enhance host cell resistance to malaria (Jensen et al, 1977). Despite concerns about the *in vivo* relevance of these culture models (Nagel et al, 1989), they have clearly shown a significant and reproducible reduction in the growth and replication of *P. falciparum* in HbAS and HbSS erythrocytes as compared with HbAA cells (Aidoo et al, 2002). Importantly, recapitulation of malaria resistance *in vitro* allows us to test various hypotheses by determining how specific experimental manipulations affect parasite growth. In addition to the erythrocyte sickling, numerous erythrocyte-based hypotheses
have been proposed to explain why sickle cell erythrocytes are unfavorable host-cells for *P. falciparum*. It has been shown that progressive dehydration of erythrocytes, and the resulting increase in cellular density, is associated with both decreased invasion by *P. falciparum* merozoites (Tiffert et al, 2005), indicating that structural features of the host cell play a role in infection. Finally and most recently, increased oxidative stress and aberrant host actin remodeling by the parasite was shown to affect trafficking of parasite proteins to the surface of in HbSC and HbCC erythrocytes (Cyrlaff et al, 2011).

These theories alone (or in combination) do not fully explain the extent of the resistance observed within mature erythrocytes. Previously, the Chi lab identified that mature erythrocytes, while lacking most genetic material, did in fact possess an abundant pool of miRNA (Chen et al, 2008). This led me to examine whether human miRNA might play a role in the erythrocytic stage of malaria.
1.3 microRNA

1.3.1 miRNA Biogenesis

Figure 5: Canonical miRNA Biogenesis and Function (Mraz et al, 2005)

miRNAs are a recently discovered class of regulatory non-coding RNAs, which appear to be present in most eukaryotes. These small ncRNAs are typically 19-25nt in length (Krol et al, 2010), and are expressed initially as pri-miRNA, a 1-1.5kb RNA precursor. After cleavage by the microprocessor complex (which consists of several proteins, notably including Drosha and DGCR8), which possesses RNase III enzymatic activity, to form a small hairpin pre-miRNA (around 60nt in length). The pre-miRNA is then transported from the nucleus by exportin-5 (Figure 5). Once within the cytosol, the hairpin loop of the pre-miRNA is recognized by Dicer, which removes the loop, leaving a ~21nt dsRNA, with 2nt overhangs on both 3’ ends. These overhangs are recognized by the RISC (RNA induced silencing Complex) and the dsRNA is separated into 2 strands,
the guide strand (which identifies target mRNAs), and the target strand, which binds to the 3’ untranslated region (UTR) of mRNAs. Binding of the miRNA is largely mediated by basepair complementarity within the “seed sequence,” a 6-7 base pair sequence close to the 5’ end (nucleotides 2-7/8 following the 1st basepair at the 5’ end, which is often an adenosine) (Lewis et al, 2005). Typically, the next few basepairs (bp 9-11) do not bind to the target mRNA (forming a bulge), and then there are variable degrees of binding for the remainder of the miRNA. miRNA targets have also been observed within the coding region and 5’ UTR of target mRNAs, but these targets appear to be less functionally relevant (Krol et al, 2010).

1.3.2 miRNA Function/Inhibition

microRNA typically function via one of two mechanisms (Figure 5). In the event of perfect complementarity, the target mRNA is cleaved by the RISC complex. This is rarely seen in mammalian and other eukaryotic organisms, but is often observed for miRNA within plants (Yekta et al, 2004). Meanwhile, translational repression resulting from incomplete complementarity is the common inhibitory mechanism seen in mammalian systems. In the event of near-perfect complementarity, the miRNA will lead to a reduction of the translation rate of the target mRNA. The mechanism behind this translational repression is not fully understood, but several hypotheses have been presented. In the case of certain miRNA (let-7a is one example), the miRNA appears to prevent proteins binding the 3’UTR, which inhibits the assembly of the translation initiation complex (Humphreys et al, 2005). For other miRNA, the miRNA appears to cause the ribosome to leave the mRNA prematurely (Pillai et al, 2005). There have also
been reports that miRNA could target nascent transcripts for degradation, by an unknown mechanism (Nottrott et al, 2006), or cases where target mRNAs are targeted for degradation by a variety of mechanisms including transcript deadenylation (Behm-Ansmant et al, 2006). More recent studies have in fact suggested that the dominant mechanism of miRNA activity may be at the RNA transcript level (Guo et al, 2011), suggesting that the mechanism of miRNA action remains poorly understood.

Since miRNAs often target many different mRNAs, an individual miRNA can have a wide range of regulatory functions. Different miRNA can also form regulatory networks with their associated target mRNA. Given that multiple miRNA can bind to the same mRNA, the potential exists for extremely complicated networks of regulation (Kim et al, 2005). miRNA have been implicated in a wide range of functions, including oncogenesis (miR-17-92 cluster) (He et al, 2005), neurogenesis (miR-124) (Yoo et al, 2011), erythropoiesis (miR-451) (Pase et al, 2009), and hematopoietic lineage differentiation (miR-181, miR-223, miR-99) (Garzon et al, 2006). miRNA also show a wide range of expression patterns, from ubiquitously expressed miRNAs (let-7) to tissue specific miRNAs (miR-451 is erythrocyte-specific, while miR-122 is liver-specific) (Barad et al, 2004, Landgraf et al, 2007). Of particular interest in the role of miRNA in regulating host immune responses against pathogens (Lodish et al, 2008, Xiao et al, 2009).

1.3.3 miRNA in Host-Pathogen interactions

miRNA are involved in the host-pathogen interactions of many pathogens, including numerous viruses and parasites. For example, silencing of Dicer and Drosha,
two key ribonucleases in miRNA biogenesis, enhanced the replication of HIV-1 (Triboulet et al, 2007). While there are many examples of miRNAs involved in viral replication and host-immune responses, miRNAs also play a similar role against complex parasitic pathogens.

Both major prokaryote lineages (Archaea and Bacteria) possess Argonaute proteins, suggesting that the ability to utilize miRNA is quite widespread and may be evolutionarily conserved (Markova et al, 2006). Trypanosomes possess several proteins similar in function and sequence to Dicer and Argonaute 2 (Shi et al, 2006, Shi et al, 2007) and are predicted to possess several endogenous miRNAs (Mallick et al, 2008). *Entamoeba histolytica* also appears to encode several miRNAs, and possesses most of the protein machinery needed for miRNA function, but few direct mRNA targets have been validated (Abed et al, 2005, De et al, 2006). *Giardia lamblia* possesses homologs to Dicer and Ago2, but lacks drosha or exportin 5 homologs. In addition, a *Giardia* small RNA was encoded within the SNO30 RNA, and a number of potential targets were identified using computational approaches and reporter assay (Li et al, 2012).

*Toxoplasma*, the closest related major parasite to *Plasmodium*, has been shown to alter the levels of several host cell miRNA during its infection (Zeiner et al, 2010, Zeiner et al, 2010). In addition, Toxoplasma appears to possess an array of small RNA which function via an argonaute-like protein complex (Braun et al, 2010). The Chi lab had previously identified miRNA within mature erythrocytes, one of the host cells for *P. falciparum*, which led us to wonder whether those miRNA might play a role in malaria infection.
1.3.4. miRNA within Host Erythrocytes

Given the involvement of miRNA in other parasitic species, we naturally wondered whether erythrocytic miRNA might play a similar role. Using a technique to isolate pure erythrocytes (Sangokoya et al., 2010), we obtained a modest but reproducible amount of RNA from several mature erythrocyte samples.

**Figure 6: Analysis of total RNA content from mature erythrocytes and PBMCs.**
(A) Analysis of total RNA content from mature erythrocytes from 3 RBC and one PBMC RNA sample. (B) The miRNA expression pattern for RBC and K562 cells interrogated by miRNA microarray. (C) miRNA expression within the indicated cell types via Northern blot. From Chen et al., 2008

RNA yields were ~ 3x10^-4 pg/erythrocyte, similar to the RNA content of platelets (Bahou et al., 2004), but about half of what is present in normal human cells. Erythrocyte RNAs (Figure 6A, lane 2-4) did not contain the two distinct ribosomal rRNA signals (28S and 18S) commonly observed in nucleated cells (Figure 6A, lane 5). Instead, erythrocyte RNA was significantly enriched for small-sized RNAs less than 200 base pairs in length. Using different independent techniques (rtPCR, Northern blot, miRNA microarray), we have found abundant and diverse miRNAs in erythrocytes. When
compared with human erythroleukemic K562 cells, erythrocytes consistently had several miRNAs with increased expression, including several miRNAs in the let-7 family and two miRNAs (miR-181A, miR-223) with known roles in lineage differentiation of hematopoietic cells (Chen et al, 2004) (Figure 6B). To examine changes in miRNA expression during erythroid differentiation, we compared our array results with a study of miRNA expression in differentiating CD34+ cells (Lu et al, 2005). Almost all erythrocyte-specific miRNAs found in our array analysis were upregulated during erythroid differentiation (Fig 3B), suggesting that most erythrocyte miRNAs likely derive from erythroid cells at earlier stages of differentiation. The expression of these miRNAs in erythrocytes was further confirmed with Northern blots and those miRNA were found to be mostly in their ~22nt mature form (Figure 6C).

1.3.5 Dysregulated miRNA expression in Sickle Cell erythrocytes

Dysregulated miRNA composition within in HbSS erythrocytes has previously been shown to contribute to several erythrocyte phenotypes, including defective terminal differentiation (Chen et al, 2008) and decreased tolerance for oxidative stress (Sangokoya et al, 2010). Given that previous studies had demonstrated that HbSS miRNAs have altered levels of miRNA expression (Chen et al, 2008), we hypothesize that the dysregulation of erythrocytic miRNA might play a role in malaria resistance. To demonstrate that miRNA expression is altered in SCD, previous studies within the Chi Lab compared the miRNA gene expression in 12 SCD (HbSS) patients and 7 race/gender-matched normal healthy donors (HbAA) (Chen et al, 2008).
Figure 7: Global expression analysis of HbAA and HbSS miRNAs by miRNA microarray. (A) Global miRNA expression of 7 HbAA and 12 HbSS erythrocytes as evaluated by miRNA microarrays. (B) Two miRNAs, miR-451 and miR-223, were higher in (at least a subset) of HbSS patients. From Chen et al, 2008.

Unsupervised hierarchical clustering produced consistent groupings of all 12 HbSS patients separate from the 7 HbAA donors into two large distinct branches (Figure 7A), showing that HbSS and HbAA erythrocytes have consistent and dramatic differences in their miRNA expression patterns. Of particular relevance to this dissertation is the enrichment of two miRNA, miR-451 and miR-223, both of which are individually enriched in (at least a subset of) HbSS patients (Figure 7B).
2. Erythrocytic miRNA affect the lifecycle of *P. falciparum* through a novel mechanism of action

2.1 Introduction: miRNA in *Plasmodium falciparum*

At the beginning of this study, the presence and functionality of miRNA and RNAi within *P. falciparum* was unclear. Analysis of the genome of *P. falciparum* failed to identify candidate Dicer or RISC complex subunits via sequence homology (Hall et al, 2005). Several previous studies have also cloned the total small RNA pool in order to determine the presence of any endogenous miRNA within *P. falciparum*. Instead of finding any parasite miRNAs, they only found the presence of several human miRNA within the parasite (Rathjen et al, 2007, Xue et al, 2008). Both of these studies ascribed the presence of these miRNA to host erythrocytic contamination.

In addition to the lack of endogenous miRNA, studies attempting to perform systematic RNAi knockdowns in *P. falciparum* have largely been unsuccessful (Baum et al, 2009). However, individual examples of RNAi have reported successful gene knockdown and alteration of parasite growth. For example, several groups have reported successful siRNA knockdowns, including that of dihydroorotate dehydrogenase (DHODH), a necessary component of pyrimidine biosynthesis (McRobert et al, 2002).

Host cell miRNA have been recognized to play a role in malaria infection however. One study has shown that a Dicer knockout strain of *Anopheles gambiae* is far more susceptible to *Plasmodium* than wild-type mosquitoes, though the specific targets of the miRNA, and whether they are parasitic or mosquito in origin, remain unclear (Winters et al, 2007). In addition, *P. chabaudi* has been shown to modulate the levels of hepatocyte miRNAs during the liver-stage of infection (Delic et al, 2011). miRNA expression within
the brain, in particular miRNAs miR-27a, 150, and let-7i, has been shown to modulate the severity of cerebral malaria in mouse models (Hunt et al, 2011). Because significant host cell material is actively taken up by the parasite (Francis et al, 1997) and human miRNA were detected in studies of small RNA in *P. falciparum*, these findings provided the rationale to examine the role of erythrocyte microRNAs in regulating the parasites’ gene expression and phenotypes.

### 2.2 Human miRNAs are translocated into *P. falciparum*

To examine the possibility that erythrocyte miRNAs play a role in the erythrocyte-parasite interaction, we first determined whether erythrocytic miRNAs were present in parasites during the intraerythrocytic development cycle (IDC). Since *P. falciparum* is an intracellular parasite during the intraerythrocytic stage of development, infected erythrocytes were first lysed with saponin and washed thoroughly to remove the host erythrocyte cytosol. Using multiplex real-time PCR assays to assay miRNA levels within purified *P. falciparum* parasites, we detected the presence of ~100 human miRNAs within parasites (Table 1), out of ~350 assayed.
Table 1: The Relative Composition of individual miRNAs within both uninfected erythrocytes and purified parasites. The percentage composition of individual miRNAs within a 336 human miRNA pool for the uninfected erythrocytes, *P. falciparum* parasites at 8 and 36 hours after infection. N.D.=Not Detected

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We were particularly interested in differences between the miRNA profiles between host erythrocytes and isolated *Plasmodium* parasites. Given that the pool of miRNA within mature erythrocytes is fixed in composition (due to the lack of novel miRNA transcription), these observed differences in miRNA abundance begin to suggest miRNAs which undergo active translocation.
Among top 12 the miRNAs found in uninfected normal erythrocytes, three miRNAs (miR-451, miR-223, and miR-19b) were significantly enriched within the parasite (Figure 8).

**Figure 8:** Levels of assayed miRNAs in either Uninfected erythrocytes or purified *P. falciparum* at 8 and 32 hours post-infection. miRNA levels are expressed as a percentage of the total miRNA pool.

We believe that the observed enrichment of miR-451 is the result of increased miRNA translocation as its level within mature erythrocytes does not change over the course of 48 hours (Figure 9A). miR-451 makes for an interesting miRNA to undergo parasitic enrichment, since it is a highly abundant erythrocyte-specific miRNA and has been implicated in erythropoiesis (Chen et al, 2008).

In order to further verify that intra-parasitic miRNAs represented fully intact mature human miRNAs, we performed Northern blots for miR-223 and miR-451 (Figure 9B). Indeed, intra-parasitic miRNAs were of similar size compared to those in either HbAA erythrocytes or whole blood. Importantly, host-cell miRNAs were not likely to represent a contaminant, as the abundant *Hb-β* transcript was not detected in purified parasites (Figure 9C), while the parasite specific UBC13 gene was detected.
Figure 9: Human miRNA within the Plasmodium parasite are intact and relatively free of host cell miRNA contamination

(A) Levels of miR-451 in uninfected RBCs, RBC cytosol post-saponin treatment (Saponin lysate), and saponin-lysed enriched parasites (Saponin parasites) at 8 and 32 hours post infections. Values are normalized to the 8 hour level and by cell number.

(B & C) RNA was extracted from uninfected erythrocytes (RBC), uninfected blood, and from purified *P. falciparum* at 32 hours (T3) and 40 hours (T4) post erythrocyte invasion. Subsequently, RNA was analyzed by Northern blotting for miR-223 and miR-451 (B) or by RT-PCR for *Hb-β* and *PfUBC13* (C).

(D) miR-451 levels in HbAA, HbAA+miR-451 and HbSS erythrocytes in the RBC membrane ghosts or complete erythrocyte (n=4; mean +/- s.e.m.).

(E) miR-451 levels in uninfected HbAA and HbSS erythrocytes after saponin lysis (for resulting membrane ghosts and RBC cytosol) without or with RNAse A treatment (n=3; mean +/- s.e.m.).

(F) miR-451 levels in enriched parasites from HbAA, HbAA +miR-451 and HbSS erythrocytes without or with RNAse A treatment (n=4; mean +/- s.e.m.).

(G) Levels of 18S rRNA in purified parasites (RNAse A-treated and untreated) grown in HbAA and HbSS erythrocytes (n=4; mean +/- s.e.m.).

(H) Levels of miR-451 and miR-16 in Saponin-lysed (Saponin) versus Saponin + Methyl-Beta-cyclodextrin (MBCD) treated parasites.
Moreover, the potential for miRNA contamination from host erythrocytes (as opposed to parasite miRNA uptake) was further examined a variety of techniques including 1) saponin lysis, which disrupts the erythrocyte membrane (Figure 9D), 2) saponin lysis combined with RNaseA treatment of host cell miRNAs (Figure 9E-G), or 3) Methyl-Beta-cyclodextrin lysis, which disrupts the parasitophorous vacuolar membrane (Figure 9H). If parasitic miRNAs were the result of erythrocyte contamination, these treatments would significantly reduce miRNA content. However, no such decrease occurred for any of these treatments, further indicating miRNAs are indeed located within the parasite. These results would appear to both confirm and extend the previous identification of miR-451 and other microRNAs in *P. falciparum* (Rathjen et al, 2007, Xue et al, 2008).

In order to validate the miRNA enrichment across the IDC, we extracted RNA at 4 points (8, 16, 32 and 40 hours post-infection) from purified parasites (after saponin-lysis and RNaseA treatment), and examined two miRNAs: miR-451 and miR-181a, by real-time PCR (Figure 10A). We also examined one additional miRNA, let-7i. Let-7i was absent from the TLDA, but given its purported involvement in modulating cerebral malaria and its presence in mature erythrocytes (Chen et al, 2008), we investigated whether let-7i was translocated within the parasite. Let-7i was at its maximum level after 16 hours of parasite growth, while miR-451 reached its highest levels after 32 hours. Again, given that erythrocytic miRNA levels are largely unchanged during *in vitro* culturing (Figure 9A), the differences in miRNA abundance across the parasite lifecycle further suggest active and sequence specific uptake of miRNA by the *Plasmodium* parasite.
Previously, we found that miR-451 and miR-223 levels were dramatically elevated in HbSS erythrocytes compared to HbAA erythrocytes (Chen et al, 2008). Here we found that HbAS erythrocytes also had a modest elevation in miR-451, though less than that of HbSS (Figure 10B). Additionally, let-7i levels were also elevated in HbSS and HbAS erythrocytes Figure 11A).

Figure 10: miRNA accumulate across the lifecycle and higher erythrocytic miR-451 levels in HbSS and HbAS erythrocytes lead to higher intraparasitic levels of miR-451.
(A) Levels of three indicated miRNAs shown in parasites at 8, 16, 32, and 40 hours post-infection.
(B) Relative levels of erythrocytic miR-451 in untreated HbAA, HbAS and HbSS erythrocytes (normalized by cell number, n=6; mean +/- s.e.m.).
(C) Relative levels of erythrocytic miR-451 in untreated HbAA and HbSS erythrocytes and with indicated treatments (normalized by cell number, n=6; mean +/- s.e.m.).

To study the effect of parasitic translocation of various miRNAs, we established a system to introduce miRNAs into host erythrocytes. We achieved ~50% transfection efficiency (Figure 11B,C), and all transfected miRNA appeared to be both stable and enriched to a similar degree within the RBCs (Figure 11D).

Transfection of HbAA erythrocytes with miR-451 increased HbAA miR-451 to levels similar to HbSS erythrocytes (Figure 10C). Additionally, HbSS levels of miR-451 were inhibited by antisense 2’O-methyl-451, but not by antisense 2’O-methyl-181a (Figure 10C).
Figure 11: Efficiency of Transfection of exogenous miRNAs into mature erythrocytes. 
(A) Intra-erythrocytic let-7i levels in the indicated erythrocytes and treatments. (n=6; mean +/- s.e.m.).
(B) The characteristics of transfected erythrocytes are shown by forward (x-axis) and side scatter (y-axis) using flow cytometry and a representative histogram of FITC intensity (FL1-H) after transfection with unlabeled (purple) and FITC-labeled (green) synthetic oligonucleotides. 
(C) The percentage of erythrocytes successfully transfected using FITC-labeled synthetic oligonucleotides (n= 12; mean +/- s.e.m.).
(D) Enrichment of miRNA (determined by RT-PCR) of the listed miRNAs in the transfected RBCs at 48 hours (2 days) and 144 hours (6 days) post-transfection (n=4; mean +/- s.e.m.).

While we were reasonably confident that human miRNA were present within the parasite, we wanted to confirm uptake via a non-PCR based approach. Thus, we assessed the cellular localization of miR-451 within the parasite using miRNA immunofluorescence. Erythrocytes were transfected with biotinylated miR-451 and miR-181a. Then, after saponin lysis of the host erythrocyte, miR-181 was largely undetectable with FITC-labeled streptavidin, while miR-451 was readily observed (Figure 12). MiR-451 localization was observed within the parasitophorous vacuolar membrane (PVM), labeled by the PVM marker EXP1.
Figure 12: **Erythrocyte miRNAs are translocated into *P. falciparum*.** Detection of miRNA localization in uninfected and infected (Saponin-lysed) erythrocytes indicating transfected miRNA (green), the PVM (EXP1-red), and parasite nuclei (DAPI-blue).

In combination with the previous studies of miRNA content after various techniques to remove contaminating erythrocytic miRNA, and given the dynamic nature of miRNA composition within the parasite, this immunofluorescence data strongly demonstrate the presence of human miRNA within the malaria parasite.
2.3 Elevated Levels of specific miRNA confer resistance to malaria infection

The presence of higher levels of specific miRNAs in both HbAS and HbSS erythrocytes, as well as the dynamic nature of miRNA uptake, implies a functional role for those miRNA. To formally test this possibility, we transfected HbAA erythrocytes with miR-451, miR-223, miR-16, let-7i, miR-181a or a ssDNA oligo and first assessed their ability to affect parasite growth. We found that the transfection of HbAA erythrocytes with ssDNA, miR-181a or the abundant miR-16 had no effect upon parasitemia (Figure 13A). In contrast, transfection with miR-451, miR-223, or let-7i led to a markedly reduced parasitemia (Figure 13A) and decreased hypoxanthine incorporation (a measure of proliferation) by ~25% (Figure 13B). These changes are not associated with significant changes in the viability or characteristics of erythrocytes (Figure 13C-E) or parasite invasion rate (Figure 13F).
Figure 13: Elevated miRNA levels confer resistance to *P. falciparum*.

(A) Normalized parasitemia at different time points post-infection in HbAA erythrocytes transfected with control DNA or the indicated miRNAs (n=24; mean +/- s.e.m., except miR-16 and let-7i, which are n=8).

(B) Parasite proliferation measured by [3H]-hypoxanthine incorporation in HbAA erythrocytes transfected with the indicated miRNAs (n=4; mean +/- s.e.m.).

(C) Relative level of free hemoglobin immediately post-transfection, normalized against mock-transfected RBCs (n=3; mean +/- s.e.m.).

(D) Relative mean corpuscular volume (MCV) 2-hours post-transfection, normalized against mock-transfected RBCs (n=3; mean +/- s.e.m.).

(E) Relative mean cellular hemoglobin concentration (MCHC) 2-hours post-transfection, normalized against mock-transfected RBCs (n=3; mean +/- s.e.m.).

(F) Parasitemia was calculated after the first invasion cycle (day 2 (D2)) in erythrocytes treated with control DNA or the indicated synthetic miRNA (n=27; mean +/- s.e.m.).
These phenotypic changes are accompanied by increased intra-parasitic miRNA levels (Figure 14A-B). Additionally, combined over-expression of miR-451 and miR-223 was able to reduce infection rate by 46% (Figure 13A), with similar effect seen for additional combination of miR-451, let-7i, and miR-223 (Figure 14C).

We also tested how different molecular configurations of small RNA affected the anti-parasitic activities of miR-451 and found that only a mature, single-stranded, sense miR-451 was capable of reducing parasitemia (Figure 14D-E). These findings are of particular import since previous studies of miRNA and RNAi in *P. falciparum* utilized dsRNA constructs, which also exhibit no anti-parasitic activity in our study.

In addition, it appears that the seed sequence is necessary for this effect upon parasite growth. A chimeric miRNA with a miR-181a mature sequence, but substituted with a miR-451 seed, showed similar decreases in parasite growth as those observed by miR-451 (Figure 14F), while the reciprocal miRNA, with a miR-451 mature sequence but a miR-181a seed exhibited no effect upon parasite growth. Collectively, these results indicate the anti-parasitic activities of these miRNAs exhibit strong sequence specificity.
null
Figure 14: The anti-parasitic effect of miR-451 over-expression is limited to the single-stranded mature form of miR-451 and elevated levels of miR-451 and let-7i contribute to malaria resistance of HbSS and HbAS erythrocytes.

(A) Intraparasitic miR-451 levels in parasites grown in untreated HbAA, HbAS or HbSS erythrocytes, HbAA erythrocytes transfected with indicated miRNA and HbSS erythrocytes transfected with antisense 2’O-methyl oligonucleotides, normalized against total cell number and HbSS (n=6; mean +/- s.e.m.).

(B) Intra-parasitic let-7i levels in parasites grown in the indicated erythrocyte treatments. (n=6; mean +/- s.e.m.).

(C) Normalized parasitemia at different time points after HbAA erythrocytes were transfected with the indicated combinations of miRNAs (n=3; mean +/- s.e.m.).

(D) The sequences of the miR-451 variants tested in panels D and E.

(E) Parasitemia over a 10 day period when propagated in HbAA erythrocytes transfected with various synthetic miR-451 variants. Parasitemia was calculated and normalized as a percentage of mock-transfected RBCs over 10 days of growth (n=7, except for miR-451 seed which is n=3; mean +/- s.e.m.).

(F) Normalized parasitemia at different time after HbAA erythrocytes were transfected with the chimeric miRNAs (n=3; mean +/- s.e.m.).

(G) Normalized parasitemia at different time points after HbAA erythrocytes were transfected with control DNA or the indicated antisense 2’OMe-miRNAs (n=4; mean +/- s.e.m.).

(H) Normalized growth rates of parasites propagated in HbAA, HbAS or HbSS erythrocytes transfected with the indicated antisense 2’O-methyl oligonucleotides to knock down specific miRNAs (n=4 mean +/- s.e.m.).

(I) Normalized growth rates of parasites propagated in HbAA, HbAS or HbSS erythrocytes transfected with the indicated antisense 2’O-methyl oligonucleotides to inhibit specific miRNAs (n=18 except for HbSS miR-451+miR-223, where n=5 and in all HbAS samples where n=6; mean +/- s.e.m.).

(J) Normalized parasitemia at different time points after invading HbAA erythrocytes were transfected with both the indicated miRNAs and the antisense 2’OMe-miRNAs. (n=4; mean +/- s.e.m.).

Since miRNA overexpression in HbAA erythrocytes could reduce malaria growth, we then asked if the higher endogenous levels of specific miRNAs in either HbSS or HbAS erythrocytes were indeed responsible (at least in part) for the enhanced malaria resistance seen in these host erythrocytes. Either miR-451 or let-7i were inhibited via antisense 2’O-methyl-oligonucleotides in HbAA, HbSS and HbAS erythrocytes (Figure 14A-B). Inhibition of microRNAs in HbAA erythrocytes had a marginal effect on parasite growth rate (Figure 14G). In contrast, inhibition of miR-451, and to a lesser degree let-7i, led to increased parasite growth in both HbSS and HbAS erythrocytes (Figure 14H-I). Additionally, simultaneous inhibition of both miR-451 and miR-223...
appeared to have an additive effect that increased HbSS parasite growth to ~50% of HbAA erythrocytes (Figure 14H). Finally, the sensitivity of P. falciparum in HbAA erythrocytes could be negated by sequence-specific inhibition of miR-451 (Figure 14J), suggesting that the alteration of growth rate due to different levels of miR-451 is sequence specific. These data indicate that host miRNAs contribute significantly to the malaria resistance of HbAS and HbSS erythrocytes.

As for the efficacy of miR-451, it is important to evaluate the data in the context of ~50% efficiency for miRNA enrichment of transfected RBCs (detailed in methods and Figure 11B,C). Consequently, a 2-fold and 1.5 fold increase in parasitemia upon miR-451 inhibition, in HbSS and HbAS respectively, illustrates a significant effect since only half of the host cells are modulated by such genetic intervention.

2.4 Overexpression of miR-451 does not generate significant changes in parasite transcriptional profile

miRNA function by regulating the mRNA stability and translational efficiency of target mRNAs. However, a recent study suggests that the dominant effect of miRNA inhibition is to destabilize mRNA transcripts (Guo et al, 2010). In collaboration with Manuel Llinas at Princeton University, we performed a series of microarray experiments in which we overexpressed miR-451, miR-223, and miR-181a in mature erythrocytes, infected with P. falciparum, and then after 96-hours extracted purified (via saponin-lysis) parasite total RNA. Given the differences in miRNA abundance across the parasite lifecycle, we sought to identify changes in the mRNA levels of parasites at four points (8H, 16H, 32H and 40H post-infection) across the 48-hours IDC lifecycle. Thus, we
extracted RNA at those 4 timepoints, and using mock-transfected erythrocytes as a negative control, profiled parasite mRNAs on spotted *P. falciparum* cDNA microarrays.

**Figure 15: Parasite mRNA expression in response to erythrocytic miRNA overexpression.** Arrays are shown for 4 time points (8, 16, 32, and 40 hours). Left is miR-181, middle is miR-223 (32 hour array failed), and Right is miR-451. Arrays have been zero-transformed against the average of 3x mock transfected.

**Table 2: Summary of results from miRNA overexpression Experiment (Figure 15)**

<table>
<thead>
<tr>
<th>Upregulated/Downregulated at 32 Hours</th>
<th>Number of Genes showing 2-fold variation from mock</th>
<th>Number of genes also dysregulate at 40 hours</th>
<th>Number of genes with miR-451 exclusive dysregulation (versus miR-181)</th>
<th>Number of genes of known/unknown function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Upregulated</td>
<td>333</td>
<td>90</td>
<td>90</td>
<td>22/68</td>
</tr>
<tr>
<td>Downregulated</td>
<td>319</td>
<td>6</td>
<td>2</td>
<td>0/2</td>
</tr>
</tbody>
</table>
For miR-451, we chose to focus on parasite RNA at 32 and 40 hours post-infection, since those were the two points during the erythrocytic lifecycle which exhibited the highest levels of intraparasitic miR-451. As summarized in Table 2, miR-451 overexpression led to upregulation of a modest number of parasite mRNA transcripts. However, miR-451 did not significantly downregulate any annotated parasite transcripts, which suggested to us that miR-451, consistent the lack of miRNA protein machinery in *P. falciparum*, may function by a different mechanism than the canonical miRNA pathway. As further confirmation (and in collaboration with Bill Majoros), we examined the *P. falciparum* transcriptome for miR-451 conserved seed matches which could suggest potential miR-451 targets. However, we identified no conserved seed matches (between 4 species of *Plasmodium - falciparum, vivax, berghei* and *yoelli*) for miR-451. As a control, miR-181a did have a number of conserved sequences complementary to its seed sequence. The lack of candidate mRNA targets from this study caused us to reassess how human miR-451 functioned within the parasite.
2.5 Human miRNAs form chimeric fusion RNAs with *P. falciparum* mRNA

The lack of both inhibition of parasite mRNA transcripts and conserved seed matches for miR-451 suggested to us that these miRNA may be working via a non-canonical mechanism. Although no sequences in the *P. falciparum* genome appear to have significant homology to miR-451, a more detailed analysis of the various genomic databases for *P. falciparum* led us to identify 21 *P. falciparum* ESTs in PlasmoDB whose 5’ ends were identical or nearly identical to the sense miR-451 via BLAST (Table S2).

Surprisingly, the miR-451 sequence appeared to be contiguous at the 3’ end with several *P. falciparum* EST sequences, but was completely absent from the *P. falciparum* genomic DNA sequence. In total, we identified 6 human miRNAs in 35 *P. falciparum* ESTs (and four *P. vivax* ESTs), of which miR-451 occurred most frequently (Table S2) in both species (*P. vivax* only exhibited miR-451 chimeric RNA). These unexpected findings suggest that miRNAs may function during the parasite lifecycle by covalently integrating into *Plasmodium* mRNAs. Of even greater interest is that a comparable analysis of the EST databases for two rodent malaria strains (*P. berghei* and *P. yollei*) exhibited no fusion transcripts, suggesting that if these chimeric transcripts do exist, they may be human malaria-specific.
Table 3: List of *P. falciparum* ESTs in PlasmoDB that represent cross-species fusion RNAs between the indicated human miRNA and annotated *P. falciparum* genes. ESTs XPf2n3060 and XPf2n4817 contain sequences which blast to 2 separate regions of the genome. The sequence directly associated with miR-451, *PKA-R* and Histone H4, are listed.

<table>
<thead>
<tr>
<th>MicroRNA</th>
<th>Plasmodium EST</th>
<th>Chromosome</th>
<th>Location</th>
<th>Adjacent Coding Regions</th>
</tr>
</thead>
<tbody>
<tr>
<td>mir-451</td>
<td>XPf2n4817</td>
<td>MAL10</td>
<td>1203600-1203605(+)</td>
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To investigate this astounding possibility, we first performed northern blots on parasite RNA using locked nucleic acid (LNA) detection probes for miR-451, let-7i and miR-181 (once again negative control). A number of parasite transcripts predominantly >1 kb were detected by the miR-451 and let-7i probes, while the miR-181 probe failed to detect any transcripts (Figure 16A,D). The presence of these larger transcripts is not likely to represent non-specific hybridization to rRNA, as Poly-A-purified RNA showed a similar pattern of miR-451 hybridization (Figure 16B). Compared to HbAA, parasites
grown in HbAS and HbSS erythrocytes showed increased hybridization signals with miR-451 and let-7i probes (Figure 16C,D). This is confirmed by densitometry analysis of parasites derived from the various erythrocyte genotypes. These results suggest that miR-451 and let-7i sequences indeed exist within parasite mRNA transcripts and the degree of incorporation of human miRNA into parasite transcripts correlates with miRNA levels within the host erythrocyte.

Figure 16: Erythrocytic miRNA sequences are present in larger molecular weight RNA. (A) Northern blot of total RNA from human mammary epithelial cell (HMEC), blood, synthetic miR-451, and purified parasite (3D7) probed for miR-451 or miR-181 via LNA probe. Amounts of RNA were visualized by ethidium bromide staining. (B) Northern blot of polyA+ (Upper middle) RNA from human mammary epithelial cell (HMEC), blood, synthetic miR-451, and purified parasite (3D7) were probed for miR-451 (upper panel) and PKA-R (lower panel). The postulated size of the miR-451-PKA-R is indicated with a star, while the expected positions of the *Plasmodium* rRNAs are indicated with arrows. (C) Northern blot of total RNA from whole blood and purified parasite (3D7) derived from HbAA, HbAS, and HbSS cells probed for miR-451. RNA amounts were visualized by ethidium bromide staining. The two bands indicated with ‘a’ and ‘b’ have been quantified (normalized to 18s RNA levels). (A) Northern blot of total RNA from blood, and purified parasite (3D7) derived from HbAA, HbAS, and HbSS erythrocytes probed for let-7i. Amounts of RNA were visualized by ethidium bromide staining. The postulated size of let-7i-Rex1 (based on 5’RACE data) is indicated with a star, while the expected positions of the *Plasmodium* rRNAs are indicated with arrows.
The results of the northern blot do suggest the presence of miR-451 and let-7i chimeric transcripts, but they do not allow for the identification of specific mRNA targets. In order to experimentally verify that specific \textit{P. falciparum} transcripts in PlasmoDB were targeted by miR-451, we first performed 5’-RACE-PCR on potential targets suggested by the EST data. This identified miR-451-modified transcripts for the regulatory subunit of cAMP-dependent Protein Kinase (\textit{PKA-R, PFL1110c}) and Phosphoethanolamine N- methyltransferase (\textit{PEAMT, MAL13P1.214}), which were subsequently verified by RT-PCR followed by Sanger sequencing (Figure 17A,B) using gene specific reverse primers and miRNA-specific forward primers.
Figure 17: Confirmation of miRNA-mRNA fusion by a variety of sequencing-based techniques.

(A,B,C) Sequence alignment for the miR-451-PKA-R fusion (A), the miR-451-PEAMT fusion (B), or the let-7i-REXI fusion (C) as determined by indicated experimental approaches.

We further tried to circumvent potential artifacts by using RNA-Seq, which allow for the determination of both the identity and abundance of miR-451 fusion transcripts in a relatively unbiased fashion. We performed RNA-Seq using an Illumina Genome Analyzer II for parasites grown in HbAA erythrocytes. We generated a total of ~15 million reads, and from those we found that 85% of the total reads mapped to the *Plasmodium* genome, based on a BLAT alignment of at least 90% identity required. We then further analyzed these reads for all possible miRNA-mRNA fusions by looking for reads in which the first 10nt had perfect sequence complementarity to a human miRNA, while the last 30nt had near-perfect complementarity (we allowed for 2 mismatches) to the malaria transcriptome. We did not BLAT the 10nt between the miRNA and mRNA regions due to potential differences in miRNA size, either from differences in mature
miRNA sequence length or RNA fragmentation of the human miRNA. From these reads, we identified eight overlapping sequences consistent with fusion transcripts formed between human miR-451 and PKA-R (Figure 17A). However, we failed to detect any fusion transcripts between miR-451 and PEAMT, suggesting that this fusion transcript exists at very low abundance in HbAA erythrocytes. Closer examination of the sequences discovered by 5’-RACE-PCR and RNA-Seq analysis indicates the presence of two PKA-R transcript forms, the shorter of which lacks exon 1. The shorter form is likely to be functionally relevant however, as it contains an overlapping open reading frame with the annotated form of PKA-R and retains all predicted functionally relevant amino acid residues. Moreover, multiple mass spectrometry studies of PKA-R during a variety of Plasmodium life cycle stages show peptide hits only from exon 2 and beyond, suggesting that the shorter transcript may be the dominantly translated form (Lasonder et al, 2002, Lasonder et al, 2008). Likewise, next generation sequencing of PKA-R shows significantly decreased read density for the annotated first exon, further suggesting that the short form may be the dominantly transcribed form (Otto et al, 2010). Interestingly, only the shorter form of PKA-R formed a chimeric RNA with miR-451.

Somewhat surprisingly, the junction between the miR-451 and PKA-R transcripts consistently contained 14, or less commonly 6, nucleotides that were not present at the 5’ end of the annotated PKA-R transcript (Figure 17A). Notably, these junction sequences were identical to a short stretch of sequence near the end of exon 2 of PKA-R. Both Illumina and 5’ RACE show the presence of this “linker” even in the absence of miR-451 (Figure 17A), leading us to believe that it is a part of PKA-R itself. However, this
sequence is absent from PKA-R’s genomic DNA sequence, suggesting that this may represent intermediate processing or that the mRNA is modified.

Given the unexpected nature of these results, we sought to firmly rule out that miR-451:PKA-R fusion transcripts were artifacts introduced by template switching during cDNA synthesis. To irrefutably show the existence of these fusion transcripts, we performed Ribonuclease Protection Assays (RPAs) in which part of the target mRNA is protected from RNase digestion by hybridization to a target probe. We used a PKA-R specific probe of 63nt (58nt PKA-R+5 non-complementary nucleotides) and a miR-451:PKA-R fusion-specific probe of 85nt (22nt miR-451 + 58nt PKA-R+5 non-complementary nucleotides), both of which contained a non-complementary overhang to distinguish free probe from hybridized probe-target couples (schematic: Figure 18 A-C: Figure 18 D-F).
Figure 18: Ribonuclease Protection assays of miRNA-mRNA fusions in *P. falciparum*.

(A) RNA from parasites grown in either HbAA or HbSS erythrocytes was incubated with a 58 nucleotide 451-PKA-R probe (58nt PKA-R specific) and subsequently subjected to RPA. Both miR-451 modified and unmodified PKA-R will produce equal size (58nt) products.

(B) RNA from parasites grown in either HbAA or HbSS erythrocytes was incubated with an 85nt nucleotide 451-PKA-R probe (22nt miR-451 + 58nt PKA-R specific + 5nt non-complementary) and subsequently subjected to RPA. MiR-451 modified and unmodified transcripts will produce 80 nt and 58 nt products respectively.

(C) Control to show the 85 nt probe did specifically differentiate between miR-451 modified and unmodified PKA-R transcript, RNaseH digestion post-incubation with a 22 nt probe recognizing the miR-451 was performed, which was followed by incubation with an 85nt nucleotide 451-PKA-R probe (22nt miR-451 + 58nt PKA-R specific + 5nt non-complementary) and subsequently subjected to RPA. Both miR-451 modified and unmodified PKA-R will produce 58nt products.

(D) Detection of chimeric miR-451-PKA-R transcripts using Ribonuclease Protection Assays (RPA) with RNA from uninfected whole blood (WB) and purified parasites from HbAA, and HbSS erythrocytes. RNA was hybridized to a PKA-R (PKAR) or miR-451-PKAR (451-PKAR) probe, digested with RNaseA/T1 and run on gel. One RNA sample was pre-hybridized to miR-451 DNA probe, digested with RNaseH, subsequently hybridized with a 451-PKA-R probe and digested with RNaseA/T1 (lane 4). Undigested miR-451-PKA-R probe was also included (NR).

(E) Additional replication of the miR-451 PKA-R fusion with the indicated treatments

(F) Detection of chimeric miR-451-PEAMT using RPA as in (D).

(G) The use of similar RPA strategy as in (H) to detect chimeric let7i-REX1.
As expected, the PKA-R probe detected an unmodified PKA-R transcript fragment ~58nt in length in *P. falciparum*-infected HbAA erythrocytes (Figure 18D, lane 1). Similarly, the miR-451:PKA-R fusion-specific probe also detected this 58nt fragment (protected PKA-R transcript), but additionally detected a 80nt fragment (protected miR-451:PKA-R fusion transcript). Notably, this 80nt fragment was more abundant in *P. falciparum*-infected HbSS compared to HbAA erythrocytes (Figure 18D,E, lanes 3 vs. 7), suggesting that the fusion transcript is more abundant in HbSS cells. Indeed, transfection of HbAA erythrocytes with miR-451 (but not miR-181a) also increased abundance of the 80nt fragment (Figure 18D, lane 5, Figure 18E). Finally, inhibition of miR-451, but not miR-181a, in HbSS erythrocytes led to a decrease in the abundance of the 80nt fragment (Figure S4H). To further prove that this 80nt fragment represents a miR-451-PKA-R fusion transcript, we preceded our RPA by incubating *P. falciparum* mRNA from HbAA erythrocytes with a 22nt antisense miR-451 DNA probe, followed by digestion of the resulting RNA/DNA duplexes with RNase H (Schematic: Figure 18C). If miR-451:PKA-R fusion transcripts are present, hybridization to the 22nt miR-451 DNA probe followed by RNase H digestion will result in removal of the miR-451 moiety from the fusion transcript, yielding an unmodified PKA-R transcript. Indeed, Figure 18D (lane 3 vs. 4) shows that the RNase H treatment abolished the 80nt fragment (miR-451:PKA-R), but did not affect the 58nt fragment (unmodified PKA-R). Also of note is that the control PKA-R probe hybridized largely to a 58nt sequence rather than a 44nt sequence, suggesting that the unannotated 14nt region is also present within the PKA-R transcript in the absence of fused miR-451. Similar results were obtained in RPAs designed to detect a miR-451:PEAMT fusion transcript (Figure 18F).
In addition to miR-451, the overexpression of miR-223 and let-7i also reduced parasitemia. While neither our Illumina data, which in fact identified no other miRNA-mRNA fusion candidates with full-length miRNA beyond miR-451-PKA-R, nor the existing EST data suggested any fusion targets for miR-223, there was one potential fusion target for let-7i, Ring-exported factor 1 (PFI1735c, present in 2 ESTs – Table 3). This gene has been implicated in Mauer’s cleft assembly and red cell remodeling (Hanssen et al, 2008), both of which are processes which have been shown to be impaired in HbSS parasite development. Given that these ESTs were located a significant distance upstream of the *REXI* coding sequence (~1kb 5’ of the start codon), we confirmed the presence of this fusion transcript by RT-PCR and 5’ RACE (Figure 17C). The potential of let-7i to form fusion RNAs and affect parasite growth is especially interesting because like miR-451, let-7i is present at higher levels within both HbAS and HbSS erythrocytes and accumulates in the parasite (Figure 11A, Figure 14B). RPA results for let-7i-*REXI* showed similar results to miR-451, where higher levels of let-7i led to higher fusion rates (Figure 18G). Hence, the findings from our RPAs conclusively verified both the existence of host-parasite fusion transcripts and that the extent of modification is sensitive to host cell miRNA levels.

One question that immediately comes to mind is that given the (apparent) cytosolic localization of erythrocytic miRNAs (Figure 12), where does this splicing occur. Given that most mRNA splicing occurs in the nucleus, this would suggest a rather novel mechanism of gene splicing for these host cell miRNA. However, RNA modification and splicing have been shown to occur outside the nucleus in several organisms. For example, *Trypanosoma brucei* has been shown to covalently modify cytosolic tRNAs
during transport from the cytosol to the mitochondria (Schneider et al, 1996). Also, in mammalian cells, XBP1, a key transcription factor of the unfolded protein response, is spliced in the cytosol in response to ER stress in several studies (Back et al, 2006, Uemura et al, 2009). In addition to splicing, there are numerous examples of RNA editing within non-nuclear compartments, including in plants (C->U and U->C modifications), humans (ApoB) and mammalian mRNAs (Adenosine deaminase activity – ADARs). In summary, while we do not know the exact mechanism involved, the presence of various cytoplasmic RNA editing machinery in numerous other organisms don’t completely rule out the possibility the fusion process could be non-nuclear.

2.6 Host cell miRNA levels regulate the extent of chimeric RNA fusion

Having demonstrated the presence of human miRNA integrated into parasite transcripts, we next wanted to examine the extent of integration of miR-451 and let-7i. Based on Illumina sequencing of *P. falciparum* from HbAA erythrocytes, we estimated that miR-451 fused to ~3% of total PKA-R transcripts (Figure 19A). Since the growth inhibition from miR-451 was dependent upon host cell miRNA levels, we first wanted to examine whether the rate of miRNA-mRNA fusion was also correlated with host cell miRNA levels. In order to assess the fusion rate of miR-451 to *PKA-R* and its relation to host erythrocyte miRNA levels, we incubated total parasite mRNA with miR-451:*PKA-R* specific probes and digested the resulting double-stranded complexes with RNase H to remove the miR-451:*PKA-R* junction. Subsequently, we performed RT-PCR with a 5’ primer against a *PKA-R* sequence that is partially targeted by the miR-451:*PKA-R* junction-specific probe. Thus, comparison of the amount of *PKA-R* transcript that is
degraded with the miR-451:PKA-R probe versus control digests with a hypothetical miR-181:PKA-R junction probe revealed the extent of fusion between miR-451 and PKA-R.

Figure 19: Cellular levels of miR-451 fusion transcripts. (A) The percentage of total PKA-R transcripts modified by miR-451 in the RNA-Seq data is shown when we allowed for 0, 1, or 2 mismatches in the PKA-R sequence. (B, C, D) Percentage of iR-451-PKA-R, miR-451-PEAMT (B) and let-7i-REXI (C) fusion which are sensitive to RNaseH digestion in the indicated erythrocytes and treatments. Fusion rate is calculated as the percentage of degradation of target transcripts after hybridization to a miR-451-PKA-R (B), miR-451-PEAMT (C) or let-7i-REXI (D) junction probes followed by RNaseH digestion. The remaining amount of remaining transcripts was determined by RT-PCR and normalized against an RNaseH digestion with a hypothetical miR-181 probe (n=3; mean +/- s.e.m.).

This method indicates that ~5% PKA-R mRNA was modified by miR-451 in HbAA erythrocytes, while in HbSS erythrocytes this was nearly 35% (Figure 19B). The rate of miR-451 modification for PKA-R decreased to 23% when miR-451 was inhibited in HbSS erythrocyte (Figure 19B). In addition, overexpression of miR-451 in HbAA
erythocytes increased the incidence of miR-451-PKA-R fusion to ~20% (Figure 19B). While this is comparable to fusion rates detected in HbAS grown parasites, it is less than in HbSS grown parasites, suggesting that total miR-451 levels are not the sole determinants of miRNA-mRNA fusion rates.

Far less PEAMT or REX1 mRNA was respectively modified by miR-451 and let-7i, but modification levels were again higher in HbSS (~5%) compared to HbAA (<1%) erythrocytes and sensitive to inhibition of respective host miRNAs (Figure 19C,D). Taken together, these findings suggest that the miRNA levels in host cells may drive its rate of incorporation into parasite mRNA.

We further tested this notion by transfecting HbAA erythrocytes with miR-451, and then infected with *P. falciparum* and assayed the relative levels of miR-451-modified parasite transcripts by RT-PCR. Transfection of miR-451 clearly results in increased formation of miR-451:PKA-R and miR-451:PEAMT fusion transcripts (Figure 20A). The fusion rate for HbAA transfected with miR-451 is lower than observed in HbSS erythrocytes. Again, this discrepancy suggests that all of the transfected miRNAs might not be available for the fusion process or there are other cellular signals which act with higher miR-451 levels in HbSS cells to further increase the fusion rate. Conversely, knockdown of miR-451 levels in HbSS erythrocytes decreased the formation of fusion RNA (Figure 20A). Similar results were found for miR-451:PKA-R fusion transcripts in HbAS erythrocytes (Figure 20B) and for let-7i:REXI (Figure 20C).
Figure 20: **Enrichment of miRNA-mRNA fusion is correlated with increasing miRNA level.**

(A) Levels of miR-451 modified PKA-R and PEAMT as determined by qPCR. Treatments were identical to (n=7; mean +/- s.e.m.)

(B) miR-451-PKA-R levels within HbAS erythrocytes as determined in (B). n=6; (mean +/- s.e.m.)

(C) Relative level of let-7i-REX1 as determined by RT-PCR for the indicated erythrocytes and treatments (n=5; mean +/- s.e.m.).

These findings mirror and extend those shown in Figures 16, 18 and 19.

Collectively, these data firmly establish that the levels of miRNA in the host erythrocytes will determine the degree of host:parasite transcript fusion.
2.7 miRNA present within chimeric miRNA-mRNA fusions are erythrocytic in origin

While we have established that erythrocytic levels of miR-451 did modulate the extent of miR-451 fusion, we wanted to further validate the connection between host erythrocyte miRNA and the resulting chimeric miRNA-mRNA fusions. To establish host miRNAs as the source of miRNA within the chimeric transcripts, we transfected 5’-desthiobiotin-miR-451 (5’Db-miR-451) and 5’Db-miR-181 into HbAA cells followed by infection with *P. falciparum*. After four days we purified (via saponin-lysis) and extracted total parasite RNA, captured Desthiobiotin-bound RNAs with streptavidin, eluted and analyzed the presence of miR-451:mRNA fusion transcripts (schematic: Figure 21A). We chose to use Desthiobiotin-conjugated miRNAs, in place of the easier to synthesize biotinylated form, because Desthiobiotin has a reduced binding affinity for strepavidin compared to biotin. This allowed us to specifically elute the desthiobiotin-tagged RNAs via competition with excess biotin.

Based on the recovery of miR-451 from the host cell lysate after saponin-lysis, the parasite uptakes ~22% of the biotinylated miRNA in the erythrocyte (Figure 21B) and, RT-PCR analysis indicated that contrary to free miR-451 or 5’Db-miR-181, transfection of 5’Db-miR-451 followed by capture of fusion transcripts by strepavidin resulted in enrichment of *PKA-R* and *PEAMT* fusion transcripts (Figure 21C), while transfection of 5’Db-let-7i resulted in enrichment of *REX1* (Figure 21D). This effect was gene-specific, as no enrichment was observed for *Falcipain 3* (Pf11_0162), *Rhop2* (PFI1445w), or *Plasmepsin IV* (Pf14_0075) (Figure 21C). Notably, the addition of excess 5’Db-miR-451 to the lysate prior to RNA extraction and biotin capture did not alter the recovery of *PKA-R* (Figure 21E), reaffirming that these fusion transcripts are exclusively generated *in vivo*. 
Figure 21: miRNAs present in chimeric Plasmodium are erythrocytic in origin.
(A) Strategy to capture modified miRNA-mRNA: RNA oligonucleotides modified by
desthiobiotin at their 5’ end were transfected into RBCs, which were subsequently used to
propagate malaria parasites. The tagged microRNAs translocate into parasites and modify the P.
falciparum target RNAs during the 96 hour infection. After lysing the erythrocyte with saponin,
the parasite RNA is isolated and modified microRNA is captured with streptavidin beads. The
bound microRNAs and resulting fused P. falciparum target RNAs are eluted with excess biotin.
(B) Relative level of biotin-miR-451 recovered from the parasite relative to the host erythrocyte.
Parasites grown in biotin-miR-451 transfected erythrocytes were isolated by density gradient
centrifugation and the percentage of biotin-miR-451 recovery was determined in purified
parasites (saponin-lysed) compared to total infected RBC by RT-PCR (n=3; mean +/- s.e.m.).
(C) Levels of unmodified transcripts (Plasmepsin IV, Falcipain 3, and Rhop2) and miR-451-
modified transcripts (PKA-R, and PEAMT) captured by unmodified miR-451, desthiobiotin (Db-
miR-181, and Db-miR-451 (Normalized vs. 18S rRNA, n=5; mean +/- s.e.m.).
(D) Levels of PKA-R enriched by desthiobiotin (Db) pulldowns from parasites grown in
erythrocytes transfected with miR-451 (Mock), Db-miR-181 (miR-181), or Db-miR-451 (miR-
451) in the presence or absence of excess Db-miR-451 (+DbmiR-451 samples) added to the
parasite lysate prior to biotin pulldown (Normalized vs. 18S rRNA, n=4; mean +/- s.e.m.).
(E) Relative levels of REX1 enriched by desthiobiotin pulldowns from parasites grown in
erythrocytes transfected with unmodified let-7i (mock) and, biotinylated forms of miR-181, miR-
451, and let-7i as eluted from the capture assay in figure E (Normalized vs. 18S rRNA, n=4;
mean +/- s.e.m.).
2.8 miRNA require a free 3’ hydroxyl group to undergo miRNA-mRNA fusion

Since 5’-blocked miR-451 retains its ability to invade parasite mRNAs (Figure 12, Figure 21B), we hypothesized that a free 3’ end of miR-451 may be necessary to form chimeric RNA. To test this possibility, we covalently linked biotin to either the 5’ or 3’ end of miR-451 to determine the effect of terminal modification on anti-plasmodial activity and target mRNA fusion.

Figure 22: miRNA fusion requires a free 3’ hydroxyl group.
(A-C) Levels of miR-451 modified PKA-R (A) and PEAMT (B), as well as parasitemia (C) after erythrocyte transfection with unmodified miR-451 and miR-181, or with 5’ or 3’ Biotin (Bi)- and Phosphate (P)-labeled miR-451 (n=4; mean +/- s.e.m.).

While 5’ biotin-miR-451 and unmodified miR-451 strongly reduced parasite growth while increasing 5’ PKA-R modification, the 3’biotin-miR-451 lost both activities (Figure 22A-C). We were concerned that the large size of the biotin group might be inhibiting miR-451 fusion via a non-specific steric-hindrance-based effect, so we
synthesized a miR-451 variant with a smaller 3’P group. This 3’-modified miR-451 also lost all effect upon chimeric RNA formation or parasite growth. Thus, these data indicates that miR-451 requires a free 3’ hydroxyl group for integration with the target parasite mRNAs.

2.9. Higher Levels of miR-451 increase the frequency of gametocytogenesis

Precise modulation of cyclic AMP-dependent kinase signaling plays a major role in the parasite’s life cycle (Beraldo et al, 2005). Both cAMP-dependent catalytic (PKA-C) and regulatory (PKA-R) subunits have been identified in *P. falciparum*, where regulation of PKA-R levels is crucial for parasite survival (Li et al, 2000). We speculated that miR-451-mediated suppression of PKA-R protein levels should result in increased PKA-C activity, which has been implicated in the induction of gametocytogenesis of *P. falciparum* (Trager and Gill, 1989).

Since the most frequent target of miR-451 was PKA-R, we wondered if miR-451 fusion might have an effect on parasite gametocytogenesis. Thus, we analyzed the amount of sexual stage parasites in mock-, miR-181, and miR-451 transfected erythrocytes, where we observed a 2.5-fold increase in parasites which entered sexual stages (Figure 23).
Figure 23: **High miR-451 levels result in increased number of sexual stage parasites.** Normalized percentage (against mock-transfected) of sexual stage parasites grown in the indicated erythrocyte types, shown at 6 days post-transfection, as determined by Giemsa staining (n=3; mean +/- s.e.m.).

Induction of gametocytogenesis was not observed in mock- or miR-181-transfected erythrocytes. We also detected increased gametocytemia in both HbAS and HbSS erythrocytes, consistent with their higher erythrocytic miR-451 levels, as well as a previous report (Trager et al, 1999). As observed in Figure 23, the induction of gametocytogenesis was sensitive to inhibition of miR-451, but not miR-181a. Taken together, our data show that miR-451 fusion has a significant negative effect upon the activity of its fusion target. Uncovering the mechanism behind this negative effect, and thus the consequence of miR-451 fusion to PKA-R, became the next area of study.

### 2.10 miR-451 modification generates uncapped transcripts

mRNAs generally undergo a 5’ capping process in which the 5’ terminal phosphate is replaced by a 7-methylguanosine group via an unusual 5’-to-5’ triphosphate bridge. The 5’ Cap serves several regulatory functions, including protecting the mRNA from exonucleases and promoting translation. Because *PKA-R* mRNA is modified by miR-451 at the 5’ end, we investigated if miR-451:*PKA-R* fusion transcripts were properly capped.
We incubated mRNA from HbAA parasites with Terminator exonuclease, an enzyme that degrades RNA with free 5’ monophosphates. Subsequent detection of miR-451:PKA-R by RPA and RT-PCR revealed that this fusion transcript, like uncapped 28S rRNA, was susceptible to exonuclease degradation (Figure 24A,B).

Figure 24: **miR-451 modified transcripts are uncapped.**
(A) RPA for detection of miR-451-PKA-R. RPA of terminator exonuclease untreated and treated RNA from parasites grown in HbAA and uninfected whole blood (WB), with a directly synthesized 27nt RNA probe.
(B) qPCR of terminator exonuclease-treated RNA in both HbAA and HbSS derived parasites. Data is presented in a ratio of undigested/digested, and normalized against β-actin (n=3; mean +/- s.e.m.).

In contrast, normally capped PKA-R transcripts were relatively resistant to endonuclease digestion. These results suggest that miRNA-modified parasite mRNAs are uncapped.
2.11 Chimeric mRNAs are stable

Failure to properly cap 5’ termini can result in mRNA instability via increased sensitivity to 5’ exonucleases. Therefore, we assessed if miR-451 modification rendered PKA-R transcripts unstable. *P. falciparum*-infected erythrocytes transfected with miR-451, miR-181a or let-7i were treated with actinomycin D, a potent inhibitor of transcription (Reich et al, 1962). We then chased levels of miR-451:PKA-R, miR-451:PEAMT, and let-7i-REX1 relative to total PKA-R, PEAMT, and REX1 mRNAs. No significant differences were observed in mRNA decay kinetics or steady state mRNA levels for these genes (Figure 25A-F), indicating that miRNA modification does not render parasite mRNA unstable.
Figure 25: The steady-state and mRNA half-lives of miRNA fusion targets

(A,C,E) The decay half-life ($t_{1/2}$) following Actinomycin D treatment of both total and miR-451-modified PKA-R (A), PEAMT (C) and let-7i-modified REX1 (E) in P. falciparum.

(B,D,F) The steady-state levels of PKA-R (B), PEAMT (D), and REX1 (F) mRNAs determined by RT-PCR in parasites transfected with miR-181a, miR-451 or let-7i at indicated time points (n=6, mean +/- s.e.m.).
Given the apparent 5’ monophosphate structure of these fusion transcripts, the fact that there was no difference in RNA stability was rather unexpected. Given our lack of understanding of the mechanism which generates these chimeric RNA, it is difficult to speculate as to the nature of this transcript stability. However, a published study demonstrated that antisense deoxyoligonucleotides could cleave mRNA to generate transcripts lacking the 5’ cap structure, and these uncapped mRNA were stable (Hasselblatt et al, 2005). In our study, we speculate that the miRNA modification alters the secondary structure and stability of the 5’ end of the target transcript in a way that is similar, though to a lesser degree, to what is observed in many viral mRNAs. In fact, analysis using mFOLD of the 5’ end of either 451-PKA-R or PKA-R indicates that miR-451 can form a relatively strong hairpin structure with the PKA-R region of the junctional sequence with a 3-fold increase in free energy. Although this information may offer potential explanations, further experimental verification is required to test this hypothesis.

Since we did not see any differences in either RNA decay or steady-state mRNA levels, we next investigated whether miRNA fusion affected translation of the chimeric transcripts. Unfortunately, in contrast to the genome-wide approaches used to study the proteome and transcriptome, few methods are available to globally examine translational regulation in P. falciparum. To that end, we decided to develop a technique by which we could assay the translation state of the chimeric miRNA-mRNA transcripts.
3. Translational regulation in *Plasmodium falciparum*

3.1 Introduction

### 3.1.1 Polyribosome isolation by sucrose Gradient

One of the most often used and proven experimental approaches to determine the translational status of an mRNA *in vivo* is the isolation of polyribosomes based on the velocity sedimentation of cellular extracts in sucrose gradients (Warner et al, 1963), more commonly known as polysome profiling. The main principle underlying this method is that during ultracentrifugation, macromolecular complexes (in this case ribosome associated protein and RNA complexes) migrate primarily according to their mass. Since the mass of ribosomes is by far greater than the mass of their bound mRNA undergoing translation, mRNAs will sediment in the gradient according to the number of ribosomes that are bound to them. In general, migration distance in the gradient, and thus the number of ribosomes associated with a given transcript, is a good proxy for the translation status of an mRNA. These procedures are able to separate the unassociated ribosomal subunits (40S and 60S), monosomes and can resolve polysomes that differ in size by only a single ribosome (Arava et al, 2003). This approach allows both the determination ribosomal occupancy (whether mRNA is or is not associated with polyribozyme) and overall ribosomal density (the number of associated ribosomes) of any mRNAs. Such method has been used to characterize global patterns of ribosomal status in *S. cerevesiae* (Arava et al, 2003), *Drosophila* (Qin et al, 2007), *Arabidopsis* (Piques et al, 2009), and human cancers (Provenzani et al, 2006).

More recently, this technique has been adapted for use with a number of more recent genomic technologies including microarrays (Qin et al, 2007) and high throughput
sequencing (Ignolia et al, 2009). Ribosomal footprinting represents an extension of the use of these techniques, in which mRNAs associated with ribosomes are digested, isolated and sequenced, allowing for quantitation of associated transcripts. This technique has been employed in a number of contexts, but perhaps the most relevant was to examine the relative contribution of translational inhibition versus mRNA turnover (which was implicated as the dominant mechanism) as a consequence of inhibition of miRNA targets by a single miRNA (Guo et al, 2010).

Polysome profiling has also played a valuable role in several other parasitic species, such as *Trypanosoma brucei*, which showed significant changes in ribosomal occupancy at different stages of development (Brecht et al, 1998). It has also led to identification of transcripts associated with alternative post-transcriptional regulatory pathways, such as RNA-binding protein TcPUF6 in *Trypanosome cruzi* (Dallagoivanna et al, 2008). In addition, the colocalization of parasite microRNAs with heavy ribosomal fractions has been used as confirmation of microRNA activity in *Trypanosoma brucei* (Djikeng et al, 2003) and *Toxoplasma gondii* (Braun et al, 2010).
3.1.2 Previous Polyribosome Isolation Techniques in *Plasmodium*

Developing a method for polysome profiling of *P. falciparum* has met with substantial technical challenges. Previous efforts to isolate ribosomes in *Plasmodium* predominantly resulted in primarily the recovery of monosomes, with polysomes present in quite low abundance. Electron micrographs of purified ribosomes from *Plasmodium knowlesi* revealed mostly monosomes, although some rare polysomes (4-6 ribosomes) were seen (Aikawa et al, 1971). Polysome profiling of the avian malaria parasite, *Plasmodium lophurae*, similarly revealed a predominance of monosomes with rare polysomes (Wallach and Kilejian, 1982). The only polysome profile obtained for *P. falciparum* also showed a large monosome peak, but with extremely poor resolution of both ribosomal subunits and polyribosomes (Ferreras et al, 2000). In total, these earlier approaches were limited in their ability to recover malaria polysomes. In fact, these findings seemed to suggest that malarial ribosomes tended to exist as monosome (i.e. one ribosome per transcript), and that the association of multiple ribosomes on a given transcript was rare. Without a means to purify malaria polysomes, it has been quite difficult to obtain the global translational profile of the parasite or interrogate the translational regulation of specific mRNAs.

3.2 Development of Polysome Profiling in *Plasmodium falciparum*

I describe here a novel procedure isolate and purify polyribosomes from *P. falciparum*, developed in collaboration with Josh Lacsina. Due to concerns about host cell contamination, several procedures have been used to separate the *Plasmodium* parasite from the host erythrocyte. One of the most common of these is saponin lysis of
host erythrocytes, followed by purification of the parasites by centrifugation (Christophers and Fulton, 1939). For reasons that remain unclear, purification of malaria parasites by saponin lysis leads to breakdown of the polysomes into monosomes (Figure 26A). Thus, saponin lysis—which has been used for decades to isolate *Plasmodium* parasites—produces an artifactual monosome-dominated polysome profile.

To circumvent this obstacle and obtain the native polysome profile, we simultaneously lysed both the infected erythrocytes and the parasite within. Following simultaneous lysis of the erythrocytes and parasites, we purified the ribosomes and subunits from the lysate by centrifugation through a sucrose cushion, after which sample pellets can be frozen at -80\(^\circ\)C. The ribosomal pellet was then resuspended and loaded onto a continuous sucrose gradient to separate the ribosomal subunits, monosomes, and polysomes by velocity sedimentation, followed by simultaneous A\(_{254}\) detection and fractionation. We have observed that RNA yields vary considerably within the erythrocytic stage of the parasite, where trophozoite and schizont stage cultures (20+ hours post-infection) require around half of the input of early ring stage cultures (4-8 hours post-infection).

A typical A\(_{254}\) trace is shown in Figure 26B, with the direction of sedimentation from left to right on the graph (with lighter fractions to the left). The initial A\(_{254}\) is high, likely due to absorbance by hemoglobin in the early fractions. The A\(_{254}\) then drops precipitously to baseline.
The first peak is small, and corresponds to the small ribosomal subunit (40S). The second peak is slightly taller than the first, and corresponds to the large ribosomal subunit (60S). The following peak is the largest, and corresponds to the monosome (80S). Following the monosome peak are the peaks corresponding to polysomes. Each successive peak represents polysomes bearing progressively higher integral numbers of ribosomes (2, 3, 4, etc.). Typical runs allow the resolution of polysomes composed of 5-7 ribosomes, though we have resolved up to 9-mer polysomes in some runs with a larger amount of starting material (roughly 3x the recommended input). Higher order polysomes compose the remainder of the trace, and cannot be resolved under these gradient conditions. In order to confirm that the A$_{254}$ signal represents rRNA, qPCR was
performed to quantitate 18S and 28S rRNA across the gradient (Figure 26C), and the results corroborate the identities of the A_{254} profile peaks described above.

Concerns are often raised about the contamination of *Plasmodium* samples with nucleated blood cells, and in this case, contamination with host-derived ribosomes, even though ribosomes are largely absent from mature erythrocytes. In order to ensure that host ribosomal contamination is not a significant concern, we have performed the same procedure with uninfected erythrocytes, to compare the A_{254} traces of infected and uninfected erythrocytes. As expected, we observe no pellet after centrifugation through the sucrose cushion, and see no A_{254} signal above baseline after velocity sedimentation (Figure 26D). Importantly, we also observe that malaria-specific transcripts co-migrate with the polysomal fractions (Figure 27), further supporting the idea that the observed polysomes are parasitic in origin.
These studies demonstrate that substantial quantities of polysomes are present in *P. falciparum* and that the polysomes can be isolated with sufficient quality and purity for translational profiling. With this method, it is now possible to directly examine the global translational status of the parasite. This opens the door for novel investigations into how translation is regulated over the course of the malaria lifecycle and how the translational profile changes in response to varying experimental conditions. In addition to obtaining a global translational profile, we can apply this approach to investigate the translational activity of specific mRNAs.

As a proof-of-principle, we first sought to demonstrate that our method could distinguish between mRNAs with different ribosomal loading profiles. We compared the sedimentation profiles of four different parasite mRNAs at 32 hours post-infection.
Transcripts expressing both phosphoethanolamine N-methyltransferase (PEAMT, MAL13P1.214) and knob-associated histidine-rich-protein 3 (KAHRP, PfB0100c) predominantly co-sedimented with heavy polysomes. In contrast, a subset (~10%) of mRNAs encoding ring-infected erythrocyte surface antigen (RESA, PfA0110w) are associated with the 40S small subunit, suggesting that this subset of RESA transcripts initiates translation with decreased efficiency. A significant subset of cyclic AMP-dependent protein kinase, regulatory subunit (PKA-R, PfL1110c) mRNAs co-sedimented with 80S monosome (fraction #15), indicating alternative translational regulation of this subset relative to the rest of the PKA-R transcripts. Translational regulation of PKA-R is of particular interest, because this gene is involved in the transition from the asexual to sexual blood stage (Trager and Gilles, 1989), and translational regulation in the blood stage of *P. falciparum* has previously been demonstrated primarily in gametocytes (Mair et al, 2006). Therefore, polysome profiling can be applied in malaria to detect transcript-specific differences in ribosomal loading.

To correlate ribosomal loading with translational regulation, we used analytical methods developed previously (Arava et al, 2003). The malaria polysome profiles were used to calculate the average number and density of ribosomes loaded onto mRNAs of interest. While the average number of loaded ribosomes correlates with ORF length, ribosomal density is of particular interest because it is largely determined by the rates of the three stages of translation: initiation, elongation, and termination. High ribosomal density indicates efficient initiation and/or inefficient elongation or termination; conversely, low ribosomal density reflects impaired initiation and/or speedy elongation or termination. Thus, changes in ribosomal density for a specific mRNA point to shifts in
translational regulation. Alternatively, extremely high or low ribosomal density can indicate unusual mechanisms of translational regulation. Notably, ribosomal density has been shown in other organisms to decrease as ORF length increases (Arava et al, 2003).

We applied this analysis to our malaria data as follows: the A$_{254}$ polysome trace (Figure 26B) was used to determine the number of ribosomes per mRNA for each fraction. Using the qPCR data to calculate the percentage of a given mRNA in each fraction (Figure 27A), we determined the number of ribosomes loaded per mRNA as a weighted average (Table 4, second column). After collecting ORF lengths from the most recent assembly of the P. falciparum genome (Table 4, third column), we calculated ribosomal density and spacing (Table 4, fourth and fifth columns). Because a eukaryotic ribosome spans ~35 nucleotides (Arava et al, 2003), we were also able to calculate the percentage of the maximal ribosomal packing density occupied by ribosomes on each transcript (Table 4, last column).
Table 4: Ribosomal densities of indicated mRNAs

<table>
<thead>
<tr>
<th>Gene</th>
<th>Number of ribosomes per mRNA (weighted average)b</th>
<th>ORF length (nts)c</th>
<th>Number of ribosomes per 100 ntsd</th>
<th>Ribosome spacing (one ribosome per X nts)e</th>
<th>% Maximal ribosomal packing densityf</th>
</tr>
</thead>
<tbody>
<tr>
<td>PEAMT</td>
<td>5.40</td>
<td>801</td>
<td>0.67</td>
<td>148</td>
<td>23.57</td>
</tr>
<tr>
<td>KAHRP</td>
<td>4.02</td>
<td>2312</td>
<td>0.17</td>
<td>575</td>
<td>6.09</td>
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<tr>
<td>RESA</td>
<td>4.09</td>
<td>3258</td>
<td>0.13</td>
<td>797</td>
<td>4.39</td>
</tr>
<tr>
<td>PKA-R</td>
<td>3.98</td>
<td>1326</td>
<td>0.30</td>
<td>333</td>
<td>10.52</td>
</tr>
<tr>
<td>RESA, 8 h p.i.a</td>
<td>5.14</td>
<td>3258</td>
<td>0.16</td>
<td>634</td>
<td>5.52</td>
</tr>
<tr>
<td>RESA, 32 h p.i.</td>
<td>4.09</td>
<td>3258</td>
<td>0.13</td>
<td>797</td>
<td>4.39</td>
</tr>
</tbody>
</table>

All calculations adapted from (Arava et al, 2003).
a p.i. = post-infection.
b Sum of (# ribosomes per mRNA for Fraction X) × (% of mRNA in Fraction X) for all fractions.
c From the Plasmodium falciparum (3D7) chromosome sequence and annotation (version: 2010-06-01).
d (Number of ribosomes per mRNA)/(ORF length) × 100.
e 100/(Number of ribosomes per 100 nts).
f (~35 nts spanned per ribosome)/(ribosome spacing).

Our initial studies of ribosomal density in *P. falciparum* are consistent with the findings of Arava and colleagues in *Saccharomyces cerevisiae* (Arava et al, 2003). For the four genes examined in Figure 27A, we obtained densities ranging from 0.13 to 0.67 ribosomes per 100 nts (Table 4, fourth column), well within the range of 0.03 to 3.3 per 100 nts reported by Arava et al. Similar to prior findings in yeast, malaria transcripts do not display maximal ribosome loading (Table 1, final column), suggesting that initiation is rate-limiting under these experimental conditions. Finally, within our admittedly limited sample we do note a negative relationship between ORF length and ribosomal density, as seen previously. These findings demonstrate that polysome profiling makes it possible to quantitatively analyze translational regulation in malaria, providing a
powerful approach to track changes in *Plasmodium* translation at the level of individual genes.

We then applied polysome profiling to examine differences in ribosomal loading and density of a specific mRNA over the course of the *P. falciparum* lifecycle. We chose the RESA gene, which shows marked differences in transcriptional regulation across the 48 hour erythrocytic lifecycle (Bozdech et al, 2003). At 8 hours post-infection (Figure 27B) all RESA transcripts were localized in heavy polysome fractions, while at 32 hours post-infection (Figure 27A,B) a subset of RESA transcripts co-localized with the small ribosomal subunit, indicative of a decreased efficiency in translation initiation. The average density of ribosomes on RESA transcripts decreased 20% from the 8 to 32 hour time point (Table 4) reflecting the shift in a subset of mRNAs to the non-translating, small subunit-associated pool. These results demonstrate that polysome profiling is capable of revealing changes in the translation of individual mRNAs throughout the *P. falciparum* lifecycle.

### 3.3 miR-451 RNA fusion impairs ribosomal loading of chimeric mRNA

Having successfully adapted polysome profiling to the study of the erythrocytic cycle of *P. falciparum*, we compared the ribosomal loading of miR-451:*PKA-R* transcripts, compared to total PKA-R. While no ribosomes were detected in uninfected erythrocytes, ribosomes from *P. falciparum*-infected erythrocytes fractionated by velocity sedimentation into ribosomal subunits (40S and 60S), 80S monosomes, and polyribosomes (Figure 28A,B). Each ribosomal fraction was assessed for the presence of
modified miR-451:PKA-R transcripts, compared to total PKA-R mRNA. The majority of total PKA-R transcripts resided in polyribosome fractions, suggesting efficient translation (Figure 5E). However, modified miR-451:PKA-R transcripts predominantly occupied small and large ribosomal subunits fractions as well as the 80S monosome, suggesting that the translation of these modified transcripts was impaired (Figure 28C,D).
Figure 28: **miR-451 modified transcripts exhibit decreased ribosomal loading**

(A) Polysome profile of synchronized parasites at 32 hours post infection.

(B) 18S and 28S *P. falciparum* rRNA distribution in the gradient fractions. The relative migration of the small and large ribosomal subunits and the 80S monosome are indicated by the *, †, and ‡ symbols, respectively.

(C) Normalized levels of total and miR-451-modified *PKA-R* across the HbAA polysome gradient (n=4; mean +/- s.e.m.).

(D) Relative expression of miR-451-*PKA-R* across polysome gradients of HbAA and HbSS cells transfected with or without miR-451 (HbAA) or antisense 2’OMe-miR-451 (HbSS).
(E,F) Levels of total PKA-R (G) and Plasmepsin IV (H) across polysome gradients of HbAA and HbSS cells transfected with or without miR-451 (HbAA) or antisense 2’OMe-miR-451 (HbSS) (n=3 except HbSS-anti miR-451, where n=1).

(G,H) Levels of PKA-R (I) and Plasmepsin IV (J) in fractions 1-17 vs. fractions 18+ for HbAA and HbSS cells under the indicated treatments.

Because HbSS erythrocytes have high levels of miR-451 and show high levels of RNA modification, we wondered if this could result in enhanced translation repression of \textit{P. falciparum PKA-R} in HbSS cells. Thus, we analyzed the presence of miR-451:PKA-R transcripts across various fractions from \textit{P. falciparum}-infected HbSS and HbAA erythrocytes with or without transfected miR-451. Compared to non-transfected HbAA erythrocytes, HbSS and miR-451-transfected HbAA erythrocytes both exhibited enhanced accumulation of miR-451:PKA-R transcripts in the fractions with lower ribosome occupancy (Figure 28D). Importantly, this resulted in a significant shift of total \textit{PKA-R} transcripts from the polysomes to fractions with lower density (Figure 28 E,G), an effect not seen for the miR-451 non-target Plasmepsin IV (Pf14_0075) (Figure 28 F,H), suggesting that miR-451 fusion has dramatic effects on the translation of specific parasite mRNAs. Conversely, HbSS erythrocytes transfected with antisense 2’OMe-451 showed a reciprocal rightward shift of PKA-R transcript relative to HbSS alone (Figure 28 E,G), further indicating that intraparasitic levels of miR-451 are able to modulate the ribosomal occupancy of the associated fusion mRNAs.
3.4 Inhibition of Ribosomal loading results in decreased protein levels of PKA-R

Analysis of PKA-R protein levels by western blot, consistent with the polysome data, compared to non-transfected HbAA cells, showed that *P. falciparum* possessed significantly less PKA-R protein in HbSS (16% of HbAA levels), HbAS (38%) and, HbAA erythrocytes transfected with miR-451 (33%) (Figure 29). Accordingly, blockage of miR-451 increased the level of PKA-R protein in parasite-infected HbSS (4-fold) or HbAS (2-fold) erythrocytes (Figure 29).

![Figure 29: High miR-451 levels result in decreased PKA-R protein levels.](image)

These data clearly demonstrate that host miR-451 has remarkable effects on the parasite’s ability to translate modified parasite mRNAs, while not affecting general protein synthesis (Figure 30).

![Figure 30: Effect of miR-451 on global parasite translation](image)

**Figure 30: Effect of miR-451 on global parasite translation**

Normalized protein synthesis as determined by 35S-methionine incorporation in parasites grown in mock or miR-451-transfected erythrocytes or in the presence of cycloheximide (n=3, mean +/- s.e.m.).
4. Future Studies

4.1. Future Directions- mechanism underlying miR-451 chimeric RNA fusion

The most pressing area of study will be to determine the mechanism by which these miRNA-mRNA chimeric RNAs are formed. Given that *P. falciparum* does not have any putative RNA ligases, the first step will be to identify the protein(s) involved in both the uptake and fusion of these miRNAs. To do this, we initially plan to leverage our previous study using Biotinylated miRNAs in order to capture relevant miRNA-associated proteins. We will transfect Biotin/Desthiobiotinylated miRNAs into the host erythrocyte, infect with *P. falciparum*, UV crosslink the miRNA, then isolate miRNA bound complexes via strepavidin capture. In order to control for interactions which are related to the formation of chimeric miRNA-RNA transcripts, we will use the same experimental setup in Figure 15, in which we overexpress miR-451, miR-181a (as a negative control) and miR-223 (which is taken up, but does not appear to form chimeric RNAs). After analysis of associated proteins by mass spectrometry, comparison between these overexpressed miRNAs will hopefully identify the machinery involved in both miRNA uptake (miR-181a versus miR-451 and miR-223) and, more importantly, cross-species miRNA-mRNA fusion (miR-451 versus miR-223).
4.2. Preliminary evidence for “canonical” miRNA activity within *P. falciparum*

4.2.1. Introduction

This dissertation has focused on a relatively small number of erythrocytic miRNAs during the characterization of miRNA-mRNA fusions, focusing primarily of the activity of miR-451 and let-7i. However, our initial miRNA uptake data suggest that ~100 miRNAs translocate into the parasite. Since RNA-Seq and PlasmoDB EST database only implicated a few miRNAs as fusion candidates, this suggests that the vast majority are unable to covalently modify parasite mRNAs. It is possible that several more of these miRNA form chimeric transcripts, and that we either did not have sufficient transcriptomic coverage or we did not assay the appropriate host cell context in order to identify them. Given the lack of an apparent mechanism for several miRNA, I chose to first screen several miRNAs, both erythrocytic and non-erythrocytic, for their ability to affect the lifecycle of *P. falciparum*.

4.2.2 miR-122 negatively effects parasite growth via a fusion-independent mechanism

Beyond the miRNAs identified in Table 1, we also chose to investigate the anti-parasitic effect of several non-erythrocytic miRNA, since we hypothesized that they may more readily hijack existing *P. falciparum* machinery and thus cause a greater inhibition of parasite growth. In particular, we wanted to investigate whether miR-122, the most abundant miRNA in liver hepatocytes, the cell of origin of the initial invading merozoites which begin the blood stage, affected parasite growth. This is, in no small part, due to the fact that *P. berghei* regulated hepatocellular miRNA content, including miR-122 expression, during the exo-erythrocytic stage of infection (Delic et al, 2011).
Thus, we transfected a series of erythrocytic miRNAs, as well as miR-122 and several other non-erythrocytic miRNA, and determined whether those miRNA affected parasite growth. The overexpression of most erythrocytic miRNA, such as miR-15a, had extremely modest effects upon parasite growth. However, miR-122 led to a dramatic reduction in parasite growth, while transfection of miR-1 (a muscle specific miRNA not naturally found in mature erythrocytes) had no effect, suggesting that the effect of miR-122 may be sequence specific (Figure 31). This dramatic decrease in parasite growth made uncovering the mechanism behind miR-122’s anti-parasitic effect the primary focus of this part of the study.

![Figure 31: miR-451 and miR-122 overexpression within host erythrocytes.](image)

**Figure 31: miR-451 and miR-122 overexpression within host erythrocytes.**

MiR-451 and miR-122 were transfected into mature erythrocytes either unmodified or with a Biotin group covalently linked to either the 3’ or 5’ ends of the miRNA

miR-122 is fairly unique among miRNA in terms of the magnitude of its inhibition of parasite growth when expressed within mature erythrocytes. Given that miR-122 affected malaria growth in the erythrocytic stage even though it is normally present only in the hepatic stages of parasite development, we hypothesized that the introduction of parasite miRNA from the other stages of parasite development could have a similar effect.
upon parasite growth. I therefore overexpressed 5 *Anopheles* miRNAs which were shown to have altered expression levels in response to malaria infection (Winters et al, 2006). In contrast to miR-122, these miRNA did not have any effect upon malaria growth, which further suggests that miR-122 has a unique sequence-specific effect upon parasite growth.

Given the magnitude of the miR-122’s inhibition of parasite growth when expressed in mature erythrocytes, one concern was that miR-122 may be causing systemic changes to the parasite and/or host erythrocyte. However, after overexpression of miR-122, I did not observe any differences in erythrocyte characteristics, parasite morphology, initial invasion rate or developmental timing (data not shown). The only slight change that was observed was a slight shift in ribosomal occupancy, exhibited by an increase in 40S rRNA subunit fraction.

![Figure 32: Global polysome profile of HbAA with or without miR-122.](image-url)
Given that the vast majority of ribosomes were still migrating as expected within denser polysome fractions, we did not believe that global changes in protein translation could explain the growth inhibition of miR-122.

Since miR-122 is generally absent in mature human erythrocytes, we did not expect to discover any miR-122 mRNA fusions within *P. falciparum* under normal host cell conditions. However, given the hepatic stage of lifecycle of *P. falciparum* in human hepatocytes precedes the erythrocytic stage, we speculated that the high level of miR-122 in hepatocytes may modify certain parasite transcripts to form chimeric RNAs within infected hepatocytes. Then, when miR-122 is expressed in mature erythrocytes, the miRNA could potentially hijack the same splicing machinery which forms the miR-451 chimeric transcripts. To test this hypothesis, we grew *P. falciparum* parasites in human mature erythrocytes enriched with miR-122 (enrichment via electroporation), extracted purified parasite RNA and again performed Illumina transcriptome sequencing. However, after identical analysis of these new parasite transcriptome libraries as previously performed for miR-451, we did not observe any fusions of miR-122 to any *P. falciparum* transcripts.

The anti-parasitic activity of miR-451 required a free 3-hydroxyl group, putatively to covalently link to its mRNA fusion targets. If miR-122 does function by a different mechanism than the chimeric RNA fusions observed for miR-451, miR-122’s anti-parasitic activity should by independent of its 5’- or 3’-hydroxyl groups. After overexpression of miR-122 modified with either 5’ or 3’ biotin, we indeed found that the anti-parasitic activities of miR-122 were not affected by substitution of the free hydroxyl
group on either 5’ or 3’ ends, while miR-451 required a free 3’ end for its anti-malarial activity (Figure 33).

Figure 33: miR-451, but not miR-122, requires a free 3’ end for activity.

miR-451 and miR-122 were transfected into mature erythrocytes either unmodified or with a Biotin group covalently linked to either the 3’ or 5’ ends of the miRNA. These data indicate that the anti-parasitic activities of miR-122 were due to a mechanism distinct from miR-451. To tease out how miR-122 may function within *P. falciparum*, we gained some insights from additional studies, performed in collaboration with Joseph Reardon, looking into the effects of miR-15a, an erythrocytic miRNA previously shown to modestly affect parasite growth.

During our previous analysis of the malaria transcriptome looking for canonical conserved miRNA seed matches/target sites, we did not identify any conserved annotated targets for miR-451. However, we did identify one transcript that was a putative target for miR-15a (20nt complementarity with one mismatch), PFE0375c (Proton-Sterol Symporter). In order to determine whether miR-15a had any effect upon PFE0375c, we overexpressed miR-15a and measured transcript abundance of PFE0375c by qPCR. We
found that overexpression of miR-15a, but not its close homologue miR-15b or miR-451, reduced the transcript levels of PFE0375c (Figure 34). This suggested that erythrocyte miRNA can reduce the transcript levels of target mRNAs which possess significant complementarity to the microRNAs.

Figure 34: **miR-15a overexpression downregulates mRNA levels of the target PFE0375c.** A) qPCR relative quantitation of PFE0375c after overexpression of the indicated miRNAs B) mature miRNA sequence of miR-15a/b.

### 4.2.3 Mature human erythrocytes possess Ago2

The effect of miR-15a upon PFE0375c raised more questions than answers in terms of the mechanism of miR-122. In most eukaryotic organisms, a mature miRNA which decreases the abundance of an almost perfectly complementary target mRNA transcript would indicate canonical miRNA or potentially siRNA activity. However, studies of the proteome of *P. falciparum* have not identified any single protein which contains both Paz and piwi protein domains (Hall et al, 2005). Given that the activity of several erythrocytic proteins are required during the erythrocytic stage of the malaria parasite (for example PAK1 and MEK1 (Sicard et al, 2011)), we speculated that human Ago2, if
present within mature host erythrocytes, could be taken up within the parasite and, along with associated human miRNA such as miR-122, target parasite mRNA transcripts in lieu of a *P. falciparum* Ago2. 

I performed an RNA binding protein immunoprecipitation (RIP) (Keene et al, 2006) of Ago2 to determine both whether Ago2 is present and more importantly, whether it is in complex with miRNAs. As shown by western blot in Figure 35A/B, we are able to RIP Ago2 and demonstrate both that human Ago2 is present in both mature erythrocytes and *P. falciparum* and is associated with human miR-451 (Figure 35C).

![Figure 35](image)

Figure 35: **Ago2, in complex with human miR-451, is present within both uninfected RBCs and *P. falciparum***.

(A/B) Western Blots of Ago2 and either B-actin or MSP1 (as negative controls) before and after IP of Ago2 C) Relative quantitation of miR-451, compared to total input, in Ago2 pulldown or total mouse serum (negative control – NC) from both RBCs and *P. falciparum* by qPCR.

However this result comes with a significant caveat. Given that *P. falciparum* is an intracellular parasite during the erythrocytic stage of infection, it is very difficult to separate protein uptake from host erythrocytes from protein contamination simply in a
parasite protein lysate. However, it is interesting that Ago2 is present in complex with miRNA within mature erythrocytes, given the absence of most mRNAs which would be targeted by the RISC complex.

4.2.4 Canonical miRNA activity in *P. falciparum* – Future Directions

Obviously investigating the mechanism underlying the growth defect due to miR-122 still has several holes to fill in order to definitively demonstrate that Human Ago2, via miR-122, can affect *P. falciparum* mRNA transcripts. However, given that miR-122 expression exerts the largest inhibitory effect upon parasite growth, determining its mechanism of action offers the best chance to identify critical therapeutic targets within *P. falciparum*. The two most glaring holes we have identified are 1) The need to clearly demonstrate that human Ago2 does in fact translocate within the *P. falciparum* parasite, and 2) to examine miR-122/Ago2 targeting of *P. falciparum* transcripts in a global, and more unbiased fashion. For the first part, as stated above, we will perform immunofluorescence of Ago2 in infected erythrocytes to determine the localization of Ago2 within the infected cell.

As to the second point, in the past couple of years, several modified techniques for performing immunoprecipitation to isolate associated RNAs, designated PAR-CLiP, based on photo-reactive nucleotides (Hafner et al, 2010), or HITS-CLiP have been developed. Theoretically PAR-CLiP is the ideal technique to answer whether parasite mRNAs are associated with human Ago2, since the host erythrocyte is transcriptionally inactive, meaning that base-pair transitions in Ago2-associated transcripts can only occur in transcripts that are transcribed within the parasite.
However, performing Ago2 PAR-CLiP in *P. falciparum* presents a significant challenge. *P. falciparum* lacks a pyrimidine salvage pathway (Downie et al, 2008), which makes using 4-thiouridine (the more efficient option) impossible in standard lab strains. Therefore, we will have to utilize incorporation of 6-thioguanidine, which has a lower conversion efficiency and appears to cause parasite toxicity. The other possibility is to perform PAR-CLiP in strains where pyrimidine salvage has been genetically introduced, but this could introduce other problems, in particular during transcriptome alignment due to significant genomic differences between parasite strains. In addition, given the input material requirements for PAR-CLiP, we would have to generate a tremendous amount of parasite which is only possible with large-scale malaria culturing techniques using a bioreactor. This problem is compounded in the case of miR-122 transfection, since it so strongly decreases parasite growth.

Thus, we will likely perform RIP-Seq through Ago2 association (Keene et al, 2006) in the presence and absence of miR-122. While RIP-Seq does not produce specific binding site information, a recent study comparing PAR-CLiP to RIP showed that RIP performed as well (or possibly even better) to identify functionally relevant RNA targets (Mukhergee et al, 2011). Therefore, there is a strong rationale to perform RIP-Seq in the pilot experiment.

One additional difficulty with this pilot experiment is that we lack a reliable positive control. Since we are largely interested in whether human Ago2 could be mediating the effect of miR-122 upon parasite growth, we performed miRNA seed match predictions, based upon the assumption of canonical miRNA targeting, within the *P.*
*P. falciparum* transcriptome for miR-122, and observed 10 annotated, and an additional 13 hypothetical proteins, targets with seed matches for miR-122 (Table 5).

**Table 5: Predicted Annotated miR-122 targets (based on a 2-8bp seed match).**

<table>
<thead>
<tr>
<th>PlasmoDb ID</th>
<th>Gene Name</th>
<th>3' UTR seed location (bp from stop codon)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PF08_0034</td>
<td>GCN5 (histone acetyltransferase)</td>
<td>155</td>
</tr>
<tr>
<td>PF0110W</td>
<td>hyp11</td>
<td>478</td>
</tr>
<tr>
<td>PF11_0524</td>
<td>lsm4</td>
<td>496</td>
</tr>
<tr>
<td>PFD685W</td>
<td>pseudouridylate synthase</td>
<td>95</td>
</tr>
<tr>
<td>PFD1150C</td>
<td>RH4 (retic binding protein)</td>
<td>238</td>
</tr>
<tr>
<td>PF10_0399</td>
<td>rifin</td>
<td>275</td>
</tr>
<tr>
<td>PFE0570W</td>
<td>RNA pseudouridylate synthase</td>
<td>202</td>
</tr>
<tr>
<td>PFD0515W</td>
<td>rrp4 (exosome complex subunit)</td>
<td>195</td>
</tr>
<tr>
<td>MAL13P1.337</td>
<td>skp1</td>
<td>286</td>
</tr>
<tr>
<td>PFE1510C</td>
<td>triose phosphate transporter</td>
<td>469</td>
</tr>
</tbody>
</table>

We speculate that these targets will association with human Ago2 only when associated with miR-122, which would strongly suggest a sequence specific association between human Ago2 and *P. falciparum* transcripts.
5. Discussion:

HbS is one of the most common hemoglobin variants in malaria endemic areas, evidently because it provides protection against severe malaria caused by *P. falciparum*. A greater molecular understanding of how the HbS red cells continue to resist *P. falciparum* may provide novel therapeutic targets. Therefore, the intrinsic malaria resistance of sickle cell disease has been an active area of research. The enhanced malaria resistance of HbS red cell has been attributed to several factors, including enhanced phagocytosis of parasite-infected sickle cells (Williams et al, 2005, Ayi et al, 2008) and reduced parasite invasion and growth due to structural changes associated with these cells (Tiffert et al, 2005). Here we show that intrinsic miRNA levels of HbS erythrocytes also play a major role in malaria resistance. Enhanced accumulation of specific miRNAs in both HbAS and HbSS erythrocytes strongly reduced the growth of *P. falciparum*. miR-451 was particularly effective in this respect and exhibited an unusual anti-malarial activity. In particular, we demonstrate that miR-451 covalently integrated into *P. falciparum* mRNAs which are essential for parasite growth and suppressed the translation of one of these transcripts, *PKA-R*.

Although erythrocytes do not contain nuclei, these cells still have abundant levels of miRNAs. Considering the potential of host erythrocyte miRNAs to regulate gene expression of parasites, we identified more than 100 different miRNAs which translocate into *P. falciparum*. Our study and others (Rathgen et al, 2007) show that miR-451 significantly accumulated within the parasite. Notably, during erythropoiesis the expression of miR-451 is highly upregulated, resulting in high levels in mature erythrocytes (Masaki et al, 2008, Nelson et al, 2007). Thus, dysregulated erythropoiesis
and greater red cell hemolysis in sickle cell diseases may lead to altered miRNA composition in HbS erythrocytes (Chen et al, 2008, Sangokoya et al, 2010). Moreover, inhibition of these miRNAs rendered both HbAS and HbSS erythrocytes susceptible to *P. falciparum*, whereas their transfection into HbAA erythrocytes enhanced resistance. Our findings suggest that the translocation of human miRNAs into *P. falciparum* may be a novel defense mechanism in erythrocytes and an important factor of malaria resistance in sickle cell erythrocytes.

Host immune responses have been widely reported to be regulated by small RNAs, including miRNAs (Lodish et al, 2008, Xiao et al, 2009). Two host miRNAs target the histone acetylase PCAF, an important cofactor for viral transactivators, to provide protection against HIV. Host miRNAs have also been shown to directly target sequences in viral RNA genomes to suppress virus replication (Lecellier et al, 2005). In accordance with these findings from mammalian systems, miRNAs were shown to confer resistance to virus infection in plants (Qu et al, 2007), indicating that miRNAs may play a wide evolutionary role in host defenses against viruses. Importantly, our findings now reveal that host miRNAs not only confer protection against viruses, but also against a complex eukaryotic pathogen.

Our data indicate that the anti-malarial activity of erythrocyte miRNAs requires their entry and subsequent modification of parasite mRNAs. To the best of our knowledge, the formation of such cross-species chimeric transcripts has not yet been described. Given the surprising nature of these findings, we verified the existence of these chimeric mRNAs with multiple distinct experimental techniques: *(i)* Northern blotting; *(ii)* RT-PCR with and without biotin-labeled miRNAs; *(iii)* 5’-RACE PCR; *(iv)* Illumina RNA
sequencing; (v) RNase H degradation assays; and (vi) RPA assays. The combinations of these independent lines of evidence strongly indicate the presence of such cross-species chimeric transcripts.

How do erythrocyte miRNAs modify parasite mRNAs? Our data indicate that such modification requires the free 3’ end of miR-451 (Figure 22), suggesting a splicing-based mechanism. Eukaryotes are capable of joining RNAs by cis- and trans-splicing mechanisms. As opposed to cis-splicing in which specific regions of genes, most commonly introns, are removed, trans-splicing joins two distinct RNAs to form a chimeric molecule. In Caenorhabditis elegans, a mature mRNA was recently shown to be generated in a novel way by trans-splicing of two independent pre-mRNAs (Fischer et al, 2008). Similarly, in the eukaryotic parasite Giardia, HSP90 and a dynein motor protein were found to be encoded by intron-containing N- and C-terminal pre-mRNAs that are located in distinct genomic locations (Nageshan et al, 2011, Kamikawa et al, 2011). Trans-splicing of these pre-mRNAs was essential for generation of the mature mRNAs. Although this novel type of trans-splicing is reminiscent of our finding, it requires base-pairing between complementary sequences in the two pre-mRNAs. Because significant sequence overlap between erythrocyte miRNAs and their malarial target genes appears absent in this case, miRNA modification is likely regulated by a different mechanism.

Another type of trans-splicing in eukaryotes, known as splice-leader (SL) trans-splicing, requires far less sequence overlap. In this process short RNAs (45–140nt) are adjoined to a splice-acceptor site in pre-RNAs by the spliceosome (Hastings et al, 2005). It is plausible that erythrocyte miRNAs are integrated into target genes by a similar mechanism. Notably, SL trans-splicing provides a 5’-cap structure for certain transcripts
and may enhance mRNA translation efficiency by providing a hypermodified cap. SL trans-splicing is a nuclear RNA processing reaction in which a small common terminal 5’ exon (the Spliced Leader) derived from an SL RNA, is transferred “in trans” to the most 5’ exon of an independently transcribed pre-mRNA (Nilsen et al, 1993). This phenomenon was initially discovered in kinetoplasts and has also been found to be present in many nematodes, flatworm and a few species in the Phylum chordate (Nilsen et al, 1992). Among the organisms known to use this trans-splicing mechanism for gene regulation, the fractions of genes subject to SL trans-splicing vary greatly. For example, a large majority (70-90%) of nematode (Caenorhabaitis or Ascaris) genes is modified by SL 5’ trans-splicing (Maroney et al, 1995). In contrast, an unknown minority of the genes are modified this way in the flatworms Schistosoma (Davis et al, 1995). Trans-splicing appears to serve a variety of different functions, including processing of the poly-cistronic pre-mRNAs, mRNA capping addition, and polishing the 5’ end of primary transcripts to result in optimal spacing for the translation and addition of in-frame start codons.

Our understanding of the SL trans-splicing process has been enhanced by the complementary approach of in vivo genetics and in vitro biochemistry in nematodes (Nilsen et al, 1993). Interestingly, the SL trans-splicing process uses a mechanism and large RNA-protein machinery that are similar to the cis-splicing of other parasite genes. For example, the nematode SL RNA participates in trans-splicing as a Sm snRNA with its stem-loop structure and conserved binding sequence for Sm proteins. Furthermore, many snRNAs (e.g. U2, U4, U6), known for their role in cis-splicing, are also important for the trans-splicing processes. These observations lead to a working model for the
assembly of a trans-spliceosome in which SL RNA and the pre-mRNA efficiently associate in the presence of sequence complementarity and direction connection (Nilsen et al, 1992). Given the widespread observation of SL trans-splicing within the euglenorozoa, nematodes, flatworms and tunicates, SL trans-splicing is postulated to be an ancient feature in each of these groups (Davis et al, 1995, Nilsen et al, 2001). However, a similar process has not been found in any *Plasmodium* species. Our current proposal is based on our unexpected findings that the translocated erythrocyte miRNAs are covalently linked with the 5’ end of many *Plasmodium* genes in a manner similar to SL trans-splicing. Because miRNA invasion results in an uncapped transcript that experiences impaired ribosomal loading, it is plausible that erythrocyte miRNAs hijack a yet uncharacterized trans-splicing process in *Plasmodium*.

The formation of these chimeric spliced transcripts appears to represent a novel form of translation regulation within the parasite, which is also consistent with other examples of SL trans-splicing. However, here the generation of chimeric transcripts acts as a negative regulator of transcript translation, leading to decreased protein production of miRNA-targeted transcripts. This further highlights the importance of translational regulation within *P. falciparum*, and suggests, along with other studies (Mair et al, 2006), that translational regulation may represent the dominant mode of regulation of asexual vs. sexual stage fate determination.

While the importance of translation regulation becomes better recognized, there is a significant lack of methods to analyze the translational control on the genomic scale. Current studies of translational regulation in *P. falciparum* are currently limited to the assessment of protein levels. While polysome profiling has been utilized for several
decades, previous attempts to isolate polyribosomes from P. falciparum had been unsuccessful. We have adopted an unconventional lysis of infected erythrocytes and parasites followed by a two-step density centrifugation to obtain high resolution of polysome separation. This method will allow for more detailed analysis of translational regulation and unprecedented opportunities for studying how the malaria transcriptome is regulated to produce the malaria proteome. Beyond the study of individual transcripts, this technique can be readily adapted to either DNA microarrays or high-throughput sequencing to allow a global examination of the ribosomal association status of *P. falciparum* during the different stages of intra-erythrocytic life cycles, in order to identify transcripts with significant translational regulation. These results will further our basic understanding of *P. falciparum* biology.

Over the course of this study, we have wondered why miRNA dysregulation would have a negative effect upon parasite growth, given the rapid rate of resistance development observed in *P. falciparum*. Our current speculation is that the low rate of modification in HbAA erythrocytes, the normal erythrocyte cell type for the parasite, is unlikely to exert much pressure on the parasite to adapt to this hijacking of parasite gene regulation. It is even possible that the parasite might utilize such modest levels of post-transcriptional regulation to fine-tune the expression of critical genes (such as *PKA-R*).

However, the aberrant miRNA profile in HbS erythrocytes could short-circuit any normal adaptation to the host-cell environment, and in combination with the other factors of sickle cell resistance, represent too great a challenge for the parasite to adapt to.

Our data further demonstrate that miR-451 integration into *P. falciparum PKA-R* transcripts led to reduced translation of the regulatory PKA subunit. This is expected to
result in increased PKA catalytic activity. Precise regulation of PKA activity is essential during several stages of parasite development, including erythrocyte invasion and survival, sporozoite motility and hepatocyte invasion, and induction of gametocytogenesis (Trager and Gill, 1989, Ono et al, 2008, Li and Cox, 2000). Hence, disruption of this pathway by the host represents an excellent opportunity to disrupt parasite development. Indeed, miR-451-mediated suppression of \( PKA-R \) was associated with increased numbers of gametocytes, indicating that miR-451 has a profound effect on parasite biology.

![Figure 36: Schematic describing the mechanism by which elevated erythrocytic miR-451 leads to induction of gametocytogenesis in P. falciparum. Previous studies are indicated as references.](image)

As seen in the schematic in Figure 36, our data also helps to explain the additional observation that P. falciparum also undergoes increased gametocytogenesis in HbSS host-erythrocytes. Interestingly, this biological effect would result in the parasite devoting resources towards transmission in an attempt to develop within better host cell environments.

The effect of additional miRNAs which can negatively affect parasite growth while not (as far as can be determined) presents further questions. Why would the parasite be sensitive to multiple forms of miRNA regulation? While our incomplete understanding of the mechanisms behind this process render a definite answer unavailable at this time,
one possible clue is based on the host-cells of origin of specific miRNAs. Given that miR-122 is typically considered a liver-specific miRNA, it is possible that parasites utilize a canonical miRNA pathway within hepatocytes to enable parasite growth, and that this process is (similar to when miR-451 is significantly overexpressed) hijacked when miR-122 is suddenly present within mature erythrocytes. This would be consistent with reports of Plasmodium altering levels of hepatocyte miRNAs during infection (Delic et al, 2011).

In summary, our findings clearly demonstrate that enhanced expression of miRNAs provide an indispensable contribution to malarial resistance of both HbAS and HbSS erythrocytes. Furthermore, this data shows a novel miRNA activity may be utilized to defend hosts against complex eukaryotic pathogens while simultaneously allowing the parasite to adapt to altered host cell conditions.
6. Materials and Methods

Ethics Statement

All research involving human participants have been approved by the Duke institutional review board Protocol "Pro00012739: The RNA Expression Patterns in RBC Diseases".

Culture of malaria parasites

*Plasmodium falciparum* (3D7 strain) were maintained in normal human erythrocytes (type B+) in Complete Malaria Culture Media (using Albumax) at 2% hematocrit and 3% O₂/5% CO₂, according to previously published methods (Cranmer et al., 1997). RBCs were washed 3 times in 1X PBS and the white blood cell layer removed each time after centrifugation. Parasitemia was determined by Giemsa stain, and parasite cultures were maintained below 10-15% parasitemia at all times, and media was changed every 24 hours when the parasitemia was above 1% (every 48 hours otherwise). Gametocyte percentage was determined by Giemsa staining 6 days after transfection, and the percentage of gametocytes determined (in triplicate) for 250 parasites and with two independent counts per sample.

RNA extraction from malaria parasites

Parasite infected red blood cells were washed with 1X PBS and lysed in 0.15% Saponin for 30 minutes on ice. The parasite pellet was washed 3 times with 1X PBS and the erythrocyte membrane removed from the parasite pellet by suction to the extent possible. Total parasite RNA, including small RNA >10 nt, was extracted from purified parasites using Ambion’s miRVANA RNA isolation kit according to the manufacturer’s
protocols, using 600ul of lysis buffer per sample. Purity of the parasite RNA preparations was tested via rtPCR for Hb-B and PfUBC13, and RNA integrity was determined by 1% agarose gel (loading 500ng of RNA).

**Methyl-Betacyclodextrin Treatment of Parasites:**

After Saponin lysis as listed above, parasites were resuspended in 5mM MBCD for 30min at room temperature, then washed with 1x PBS twice at 4°C. The RNA from the PVM-depleted parasite was extracted using the miRVANA RNA extraction kit and RT-PCR performed as previously mentioned (for either miR-451 or 18S rRNA), and normalized using 18S rRNA (for infected erythrocytes).

**RNAses A assay to assess RBC contamination**

Uninfected or parasite-infected erythrocytes (at 32 hours post-infection – trophozoite stage) were treated with 0.15% Saponin, incubated on ice for 30 minutes with intermittent vortexing, then washed 3 times with 1X PBS. The pellet was treated with 0.5 µg RNAsse in 200 µl of PBS at room temperature for 20 minutes. The RNAsse was neutralized by washing the parasite pellet 3 times with 1X PBS supplemented with vanadyl ribonucleoside complex (VRCs - New England Biolabs) added to a final concentration of 10 mM. RNA was extracted and RT-PCR performed as previously mentioned (for either miR-451 or 18S rRNA), and normalized using cell number (for uninfected erythrocytes), or 18S rRNA (for infected erythrocytes).

**Multiplex microRNA real-time assays**
Total RNA was extracted from either uninfected erythrocytes or parasites 8 and 32 hours post invasion. The level of the human microRNAs in these samples was determined with microRNA real-time assays on Taqman Low Density Array (TLDA) cards (Applied Biosystems) which can detect 336 human microRNAs configured in a 96-well format and spotted on a microfluidic card. Eight pools of multiplex reverse transcription (required to cover all 336 miRNA and 28 small RNA controls) were performed on RNA collected from each time point. The RT samples were loaded into the TLDA cards and run on an ABI PRISM 7900HT according to the ABI user bulletins (PN4371129 and PN4351684). Due to the lack of validated endogenous controls between infected and uninfected blood, TLDA samples were analyzed by comparing the Ct value of an individual microRNA to the total weighted Ct value for the total microRNA. The experiment was later confirmed using individual miRNA real-time assays for selected miRNAs of interest.

**Individual real-time microRNA assays:**

Confirmatory assays within the parasite were performed using TaqMan microRNA assays, following the manufacturer’s protocols. Input for uninfected erythrocyte assays was normalized by using RNA from an equal number of cells (as counted by complete blood count) as indicated in Figure 1. For parasite RNA samples, Rab GTPase (PF08_0110: Rab GTPase which maintains steady expression across the lifecycle, DeRisi Lab Malarial Transcriptome Database) and 18S rRNA (data is presented normalized against 18S) were used as endogenous controls. The data was recorded using the SDS 2.2.1 software package (ABI) and the results were quantified using the ΔΔCt
method (Abruzzo et al., 2005), where ΔCt represents the threshold cycle (Ct) minus the endogenous control.

Effects of miRNA overexpression/inhibition upon parasite growth

Erythrocyte samples were obtain under the Duke IRB protocol “Pro00012739: The RNA Expression Patterns in RBC Diseases”, from patients and parents at Duke. Human erythrocytes were washed twice with RPMI and resuspended in cold complete cytomix at 50% hematocrit. Four hundred µl of RBCs (approximately 1.5x10⁹ cells) at 50% hematocrit were electroporated with 10 µg of the indicated nucleic acid oligonucleotide using a Gene Pulser II electroporator (Bio-Rad) at a setting of 310V/950 µF. The transfected erythrocytes were resuspended in complete malaria media and plated in 24 well plates. The transfection efficiency was determined using a FITC DNA oligonucleotide conjugated to Fluorescein and flow cytometry (Figure S2) (Barkan et al., 2000). Transfected erythrocytes were infected with synchronized late trophozoites (~15% parasitemia) to an approximate final parasitemia of 0.5%. Freshly transfected erythrocytes were added every 5-6 days (or if parasitemia exceeded 15%) to the infected cultures and percent parasitemia was determined by flow cytometry using YoYo-1 staining in the FL-1 channel of a FACScan. The effects of a particular treatment were determined by examining the level of parasitemia at days 2, 6, 10, and 20.

RBC integrity was determined via two methods, complete blood count (CBCs) and free hemoglobin measurement. CBCs were performed 2 hours post-transfection (immediately before infection) on a Sysmex KX-21N, and mean corpuscular volume (MCV) and Mean cellular hemoglobin concentration (MCHC), normalized against mock-
transfections and presented as relative values. Free hemoglobin concentrations were measured from media (which transfected cells were suspended in) prior to washing, and were measured via drabkins reagent (Sigma-Aldrich). Briefly, 20ul of media combined was with 100ul of drabkins reagent, then the absorbance was determined at 540nm, normalized against mock-transfected, and reported as relative values.

**The microRNA inhibition assays in HbSS erythrocytes**

HbSS and HbAS erythrocytes were obtained at the Duke Comprehensive Sickle Cell Center (CSCC) and Duke pediatric sickle cell clinic following the approval protocol from the Duke Institution Review Board, under the protocol "Pro00012739: The RNA Expression Patterns in RBC Diseases". The HbSS phenotypes are established in the CSCC with Hb electrophoresis. HbSS erythrocytes were electroporated with 2’-O-Me antisense oligonucleotides (all basepairs were modified) targeting the indicated microRNAs (Dharmacon). Due to the difficulty of obtaining patient blood and the short in vitro half-life of HbSS erythrocytes, effects of the microRNA inhibition were determined by measuring the parasitemia at 2 and 6 days after infection (as opposed the 20 day HbAA growth assays). Here, infection rate was calculated as the percentage change in parasitemia per day (D6 Parasitemia – D2 Parasitemia)/4 days), and normalized as a percentage of the HbSS growth rate.

**Hypoxanthine incorporation assay**

Parasite proliferation in the erythrocytes transfected with the indicated ssDNA/microRNAs was assessed by $^3$H-hypoxanthine incorporation assay (Chulay et al.,
microRNA transfected erythrocytes (2% hematocrit) were infected with synchronized trophozoite stage parasites. After 48 hours, the volume of erythrocytes (microRNA transfected, in the case of treated samples) required to attain 0.05% parasitemia in mock transfected cultures (control) was added to all cultures and then plated onto 96-well flat-bottomed microculture plates. At the same time, media containing $^3$H-hypoxanthine (0.5µCi/well) was added to allow incorporation. The total culture volume was maintained at 200 µl during the course of the hypoxanthine incorporation assay. Fifty percent of the media was replaced after 3 days, and after 6 days the parasites were harvested by Saponin lysis. The total radioactivity within the parasite pellet was measured with a scintillation counter.

Database search to identify *P. falciparum* ESTs containing human microRNAs

We downloaded the sequences for known human microRNAs in the miRBase database, (Release 11) from the Sanger Institute; this set included 838 unique mature microRNA sequences. We then downloaded all 77783 *P. falciparum* ESTs from PlasmoDB (version 5.4) and aligned them using BLAT to the *P. falciparum* genome to identify unalignable 5' portions of the ESTs. Unalignable 5' ends, hereafter called "overhangs", were then searched for matches to the microRNA sequences. Matches were ignored unless they occurred at the extreme 3' end of the overhang, with at most a 2 nt difference between the end of the microRNA and the end of the overhang; matches were allowed to overlap the beginning of the alignable portion of the EST by at most 2 nt. In addition to perfect matches detected in this way, we also considered "near matches" in which 1 or 2 residues differed between the microRNA and the portion of the overhang to
which it was aligned. Both matches and near-matches were required to cover the full
length of the mature microRNA sequence (i.e., ~20 nt).

**Solexa Sequencing**

mRNA-Seq samples were prepared using the provided Solexa mRNA-seq library
preparation kit (Illumina), though mRNA isolation (2 rounds) was performed using the
PolyATract kit (Promega) with 20μg of input, and then 100ng of resulting Poly-A+
purified RNA was used to generate the libraries. Solexa sequencing of *P. falciparum*
cDNA library generated 15 million sequence reads 51bp in length. Quantitation of these
Solexa reads was conceptually similar to Mortazavi et al, 2008, and analysis of the reads
was similar to the EST analysis.

**RT-PCR and sequence confirmation of the chimeric RNAs**

The abundance of chimeric 451-PKA-R in the parasite samples was quantified by
SYBR Green amplification using either internal or miR-451 specific primers. miR-451
tagged transcripts were amplified using a miR-451 forward primer and reverse primers
for PKA-R. Total transcript levels were determined using the same reverse primers along
with gene specific forward primers for PKA-R. For the annotated form of PKA-R,
forward and reverse were used which span the miR-451 insertion site. For PEAMT, a
forward primer of and a reverse primer of were used for total transcript level, and the
reverse with the miR-451 primer were used for the miR-451-PEAMT transcript levels.

The RT-PCR reactions were run on an ABI 7900XT, all primers were first verified
on agarose gels and by sequencing for correct PCR products, then tested via real-time to
ensure correct amplification (to use the RQ calculations), all biological replicates were run in triplicate (technical replicates were averaged, and the average for each biological replicate was used to compare across samples), and analyzed using SDS 2.2.1 and relative transcript levels quantified by the ∆∆Ct method.

5' RACE

Identical Poly-A purified total RNA (PolyATract – Promega) as was used in the Solexa sequencing was used for 5' RACE after extraction from asynchronous 3D7 cultures. RACE was performed using the SMARTRace kit (ClonTech), with gene specific reverse primers as listed in table S4 after reverse transcription-mediated 5’ adapter ligation of the SMARTRace oligo. Samples were run on a 1.5% TBE agarose gel, excised and gel extracted, then subcloned (Promega pGEM-T Easy) and Sanger sequenced.

Northern blot analysis

Five µg of total RNA (or 100ng of Poly-A purified RNA – PolyATract – Promega) from indicated human or parasite samples were separated on a 1.2% formaldehyde-reducing agarose gel, with synthetic miRNA (Dharmacon) corresponding to mature miR-451 or miR-181 loaded separately. RNA was transferred overnight to a Hybond-N+ membrane (Amersham/GE Healthcare) and blocked in ExpressHyb (ClonTech) for 30 min at 47°C. Membranes were subsequently incubated with 10 pmol of 32-P end labeled locked nucleic acid (LNA, Exiqon) recognizing miR-451 or miR-181
sequence, then washed membrane twice with 2x SSC/0.1%SDS and twice with 0.1X SSC/0.1%SDS, then developed.

**Ribonuclease Protection Assay**

The Ribonuclease Protection Assay (RPA) was performed using Ambion’s RPA III kit, loading 30μg total RNA on 15% TBE-urea acrylamide gels. Probes were designed via in-vitro transcription (IVT), using directly synthesized DNA templates for 85 bp probes for PKA-R and PEAMT (75nt for Let-7i-REX) (22nt of miRNA, 58nt PKA-R or 53nt PEAMT or 48nt REX, and 5 or 10 non-complementary nucleotides) and 63nt probes for PKA-R and PEAMT (53nt for REX) (58nt PKA-R or 53nt PEAMT or 48nt REX, and 5 or 10 non-complementary nucleotides) (Operon). In vitro transcription was performed using the MaxiScript T7 kit, with 1μg of DNA template and radiolabeled 32P-UTP, and the reaction mix was exactly as described by Ambion. The *in vitro*-transcription reaction was performed at 4°C for 2 hours, treated with DnaseI for 15min, then the correct length product was isolated by gel extraction (15% TBE-urea gel) and eluted overnight at 37°C. The RPAs were performed with 10-20μg of total RNA and 2x10^5 cpm of antisense probe and allowed to hybridize overnight at 42°C. A 1:100 dilution of RNase A/T1 was used, and the digestion was performed for 30min at 37°C, then products were run on a 15% TBE gel and visualized by autoradiography. As an additional control, 5ug of HbAA RNA was first treated with RNaseH (miR-451 or let-7i antisense DNA probe). For the Terminator exonuclease RPA, RNA was treated with terminator exonuclease, incubated at 37°C (30min) prior to an RPA with a directly synthesized probe.
**RNase H Digestion to determine the fusion transcript levels**

2 ug total RNA was combined with RNase H (Invitrogen) with 1 unit of RNaseOUT (Invitrogen) and 10 pmol of DNA oligos (either miR-451-PKA-R/PEAMT or a theoretical 181-PKA-R/PEAMT junction, or for the RPAs an antisense miR-451 or let-7i oligo). Samples were denatured (pre-RNaseH), incubated at 37°C (1 hour) and then 65°C (20 min). The level of gene fusion was determined via qPCR, with gene-specific forward primers overlapping with the junctional digestion probe. Rates of fusion were calculated to be the inverse of the remaining gene level are determined by qPCR (i.e. 70% of PKA-R remaining would indicate a 30% fusion rate), normalized against the gene level of the hypothetical miR-181a junctional digest.

**Biotin Capture Assays**

The RNA oligonucleotides for miR-451 and miR-181 were synthesized with desthiobiotin covalently linked to the 5’ end (Dharmacon). B+ erythrocytes were transfected with 10ug of 5’ desthiobiotin miR-451, -181, or unmodified miR-451, and infected with P. falciparum (3D7). For the measurement of Biotinylated miRNA recovery, cultured were transferred to conical tubes and washed twice with 1x PBS. Infected red blood cells were then layered onto 4ml of 70% percoll, and centrifuged at 2500g for 10min to remove uninfected red cells and ring-stage parasites. Cells were then washed twice with malaria culture media (15ml each wash), then parasites were saponin lysed and RNA extracted as previously reported. For measurements of mRNA enrichment, parasites were also plated into 6-well plates as above after transfection, and then Saponin lysed and RNA extracted (no percoll density separation).

Parasite RNA, resuspended in 500 RNP buffer (20mM KCl, 20mM HEPES, RNaseOut) and incubated with 50 ul washed (3x with RNP buffer) and packed streptavidin beads (GE
Healthcare) at 4°C for 1 hour. After three washes under high stringency conditions (20 mM KCl), 5'-desthiobiotin-miR-451 and miR-181 and their fused P. falciparum RNA targets were eluted with 2 mM biotin overnight at 4°C. The enrichment of the indicated P. falciparum transcripts in the eluted materials was quantified using real-time PCR as stated above.

**Isolation and velocity sedimentation of ribosomes**

More detail on this specific method can be found in Lacsina et al, 2011. Briefly, either uninfected blood or blood with synchronized parasites grown at 2.5% hematocrit and at least 10% parasitemia was pooled and washed in 0.2 mM cycloheximide, then lysed (Stephens and Nicchitta, 2007). Lysates were layered over a 0.5 M sucrose cushion and spun in an SW55 rotor (Beckman) for 146 min at 55,000 rpm. The supernatant and sucrose cushion was pooled and collected; the ribosome-containing pellet was re-suspended in lysis buffer. Linear 15-50% sucrose gradients were generated as previously described. The ribosome suspension was layered over these sucrose gradients and spun in an SW41 rotor (Beckman) for 3 hrs at 35,000 rpm. Fractions of ~330 µl were collected and absorbance at 254 nm (A$_{254}$) was continuously recorded using a Teledyne Isco according to the manufacturer’s instructions.

**35S-methionine translation assay**

Two hundred µl of packed erythrocytes were transfected with 5 µg of miR-451 according to the previously mentioned protocol. Late stage schizonts were added to the transfected erythrocytes. Thirty-six hours post-infection, cultures were washed with methionine-free media and incubated in 5 ml of low-methionine RPMI (10%) supplemented with 0.5 mCi of $^{35}$S-Methionine. Additionally, a positive control for
translational inhibition with 10 µM cycloheximide was also performed. After 6 hours, parasites were isolated (saponin lysis) and lysed in RIPA buffer with 3 freeze-thaw cycles to solubilize protein. The lysates were spun at 14,000 x g and the supernatant was allowed to bind to 150 µl Stratabeads (Stratagene) for 2 minutes at room temperature. The beads were washed 3 times in 1X PBS and protein was eluted with 250 µl Laemmli buffer. Two ml of scintillation fluid were added to 50 µl of eluted proteins, and counted in the scintillation counter.

**microRNA Immunofluorescence:**

RBCs transfected with Biotinylated miR-451 or miR-181 were infected with 3D7 parasites, fixed in 4 % formaldehyde and permeabilized with 0.15 % Saponin (Uninfected RBC control were fixed as above and permeabilized with 0.1 % Triton X-100), then spun-down on poly-lysine coated coverslips. Transfected miRNA were observed using Streptavidin-FITC while the parasite PVM was observed using an EXP1 antibody. The cells were rinsed and mounted in Vectashield containing DAPI.

**Western Blots for PKA-R**

A custom peptide polyclonal antibody for PKA-R was generated by Open Biosystems/ThermoFisher Scientific. Parasites were first enriched by 2 rounds of saponin lysis and subsequently lysed in RIPA buffer for 20min on ice, frozen O/N at -20°C, and then lysate was clarified by centrifugation at 16K g for 20 min at 4°C. 20ug of protein lysate (determined by Bradford assay) was loaded on a 4-20% Tris-HCl ready gel (Bio-Rad), and run at 70 volts for 10 minutes, and 200 volts for ~1 hour. The PKA-R primary
antibody was used at a 1:500 concentration for 2 hours at room temperature, the anti-rabbit secondary 1:5000 (cell signaling). Plasmepsin IV (MR4 reagent # MRA-814A) was used as a loading control.

**Terminator exonuclease digestion**

1ug (for qPCR) or 5ug (for RPA) were mixed with 10X terminator exonuclease buffer and 1 (qPCR) or 5 (RPA) units of terminator exonuclease (Epicenter Biotech) for 60 min at 37°C. Samples were then analyzed via qPCR or RPA (with 27nt synthesized probe) as indicated above.

**Ago2 Immunoprecipitation**

IP reactions were performed as described previously (Keene et al. 2006). Briefly, 5ug of anti-Ago2 antibody (mouse monoclonal, Wako) was incubated O/N with protein G-agarose beads. The beads were washed 4x, then buffer and cell lysate were added and the reactions tumbled for four hours at four degrees. After this incubation, the beads were washed again 5x, then either boiled in 2x Lamelli buffer for IP-Western experiments or had 0.5ml TRIzol added and RNA extracted for RT-PCR experiments. Identical IPs performed with beads pre-coated with pre-immune mouse serum were used as a negative control.

**Statistics**

All error bars shown in Figures are standard errors of the mean (s.e.m.), all p-values are determined by 2-tailed t-test and N-values are listed for biological replicates.
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8. Biography

Greg LaMonte was born on October 29th, 1978 in Tarzana, CA. Greg graduated from University of California at Berkeley with dual Bachelor of Arts degrees in Integrative Biology and Economics. He later received a Master of Science Degree in Biology from the University of San Francisco. He enrolled in the University Program in Genetics and Genomics at Duke University in 2005.

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