

# Translocation of Sickle Cell Erythrocyte MicroRNAs into *Plasmodium falciparum* Inhibits Parasite Translation and Contributes to Malaria Resistance

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## SUMMARY

Erythrocytes carrying a variant hemoglobin allele (HbS), which causes sickle cell disease and resists infection by the malaria parasite *Plasmodium falciparum*. The molecular basis of this resistance, which has long been recognized as multifactorial, remains incompletely understood. Here we show that the dysregulated microRNA (miRNA) composition, of either heterozygous HbAS or homozygous HbSS erythrocytes, contributes to resistance against *P. falciparum*. During the intraerythrocytic life cycle of *P. falciparum*, a subset of erythrocyte miRNAs translocate into the parasite. Two miRNAs, 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 messenger RNAs and, via impaired ribosomal loading, resulted in translational inhibition. Hence, sickle cell erythrocytes exhibit cell-intrinsic resistance to malaria in part through an atypical miRNA activity, which may represent a unique host defense strategy against complex eukaryotic pathogens.

## INTRODUCTION

In malaria-endemic regions, a number of erythrocytic polymorphisms, including sickle cell diseases, occur at relatively high

frequencies (Livincstone, 1971; Nagel and Roth, 1989). Interestingly, these mutations often provide increased protection against the malaria parasite *Plasmodium* (Aidoo et al., 2002). This enhanced resistance is thought to be conferred by multiple distinct factors. Parasitized mutant polymorphic erythrocytes have been found to be subject to enhanced phagocytosis by monocytes (Ayi et al., 2004), suggesting that *P. falciparum* is more rapidly cleared by the immune system. It has also been shown that both progressive dehydration of erythrocytes and increased cellular density are associated with decreased invasion by *P. falciparum* (Tiffert et al., 2005), indicating that structural features of the host cell play a role in infection. 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 cytoadherence ligand PfEMP-1, and leading to decreased cell adhesion, impaired rosetting, and reduced hemagglutination (Fairhurst et al., 2005). Most recently, in HbSC and HbCC erythrocytes, aberrant host actin remodeling by the parasite was shown to affect trafficking of parasite proteins to the erythrocyte surface (Cyrklaff et al., 2011). Finally, *P. falciparum* grows poorly within homozygous sickle (HbSS) erythrocytes (Friedman, 1978; Pasvol et al., 1978), indicating that intrinsic erythrocytic factors may also contribute to overall resistance at the organism and population levels. However, the nature of this intrinsic resistance remains incompletely understood.

Recent studies have implicated microRNAs (miRNAs) in the control of mammalian immune responses. miRNAs are a naturally occurring class of noncoding single-stranded RNA molecules 21–25 nucleotides in length. miRNAs are predominantly derived from primary gene transcripts and are matured by the action of RNase III endonucleases Drosha and Dicer. The mature miRNA guides the RNA-induced silencing complex

to its messenger RNA (mRNA) target where the degree of complementarity determines the posttranscriptional effects, which range from promoting mRNA cleavage and decay to translational repression. Mutation of components of the miRNA machinery or loss of specific miRNAs have been shown to profoundly compromise immune cell development and both innate and adaptive immunity, indicating that miRNA biology plays diverse roles in mammalian immune responses (Lodish et al., 2008; Xiao and Rajewsky, 2009).

Here we set out to investigate whether miRNAs play a role in establishing *P. falciparum* resistance in HbS erythrocytes. Previously, we identified an abundant and diverse population of erythrocytic miRNAs, whose expression was dramatically altered in HbSS erythrocytes (Chen et al., 2008; Sangokoya et al., 2010). As significant material exchange occurs between the host cell and *P. falciparum* during the intraerythrocytic developmental cycle (IDC) (Deutsch et al., 2001), we speculated that the altered miRNA profile within HbS erythrocytes may directly contribute to cell-intrinsic malaria resistance. Indeed, we demonstrate that a unique, noncanonical miRNA activity modulates parasite protein translation and is a major determinant of malaria resistance in HbAS and HbSS erythrocytes.

## RESULTS

### 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 the IDC parasites. Using multiplex real-time PCR assays, we detected the presence of ~100 human miRNAs within parasites (Table S1 available online and Figure 1A). Among the miRNAs found in uninfected normal erythrocytes, three miRNAs (miR-451, miR-223, miR-19b) were significantly enriched in the parasite (Figure 1A). This miR-451 enrichment is the result of increased miRNA translocation, as its level in erythrocytes does not change over the course of 48 hr (Figure S1A).

To verify that intraparasitic miRNAs represented fully intact mature human miRNAs, we performed northern blots for miR-223 and miR-451 (Figure S1B). Indeed, intraparasitic miRNAs were of similar size compared to those in either HbAA erythrocytes or whole blood. Importantly, parasitic miRNAs were not likely to represent a contaminant as the abundant *Hb-β* transcript was not detected in purified parasites (Figure S1C). Moreover, the potential for miRNA contamination from the host erythrocytes (as opposed to parasite miRNA uptake) was further examined by (1) saponin lysis, which disrupts the erythrocyte membrane (Figure S1D), (2) saponin lysis combined with RNaseA treatment of host cell miRNAs (Figures S1E–S1G), or (3) Methyl-Beta-cyclodextrin lysis, which disrupts the parasitophorous vacuolar membrane (Figure S1H). If parasitic miRNAs are the result of erythrocyte contamination, these treatments would significantly reduce miRNA content. However, no such decrease occurred for any of these treatments, further indicating that miRNAs indeed translocate into the parasite. The uptake of miRNA from the host erythrocyte is also consistent with previous studies (Rathjen et al., 2006; Xue et al., 2008).

In order to validate the miRNA uptake profile across the IDC, we extracted RNA at four points (8, 16, 32, and 40 hr after

infection), and examined three miRNAs: miR-451, let-7i, and miR-181a, by real-time PCR (Figure 1B). let-7i was at its maximum level after 16 hr of parasite growth, while miR-451 reached its highest levels after 32 hr. Such differences suggest that the miRNA uptake may be an active, dynamic process.

Previously, we found that miR-451 and miR-223 levels were dramatically elevated in HbSS erythrocytes, as compared to HbAA erythrocytes (Chen et al., 2008). Here we found that HbAS erythrocytes also had higher levels of miR-451 but not as high as HbSS (Figure 1C). Additionally, let-7i levels were also elevated in HbSS and HbAS erythrocytes (Figure S2A).

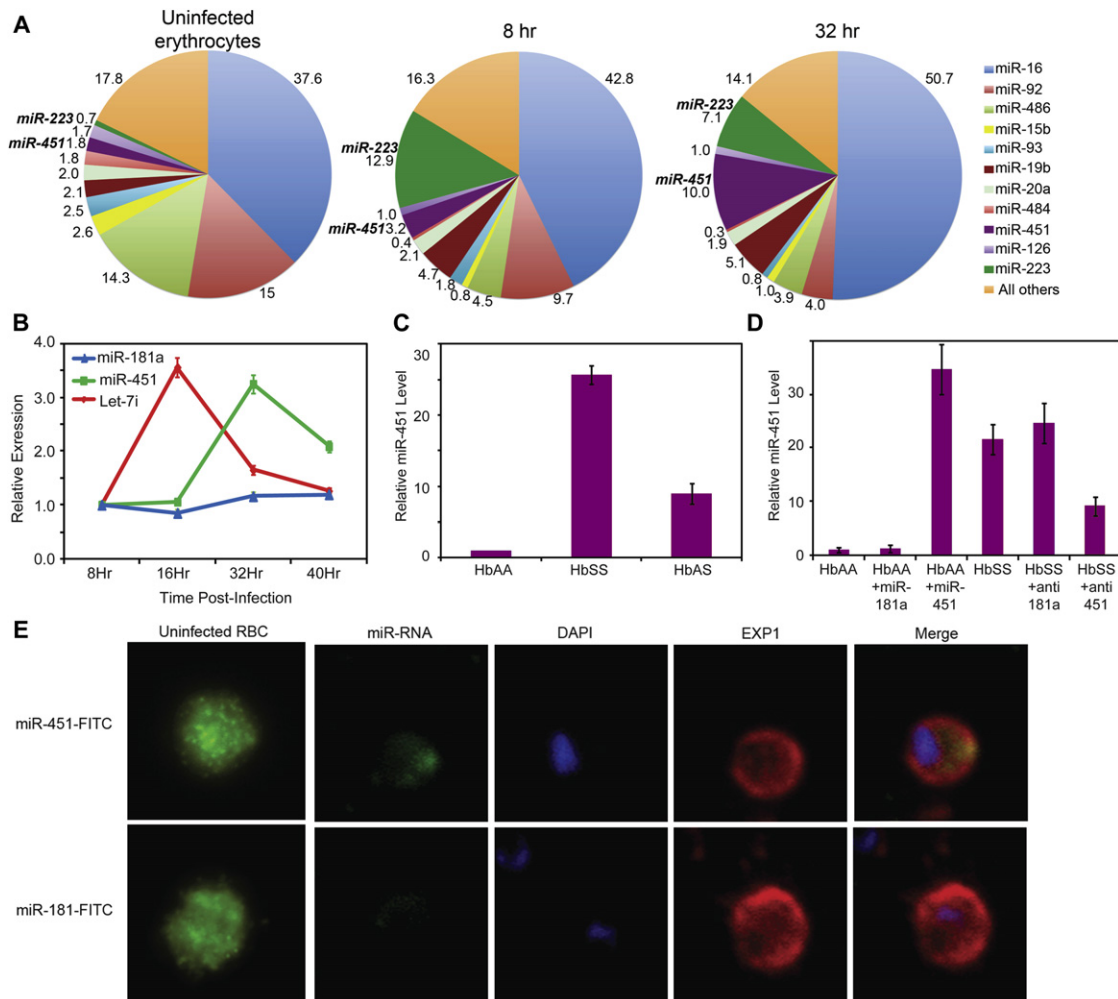
To study the effect of parasitic translocation of various miRNAs, we established a system to transfect miRNAs into host erythrocytes. We achieved ~50% transfection efficiency (Figures S2B and S2C), and the transfected miRNA were stable within the red blood cells (RBCs) (Figure S2D). Transfection of HbAA erythrocytes with miR-451 increased HbAA miR-451 to levels similar to HbSS erythrocytes (Figure 1D). Additionally, HbSS levels of miR-451 were inhibited by antisense 2'-O-methyl-451, but not by antisense 2'-O-methyl-181a (Figure 1D).

Next, we assessed the cellular location of miR-451 in the parasite. Erythrocytes were transfected with biotinylated miR-451 and miR-181a. After saponin lysis of the host erythrocyte, miR-181a was undetectable with FITC-labeled streptavidin, while miR-451 was readily observed (Figure 1E). miR-451 localization was observed within the parasitophorous vacuolar membrane (PVM), labeled by the PVM marker EXP1. Taken together, these data strongly indicate that miR-451 is transported into the parasite.

### Elevated Levels of Specific Human miRNAs Confer Resistance to *P. falciparum*

The presence of higher levels of specific miRNAs in both HbAS and HbSS erythrocytes implies a functional role. To test this possibility, we transfected HbAA erythrocytes with miR-451, miR-223, miR-16, let-7i, and miR-181a and determined their ability to affect parasite growth. Transfection of single-stranded DNA, miR-181a, or the abundant miR-16 had no effect upon parasitemia (Figure 2A). However, transfection with miR-451, miR-223, or let-7i led a markedly reduced parasitemia (Figure 2A) and decreased hypoxanthine incorporation by ~25% (Figure 2B) without significant changes in the viability or characteristics of erythrocytes (Figure S2E) or parasite invasion rate (Figure S2F). These phenotypic changes are accompanied by increased intraparasitic miRNA levels (Figures 2C and S2G). Additionally, combined overexpression of miR-451 and miR-223 was able to reduce infection rate by 46% (Figure 2A), with similar effect seen for additional combination of miR-451, let-7i, and miR-223 (Figure S2H). Moreover, only a mature, single-stranded, sense miR-451 with intact seed sequence was capable of reducing parasitemia (Figures S2I–S2K). Collectively, these results indicate the antiparasitic activities of these miRNAs exhibit strong sequence specificity.

We then asked whether the higher levels of specific miRNAs in either HbSS or HbAS erythrocytes were indeed responsible for the enhanced malaria resistance. Either miR-451 or let-7i in HbAA, HbSS, and HbAS erythrocytes were inhibited via antisense 2'-O-methyl oligonucleotides (Figures 2C and S2L–S2N). Inhibition of miRNAs in HbAA erythrocytes had a marginal effect



**Figure 1. Erythrocyte miRNAs Are Translocated into *P. falciparum***

(A) The 11 most abundant miRNAs found in the IDC of *P. falciparum* and their percentage representation in either uninfected erythrocytes or *P. falciparum* at 8 or 32 hr after infection.

(B) Levels of the three indicated miRNAs shown in parasites at 8, 16, 32, and 40 hr after infection.

(C) Relative levels of erythrocytic miR-451 in untreated HbAA, HbAS, and HbSS erythrocytes (normalized by cell number,  $n = 6$ ; mean  $\pm$  SEM).

(D) Relative levels of erythrocytic miR-451 in untreated HbAA, HbAS, and HbSS erythrocytes and with indicated treatments (normalized by cell number,  $n = 6$ ; mean  $\pm$  SEM).

(E) Detection of miRNA localization in uninfected and infected (saponin-lysed) erythrocytes indicating transfected miRNA (green), the PVM (EXP1, red), and parasite nuclei (DAPI, blue).

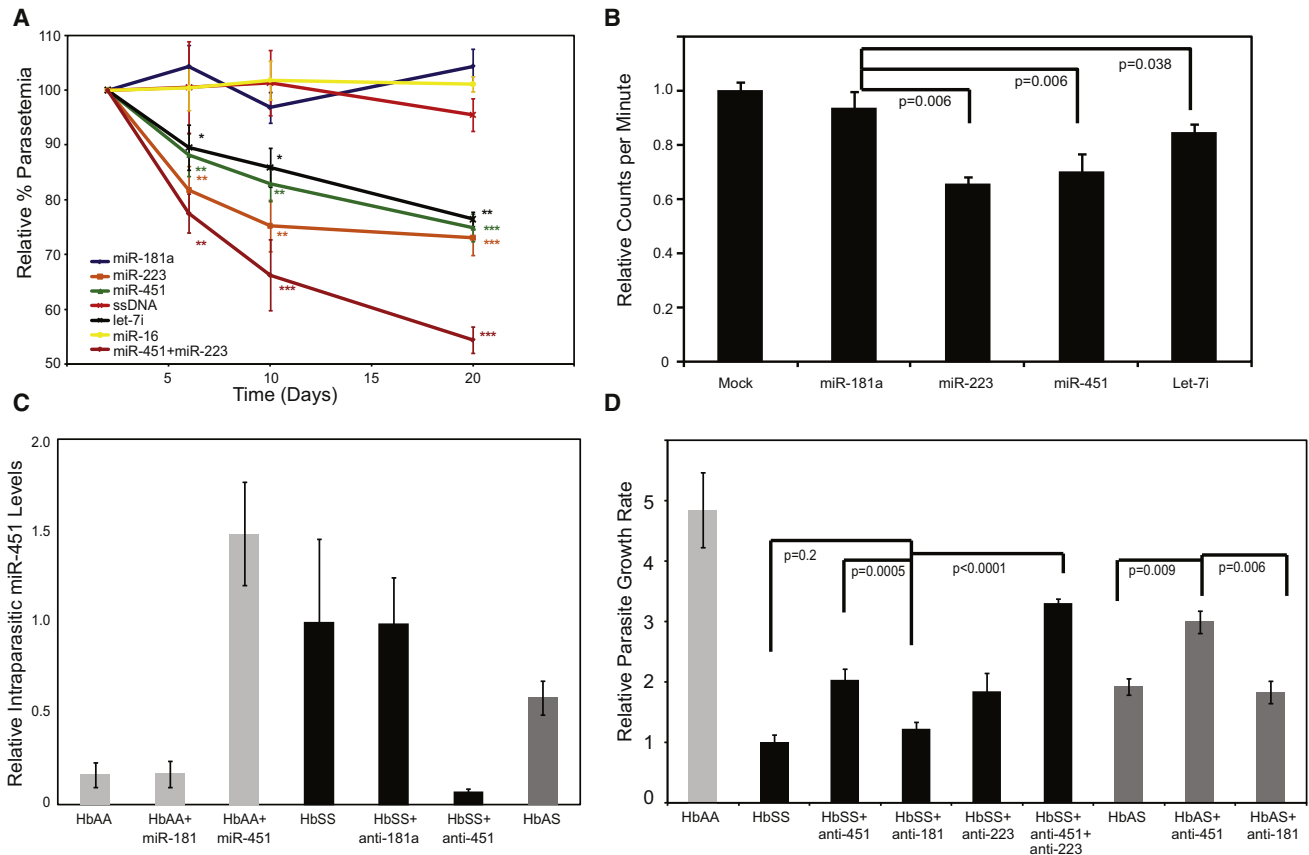
See also Figure S1 and Table S1.

on parasite growth rate (Figure S2L). In contrast, inhibition of miR-451, and to a lesser degree let-7i, led to increased parasite growth in both HbSS and HbAS erythrocytes (Figures 2D and S2M). Additionally, simultaneous inhibition of both miR-451 and miR-223 appeared to have an additive effect that increased HbSS parasite growth to  $\sim 50\%$  of HbAA erythrocytes (Figure 2D). These data indicate that host miRNAs contribute significantly to the malaria resistance of HbAS and HbSS erythrocytes.

#### Human miRNAs Form Chimeric Fusion RNAs with *P. falciparum* mRNA

Mammalian miRNAs typically regulate their target mRNAs through partial base pairing with the 3' untranslated region,

mediated by a ribonucleoprotein complex composed of Dicer/Ago proteins (Bartel, 2004). Since *P. falciparum* lacks orthologs for Dicer/Ago (Baum et al., 2009; Hall et al., 2005), it is unclear how human miRNAs might affect parasites. Although no sequences in the *P. falciparum* genome appear to have significant homology to miR-451, we identified 21 *P. falciparum* expressed sequence tags (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 seven human miRNAs in 35 *P. falciparum* ESTs (and four *P. vivax* ESTs), of



**Figure 2. Elevated miRNA Levels Confer Resistance to *P. falciparum* in HbS Erythrocytes**

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

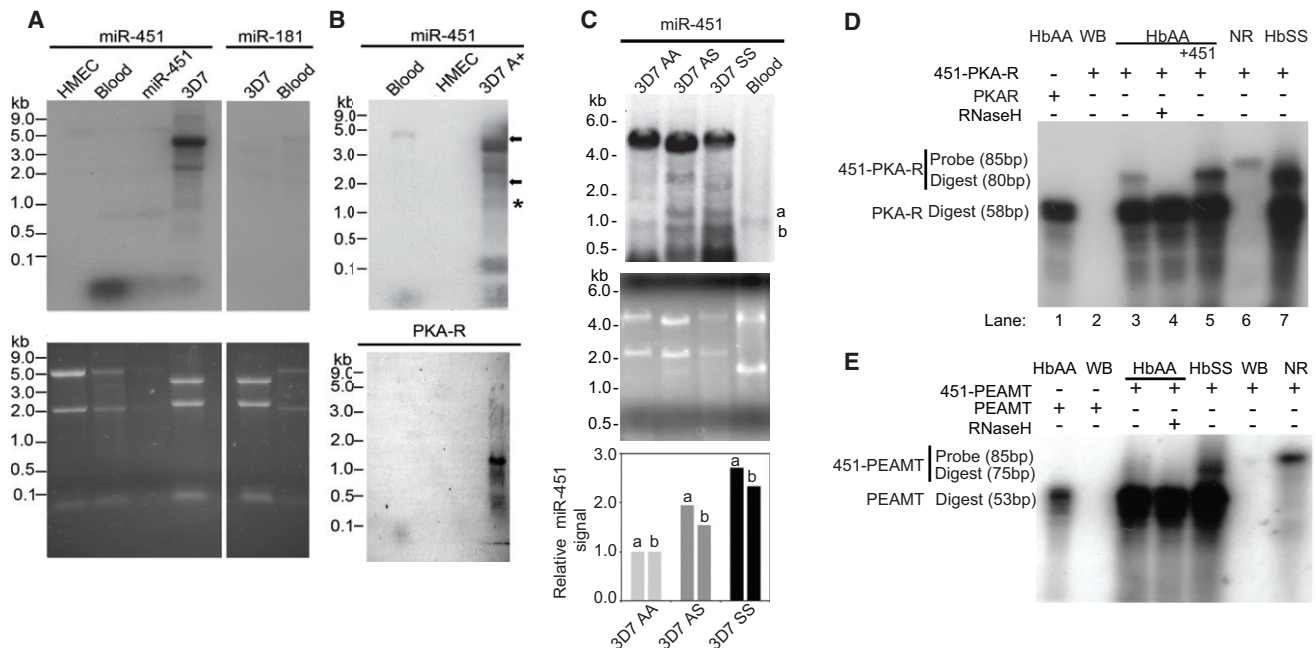
(B) Parasite proliferation measured by [<sup>3</sup>H]-hypoxanthine incorporation in HbAA erythrocytes transfected with the indicated miRNAs (n = 4; mean ± SEM).

(C) Intraparasitic miR-451 levels in parasites grown in untreated HbAA, HbAS, or HbSS erythrocytes, HbAA erythrocytes transfected with the indicated miRNA, and HbSS erythrocytes transfected with antisense 2'-O-methyl oligonucleotides, normalized against the total cell number and HbSS (n = 6; mean ± SEM).

(D) 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 ± SEM). See also Figure S2.

which miR-451 occurred most frequently (Table S2). These unexpected findings suggest that miRNAs may be covalently incorporated into *Plasmodium* mRNAs. To investigate this astounding possibility, we performed northern blots on parasite RNA using locked nucleic acid (LNA) detection probes for miR-451, let-7i, and miR-181. 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 (Figures 3A and S3A). The presence of these larger transcripts is not likely to represent nonspecific hybridization to ribosomal RNA (rRNA), as Poly-A-purified RNA showed a similar pattern of miR-451 hybridization (Figure 3B). Compared to HbAA, parasites grown in HbAS and HbSS erythrocytes showed increased hybridization signals with miR-451 and let-7i probes (Figures 3C and S3A). These results suggest that miR-451 and let-7i sequences indeed exist within parasite transcripts and the degree of incorporation of human miRNA into parasite transcripts correlates with miRNA levels within the host erythrocyte.

To experimentally verify which potential *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* (*PKA-R*, PFL1110c) and *Phosphoethanolamine N-methyltransferase* (*PEAMT*, MAL13P1.214), which were subsequently verified by RT-PCR followed by Sanger sequencing (Table S3 and Figures S3B and S3C). We further tried to circumvent potential artifacts by using direct sequencing techniques, which also allow for the determination of both the identity and abundance of miRNA-451 fusion transcripts. We performed RNA-seq using an Illumina Genome Analyzer for parasites grown in HbAA erythrocytes. From a total of ~15 million reads, we found that 85% of the total reads mapped to the *Plasmodium* genome (90% identity required), based on a BLAT alignment. From these reads, we identified eight overlapping sequences consistent with fusion transcripts formed between human miR-451 and *PKA-R* (Table S3 and Figure S3B). However, we failed to detect



**Figure 3. Human miR-451 Forms Chimeric Fusion RNAs with *P. falciparum* mRNAs**

(A) Northern blot of total RNA from human mammary epithelial cells (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<sup>+</sup> (upper-middle panel) RNA from 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* rRNA 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 18 s RNA levels).

(D) Detection of chimeric miR-451-*PKA-R* transcripts with ribonuclease protection assays (RPAs) with RNA samples from uninfected whole blood (WB) and purified parasites from HbAA and HbSS erythrocytes. RNA was hybridized to a *PKA-R* (PKAR) or miR-451-*PKA-R* (451-*PKA-R*) probe, digested with RNaseAVT1, and run on gel. Additionally, one RNA sample was prehybridized to miR-451 DNA probe, digested with RNaseH, subsequently hybridized with a 451-*PKA-R* probe, and digested with RNaseAVT1 (lane 4). As a control, undigested miR-451-*PKA-R* probe was included (NR). A detailed schematic of this strategy is shown in Figure S3.

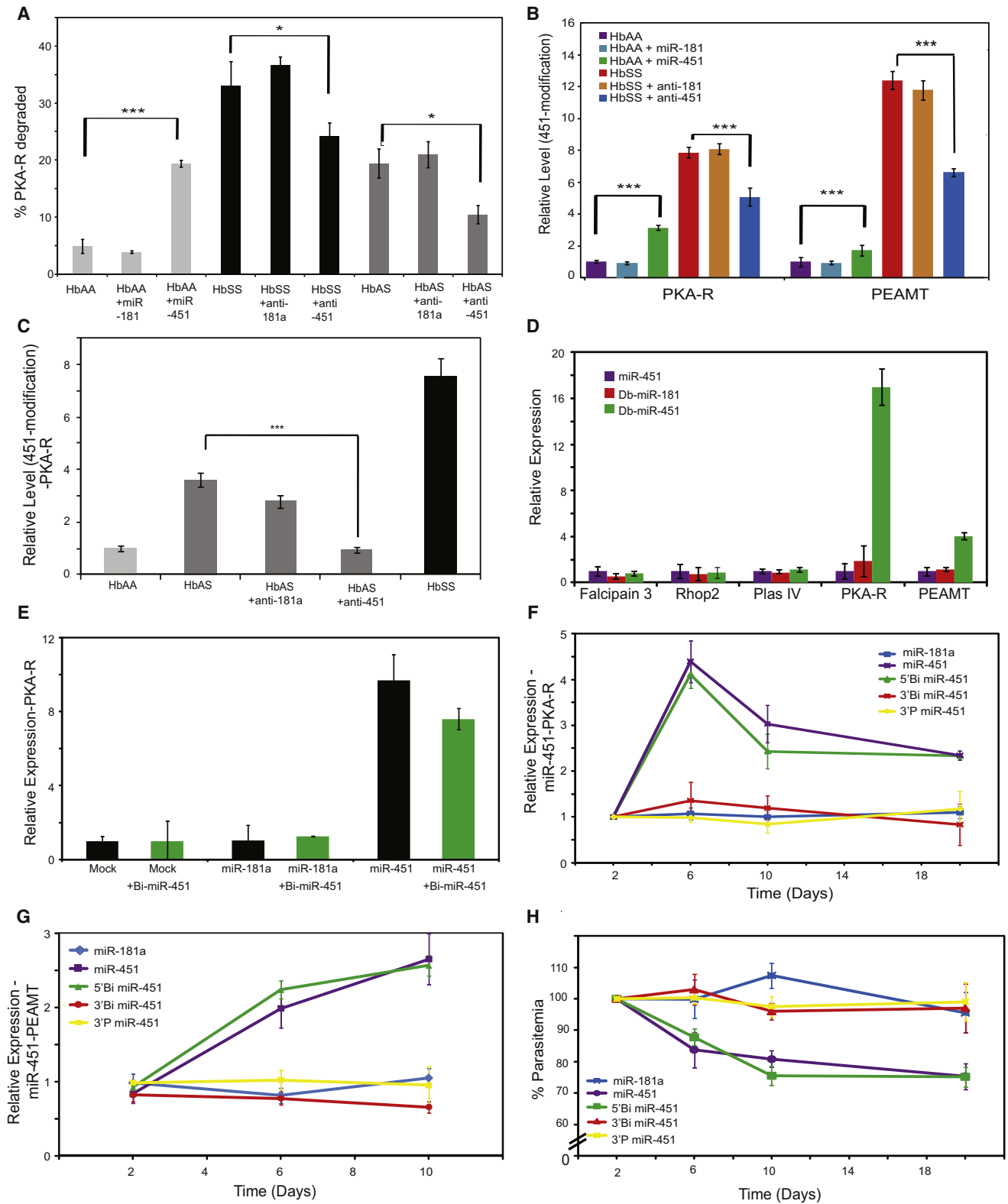
(E) Detection of chimeric miR-451-*PEAMT* with RPA as in (D).

See also Figure S3 and Tables S2 and S3.

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 (Table S3). The shorter form, which lacks exon 1, is likely to be functionally relevant, 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). 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 (Table S3 and Figure S3B). Notably, the junction sequence was 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 (Table S3), 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 complementary DNA 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 63 nt (58 nt *PKA-R* + 5 noncomplementary nucleotides) and a miR-451:*PKA-R* fusion-specific probe of 85 nt (22 nt miR-451 + 58 nt *PKA-R* + 5 noncomplementary nucleotides), both of which contained a noncomplementary overhang to distinguish free probe from hybridized probe-target couples (schematic: Figures S3E–S3G, 3D, and S3H). As expected, the *PKA-R* probe detected an unmodified *PKA-R* transcript fragment ~58 nt in length in *P. falciparum*-infected HbAA erythrocytes (Figure 3B, lane 1). Similarly, the miR-451:*PKA-R* fusion-specific probe



**Figure 4. Cellular Levels of miR-451 Fusion Transcripts**

(A) Level of miR-451-PKA-R transcripts in parasites grown in HbAA, HbAS, and HbSS cells transfected with the indicated miRNA or antisense 2'-O-me miRNA. Level is expressed as the percentage degradation of PKA-R transcripts after hybridization to a miR-451-PKA-R junction probe followed by RNaseH digestion. The remaining PKA-R transcript levels were determined by real-time PCR and normalized against an RNaseH digestion with a hypothetical miR-181-PKA-R probe (n = 4; mean ± SEM).

also detected this 58 nt fragment (protected PKA-R transcript), but additionally detected an 80 nt fragment (protected miR-451:PKA-R fusion transcript). Notably, this 80 nt fragment was more abundant in *P. falciparum*-infected HbSS compared to HbAA erythrocytes (Figure 3D, lane 3 versus lane 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 80 nt fragment (Figure 3D, lane 5, and Figure S3H). Finally, inhibition of miR-451, but not miR-181a, in HbSS erythrocytes led to a decrease in the abundance of the 80 nt fragment (Figure S3H). To further prove that this 80 nt fragment represents a miR-451-PKA-R fusion transcript, we preceded our RPA by incubating *P. falciparum* mRNA from HbAA erythrocytes with a 22 nt antisense miR-451 DNA probe, followed by digestion of the resulting RNA/DNA duplexes with RNase H (schematic: Figure S3G). If miR-451:PKA-R fusion transcripts are present, hybridization to the 22 nt 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 3D (lane 3 versus lane 4) shows that the RNase H treatment abolished the 80 nt fragment (miR-451:PKA-R), but did not affect the 58 nt fragment (unmodified PKA-R). Also of note is that the control PKA-R probe hybridized largely to a 58 nt sequence rather than a 44 nt sequence, suggesting that the unannotated 14 nt 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 3E).

In addition to miR-451, the overexpression of miR-223 and let-7i also reduced parasitemia. While neither our Illumina data 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* (PF11735c, present in two ESTs; Table S2), a gene implicated in Mauer's cleft assembly and red cell remodeling (Hanssen et al., 2008). Given that these ESTs were located a significant distance upstream of the *REX1* coding sequence (~1 kb 5' of the start codon), we confirmed the presence of this fusion transcript by RT-PCR and 5' RACE (Figure S3D and Table S3). 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 (Figures S2A and S2G). RPA results for let-7i-*REX1* showed similar results to miR-451, where higher levels of let-7i led to higher fusion rates (Figure S3I). 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.

### Host Cell miRNA Levels Regulate the Extent of Chimeric RNA Fusion

Having demonstrated the presence of miRNA integrating 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 S4A). In order to confirm the fusion rate of miR-451 to PKA-R, 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. 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 4A), which could be decreased to ~23% by miR-451 inhibition. Overexpression of miR-451 in HbAA erythrocytes increased the incidence of miR-451-PKA-R fusion to ~20%. 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 sole determinants of fusion rates. Far less PEAMT or REX1 mRNA was modified by miR-451 and let-7i, respectively, but modification levels were again higher in HbSS (~5%), compared to HbAA erythrocytes (1%), and were sensitive to inhibition of respective host miRNAs (Figures S4B and S4C). 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 directly assayed the level 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 4B). The fusion rate for HbAA transfected with miR-451 is lower than observed in HbSS erythrocytes, suggesting 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 4B). Similar results were found for miR-451:PKA-R fusion transcripts in HbAS erythrocytes (Figure 4C) and for let-7i:REX1 (Figure S4D). These findings mirror and extend those shown in Figures 3B, 3C, S3H, and S3I, firmly

(B) Levels of miR-451 modified PKA-R and PEAMT as determined by qPCR. Treatments were identical to (A) (n = 7; mean ± SEM).

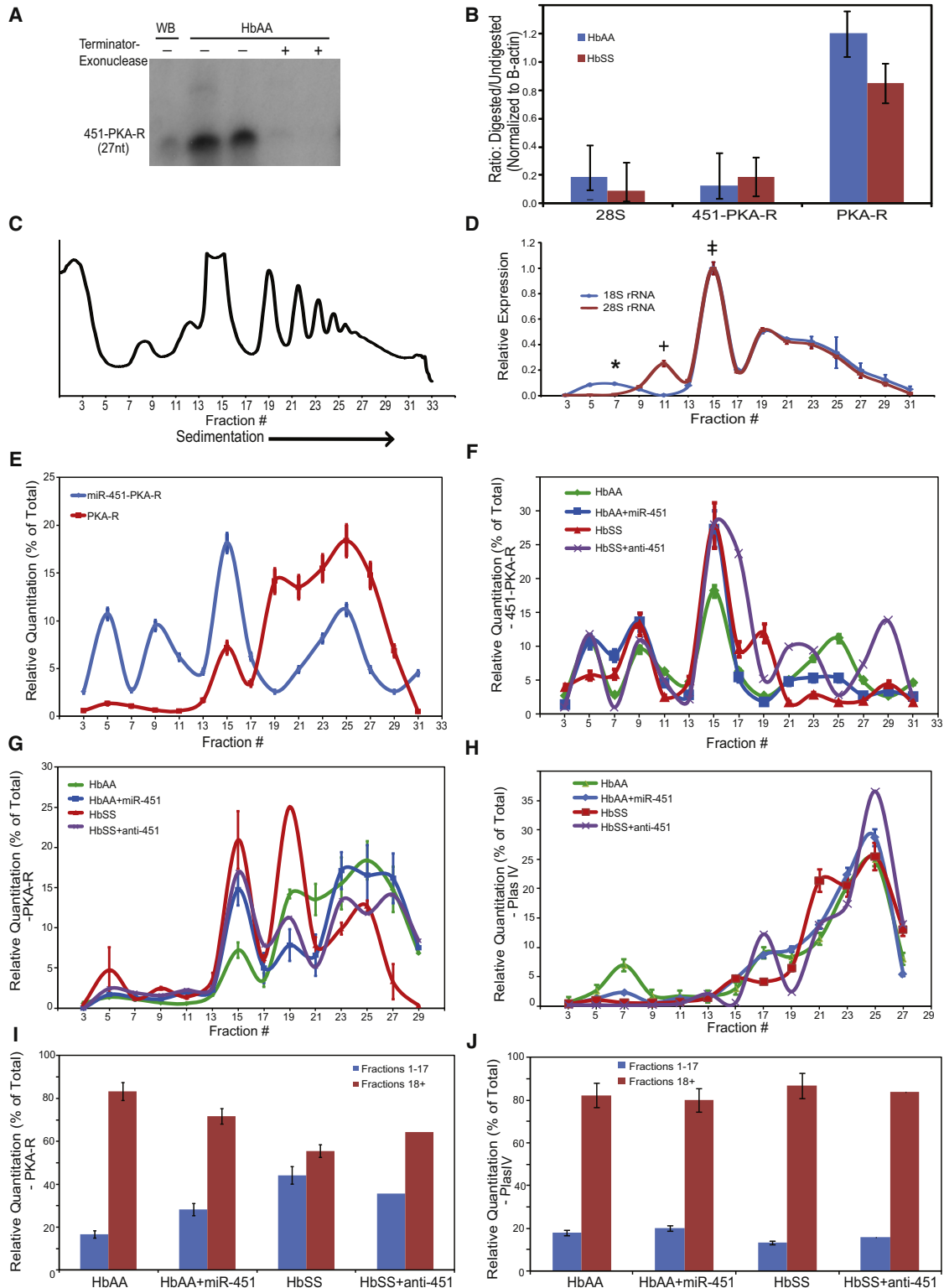
(C) miR-451-PKA-R levels within HbAS erythrocytes as determined in (B). n = 6; (mean ± SEM).

(D) Levels of unmodified transcripts (Plasmeprin 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 versus 18S rRNA, n = 5; mean ± SEM).

(E) Levels of PKA-R enriched by desthiobiotin pull-downs 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 pull-down (normalized versus 18S rRNA, n = 4; mean ± SEM).

(F-H) Levels of miR-451 modified PKA-R (F) and PEAMT (G), as well as parasitemia (H) 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 ± SEM).

See also Figure S4.



**Figure 5. miR-451 Modified Transcripts Are Uncapped and Exhibit Decreased Ribosomal Loading**

(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 27 nt 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  $\beta$ -actin (n = 3; mean  $\pm$  SEM).

(C) Polysome profile of synchronized parasites at 32 hr after infection.



establishing that the host cell level of miRNA dictates the degree of host:parasite transcript fusion.

### The Antimalarial Activity of miR-451 Requires Its Free 3' End

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 4 days, we extracted total RNA, captured Desthiobiotin with streptavidin, and analyzed the presence of miR-451:mRNA fusion transcripts (schematic: Figure S4E). The parasite uptakes ~22% of the biotinylated miRNA in the erythrocyte (Figure S4F) and, RT-PCR analysis indicated that contrary to free miR-451 or 5'Db-miR-181, transfection of 5'Db-miR-451 resulted in enrichment of *PKA-R* and *PEAMT* fusion transcripts (Figure 4D), and transfection of 5'Db-let-7i resulted in enrichment of *REX1* (Figure S4G). This effect was gene-specific, as no enrichment was observed for *Falcipain 3* (Pf11\_0162), *Rhop2* (PF11445w), or *Plasmepsin IV* (Pf14\_0075) (Figure 4D). Notably, 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 4E), reaffirming that these fusion transcripts are exclusively generated in vivo.

Since 5' blocked miR-451 retains its ability to invade parasite mRNAs (Figure 1E, 4D), 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 (or a smaller phosphate group to the 3' end: 3'P-miR-451) to determine the effect of terminal modification on antiplasmodial activity and target mRNA fusion. While 5' biotin-miR-451 and unmodified miR-451 strongly reduced parasite growth while increasing 5' *PKA-R* modification, the 3'biotin-miR-451 and 3'P-miR451 lost both activities (Figures 4F–4H). This indicates that miR-451 requires a free 3' hydroxyl group for integration with the target parasite mRNAs.

### miR-451 Modification Impairs Ribosomal Loading

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 whether 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 (Figures

5A and 5B). In contrast, normally capped *PKA-R* transcripts were relatively stable. These results suggest that miRNA-modified parasite mRNAs are uncapped.

Failure to cap 5' termini can result in mRNA instability. Therefore, we assessed if miR-451 modification rendered *PKA-R* transcripts unstable. *P. falciparum*-infected erythrocytes transfected with miR-451 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 (Figures S5A–S5F), indicating that miRNA modification does not render parasite mRNA unstable.

The 5' cap of mRNAs also plays an important role in assembling ribosomal subunits to promote translation. Thus, we investigated whether miR-451:*PKA-R* transcripts experienced difficulties in ribosomal loading. 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 (Figures 5C and 5D) (Lacsina et al., 2011). 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 the polyribosome fractions, suggesting efficient translation (Figure 5E). However, modified miR-451:*PKA-R* transcripts predominantly occupied fractions that contained the small and large ribosomal subunits as well as the 80S monosome, suggesting that the translation of these transcripts was impaired (Figures 5E and 5F).

Because HbSS erythrocytes have high levels of miR-451 and show high levels of RNA modification, we wondered whether this could result in enhanced translation repression of *P. falciparum* *PKA-R* in HbSS cells. Thus, we analyzed the presence of miR-451:*PKA-R* transcripts across various fractions from *P. falciparum*-infected HbSS and HbAA erythrocytes with or without transfected miR-451. Compared to nontransfected 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 5F). Importantly, this resulted in a significant shift of total *PKA-R* transcripts from the polysomes to fractions with lower density (Figures 5G and 5I), an effect not seen for the miR-451 nontarget *Plasmepsin IV* (Pf14\_0075) (Figures 5H and 5J), 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 (Figures 5G and 5I), further indicating that

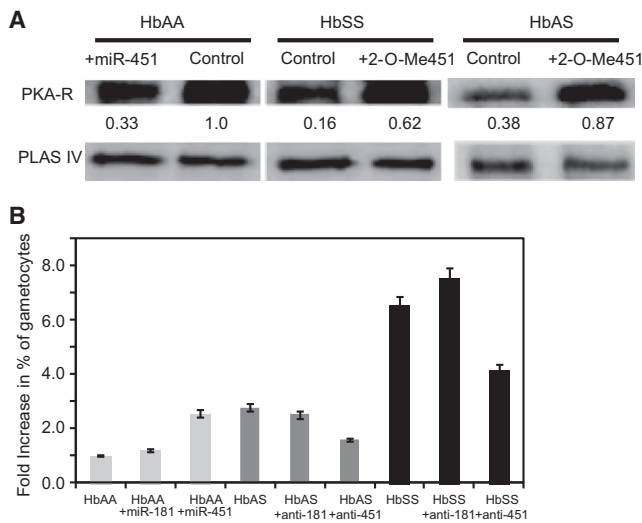
(D) 18S and 28S *P. falciparum* rRNA distribution in the gradient fractions. The relative migration of the small and large ribosomal subunits and the 80S monosome are indicated by the \*, †, and ‡ symbols, respectively.

(E) Normalized levels of total and miR-451-modified *PKA-R* across the HbAA polysome gradient (n = 4; mean ± SEM).

(F) 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).

(G and H) 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; mean ± SEM).

(I and J) Levels of *PKA-R* (I) and *Plasmepsin IV* (J) in fractions 1–17 versus fractions 18+ for HbAA and HbSS cells under the indicated treatments (mean ± SEM). See also Figure S5.



**Figure 6. High miR-451 Levels Result in Decreased PKA-R Protein Levels and Increased Number of Sexual Stage Parasites**

(A) PKA-R protein levels in parasites grown in HbAA, HbAS, and HbSS cells that were control treated or transfected with miR-451 or 2'-OMe-miR-451. Normalized ratio of PKA-R to *Plasmeprin IV* (PLAS IV) is also indicated.

(B) Normalized percentage (against mock-transfected) of sexual stage parasites grown in the indicated erythrocyte types, shown at 6 days after transfection, as determined by Giemsa staining (n = 3; mean ± SEM). See also Figure S6.

intraparasitic levels of miR-451 are able to modulate the ribosomal occupancy of the associated fusion mRNAs.

### miR-451 Overexpression Increases Induction of *P. falciparum* Gametocytes Consistent with Suppression of PKA-R

Consistent with the polysome data, western blot analysis showed that compared to HbAA cells, parasites contained significantly less PKA-R protein in HbSS, (84% reduction), HbAS (62% reduction), and miR-451 transfected HbAA erythrocytes (67% reduction) (Figure 6A). Accordingly, blockage of miR-451 resulted in a 4-fold and 2-fold increase in PKA-R protein levels in parasite-infected HbSS and HbAS erythrocytes respectively (Figure 6A). 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 S6).

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 and Cox, 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). 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 that entered sexual stages (Figure 6B). Such an induction was not observed in mock- or miR-181-transfected erythrocytes. We also detected increased gametocyte-

nia in both HbAS and HbSS erythrocytes, consistent with their higher miR-451 levels and a previous report (Trager et al., 1999). This induction of gametocytogenesis was sensitive to inhibition of miR-451, but not miR-181a. Taken together, our data show that miR-451-induced translational inhibition of PKA-R has profound effects on parasite biology and survival.

### DISCUSSION

HbS is one of the most common hemoglobin variants in malaria endemic areas, largely because it provides protection against severe malaria caused by *P. falciparum*. This enhanced resistance has been attributed to several factors, including enhanced phagocytosis of parasite-infected sickle cells (Ayi et al., 2004; Williams et al., 2005) 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 significant 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 antimalarial activity. In particular, we demonstrate that miR-451 covalently integrated into *P. falciparum* mRNAs that are essential for parasite growth and suppressed the translation of one of these transcripts, PKA-R.

Although erythrocytes do not contain nuclei, these cells have abundant levels of miRNAs. Considering the potential of miRNAs to regulate gene expression, we identified more than 100 different miRNAs that translocate into *P. falciparum*. Our study and others (Rathjen et al., 2006) 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., 2007; 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 more susceptible to *P. falciparum*, whereas their transfection into HbAA erythrocytes enhanced cellular resistance. Our findings reveal that the translocation of human miRNAs into *P. falciparum* is a 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 and Rajewsky, 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 (Niu et al., 2006; Qu et al., 2007; Simón-Mateo and García, 2006), 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 antimalarial 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 techniques: (1) northern blotting, (2) RT-PCR with and without biotin-labeled miRNAs, (3) 5' RACE PCR, (4) Illumina RNA sequencing, (5) RNase H degradation assays, and (6) RPA assays. How do erythrocyte miRNAs modify parasite mRNAs? While we have limited understanding of the process, our data indicate that such modification requires the free 3' end of miR-451 and suggest a potential splicing-based mechanism. Eukaryotes are capable of joining RNAs by *cis*- and *trans*-splicing mechanisms. As opposed to *cis*-splicing in which gene introns are removed, *trans*-splicing joins two distinct RNAs to form a chimeric molecule. In *C. elegans*, a mature mRNA was shown to be generated by *trans*-splicing of two independent pre-mRNAs (Fischer et al., 2008). Another type of *trans*-splicing in eukaryotes, known as splice-leader (SL) *trans*-splicing, occurs where short RNAs (45–140 nt) are adjoined to a splice-acceptor site in pre-RNAs by the spliceosome (Hastings, 2005). It is plausible that erythrocyte miRNAs are integrated into parasite target genes by hijacking a similar but uncharacterized RNA-editing process in *Plasmodium*.

The low rate of modification in HbAA erythrocytes, the normal host cell type for parasites, 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 posttranscriptional regulation to fine-tune the expression of critical genes (such as *PKA-R*). However, the aberrant miRNA profile in HbS erythrocytes could potentially 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 (Li and Cox, 2000, Trager and Gill, 1989; Ono et al., 2008). 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. Interestingly, this biological effect would result in the parasite devoting resources toward transmission in an attempt to develop within better host cell environments. In summary, our findings clearly demonstrate that enhanced expression of miRNAs contribute to malarial resistance of both HbAS and HbSS erythrocytes and shows that a unique miRNA activity may be utilized to defend host cells against complex eukaryotic pathogens.

## EXPERIMENTAL PROCEDURES

Sequences for primers, probes are located in Table S4, and unless otherwise stated, all kits were performed using manufacturer's protocols. Additional detail for methods is present in the Supplemental Experimental Procedures.

### Culture of Malaria Parasites

*Plasmodium falciparum* (3D7) were maintained in normal human RBCs (type B+) at 2% hematocrit in Complete Malaria Media (with Albumax) and 3% O<sub>2</sub>/5% CO<sub>2</sub>, according to previously published methods (Cranmer et al., 1997). RBCs were washed three times in 1× PBS and the white blood cells removed after centrifugation.

### RNA Extraction From Malaria Parasites

Parasite infected RBCs were washed twice with 1× PBS and lysed in 0.15% Saponin for 30 min on ice. The parasite pellet was washed thrice with 1× PBS and the erythrocyte membrane removed by suction. Total parasite RNA was extracted with Ambion's miRVANA RNA isolation kit.

### Effects of miRNA Overexpression/Inhibition upon Parasite Growth

Erythrocytes were washed twice with RPMI and resuspended in complete cytomix at 50% hematocrit. Approximately 1.5 × 10<sup>9</sup> cells at 50% hematocrit were electroporated with 10 μg oligonucleotides at a setting of 310V/950 μF. The RBCs were resuspended in complete malaria media and plated in 24-well plates. Transfected RBCs were infected after 4 hr with synchronized late trophozoites to an approximate final parasitemia of 1%. Freshly transfected cells were added every 4–6 days to infected cultures, and percent parasitemia was determined by FACS with YoYo-1 staining.

### Quantitation of miRNA and mRNA Transcript Levels

Global miRNA profiling was performed with total RNA, from either uninfected erythrocytes or parasites 8 and 32 hr after infection, with TaqMan Tiled Low-density arrays (TLDA-ABI) profiling 336 miRNA and 28 endogenous controls (on ABI 7900HT). Samples were normalized by comparing the Ct value of an individual miRNA to the total weighted Ct value for all miRNAs.

Individual miRNA expression assays were performed with TaqMan miRNA assays, with input normalized either by equal cell number (uninfected RBCs) or Rab GTPase (PF08\_0110) and 18S rRNA (parasite RNA). Individual mRNA assays were performed with SYBR Green (ABI), and for miR-451 fusions, a common reverse primer and miR-451 or internal forward primers were used. Individual assays were quantified with the ΔΔCt method (Abruzzo et al., 2005). mRNA half-life was determined using Actinomycin D similar to a previous study (Shock et al., 2007).

### RNase H Digestion to Determine the Fusion Transcript Levels

Total RNA (2 μg) was incubated with RNase H (Invitrogen) with 1 unit RNase-OUT (Invitrogen) and 10 pmol DNA oligos (either miR-451-PKA-R/PEAMT or a theoretical 181-PKA-R/PEAMT junction). Samples were denatured (pre-RNaseH) and incubated at 37°C (1 hr). The level of gene fusion was determined via qPCR as above.

### Ribonuclease Protection Assay

Probes were designed for in-vitro transcription (IVT) via direct DNA synthesis of ds template (Operon), using 85 bp probes for PKA-R and PEAMT (75 nt for Let-7i-REX) (22 nt of miRNA, 58 nt PKA-R or 53 nt PEAMT or 48 nt REX, and 5 or 10 noncomplementary nucleotides) and 63 nt probes for PKA-R and PEAMT (53 nt for REX) (58 nt PKA-R or 53 nt PEAMT or 68 nt REX, and 5 or 10 noncomplementary nucleotides) then transcribed with the MAXIscript T7 kit (Ambion) and <sup>32</sup>P UTP. After IVT, probes were run on a 15% TBE-urea gel, gel extracted, and eluted overnight. RPA was performed with Ambion's RPA III kit, loading 20 μg total RNA on 15% TBE-urea acrylamide gels. As an additional control, 20 μg HbAA RNA was first treated with RNaseH (antisense miR-451 or let-7i junction DNA probe). For the Terminator Exonuclease RPA, RNA was treated with terminator exonuclease, incubated at 37°C (30 min) prior to an RPA with a directly synthesized probe.

### Northern Blot Analysis

Total RNA (5 μg) (or 100 ng Poly-A purified RNA; PolyAtract, Promega) from the indicated human or parasite samples was separated on a 1.2% formaldehyde-reducing agarose gel. RNA was transferred overnight and blocked in ExpressHyb (ClonTech) for 30 min at 47°C. Membranes were subsequently incubated with 10 pmol <sup>32</sup>P end-labeled LNA (Exiqon) recognizing miR-451, let-7i, or miR-181 sequence, then washed and developed.

**In Vivo Capture Assay**

As described in Figure S4E, RNA oligonucleotides for miR-451 and miR-181 were synthesized with desthiobiotin covalently linked to the 5' end (Dharmacon). RBCs were transfected with Db-451, Db-181, or unmodified miR-451 and infected with *P. falciparum*. Parasite RNA was extracted after 4 days and incubated with streptavidin beads (GE Healthcare) (4°C, 1 hr). After washing with 20mM KCl, RNAs were eluted with 2 mM biotin (4°C, overnight). Enrichment of indicated *P. falciparum* transcripts was quantified with qRT-PCR.

**Determination of Translational Regulation of Fusion Transcripts**

Parasite total RNA collected and analyzed as previously published (Lacsina et al., 2011). Polysome results were confirmed via western blots for PKA-R. Global translation rates were determined via <sup>35</sup>S-methionine translation assay.

**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."

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes Supplemental Experimental Procedures, six figures, and four tables and can be found with this article online at <http://dx.doi.org/10.1016/j.chom.2012.06.007>.

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