Transcriptomics and proteomics reveal two waves of translational repression during the maturation of malaria parasite sporozoites

Scott E. Lindner1,7,8*, Kristian E. Swearingen2,7, Melanie J. Shears3,6, Michael P. Walker1, Erin N. Vrana1, Kevin J. Hart1, Allen M. Minns1, Photini Sinnis3, Robert L. Moritz2 & Stefan H.I. Kappe4,5,8*

Plasmodium sporozoites are transmitted from infected mosquitoes to mammals, and must navigate the host skin and vasculature to infect the liver. This journey requires distinct proteomes. Here, we report the dynamic transcriptomes and proteomes of both oocyst sporozoites and salivary gland sporozoites in both rodent-infectious Plasmodium yoelii parasites and human-infectious Plasmodium falciparum parasites. The data robustly define mRNAs and proteins that are upregulated in oocyst sporozoites (UOS) or upregulated in infectious sporozoites (UIS) within the salivary glands, including many that are essential for sporozoite functions in the vector and host. Moreover, we find that malaria parasites use two overlapping, extensive, and independent programs of translational repression across sporozoite maturation to temporally regulate protein expression. Together with gene-specific validation experiments, these data indicate that two waves of translational repression are implemented and relieved at different times during sporozoite maturation, migration and infection, thus promoting their successful development and vector-to-host transition.
Malaria remains one of the great global health problems today, taking a large toll on people in the tropics and subtropics. This disease, caused by *Plasmodium* parasites, affects over 200 million people annually and kills over 400,000 (WHO World Malaria Report 2018). While a protein-based subunit vaccine (RTS,S) has recently been licensed and is being used for pilot implementation in three Sub-Saharan African countries, its protection has been limited and relatively short-lived in clinical trials. Developing an effective and long-lasting malaria vaccine that prevents infection remains a chief goal that has yet to be achieved. Accomplishing this goal will require greater knowledge of the basic biology and transmission dynamics of the gametocyte stages as well as pre-erythrocytic sporozoite stages and liver stage parasites. Promising whole-parasite vaccine candidates, based on the sporozoite form of the parasite, are on the horizon and might get closer to realizing a protective vaccine.

*Plasmodium* parasites are transmitted between mammalian hosts by female *Anopheles* mosquitoes (reviewed in ref. 3). Following uptake of male and female gametocytes by the mosquito during a blood meal from an infected host, these parasites activate into gametes in the midgut and fertilize by fusion to form a zygote, which then develops into a motile ookinete. This stage burrows through the midgut wall and establishes an oocyst under the basal lamina. Within each oocyst, the parasite undergoes sporogyony to produce up to five thousand oocyst sporozoites, which are released and selectively infect the salivary glands. Oocyst sporozoites are weakly infectious if injected directly into a naive mammalian host, but become highly infectious following proteolytic rupture of the oocyst wall and their transit through the mosquito hemocoe. Sporozoites further gain infectivity after invasion of the salivary glands. Interestingly, salivary gland sporozoites lose infectivity for the salivary glands, which was demonstrated by experimentally injecting them into the hemocoe of uninfected mosquitoes. Within the glands, sporozoites await transmission as long-lived, poised salivary gland sporozoites, which occurs when the mosquito takes its next blood meal and injects these sporozoites into the skin. Sporozoites then exit the bite site in the skin, locate and enter the vasculature, and passively travel to the liver. Here, they infect hepatocytes and thus initiate the life cycle progression in the mammalian host. Relatively few sporozoites are injected during a mosquito bite and form a liver stage parasites. Thus, this transmission bottleneck has been the focus of intervention efforts using drugs, subunit vaccines, and attenuated whole-parasite vaccines.

Fundamental studies of sporozoite biology have informed efforts to inhibit and/or arrest the parasite during pre-erythrocytic infection. For example, in rodent malaria parasites some transcripts are upregulated in infective (salivary gland) sporozoites (UIS genes), a phenomenon that was originally determined for 23 currently annotated genes by subtractive cDNA hybridization. With the advent of microarray-based transcriptomics, a renewed effort to identify both UIS and upregulated in oocyst sporozoites (UOS) genes identified 124 UIS and 47 UOS genes. Interestingly, only 7 of the original 23 UIS genes were confirmed in this expanded study. However, these UIS genes (UIS1, UIS2, UIS3, UIS4, UIS7, UIS16, and UIS28) have proven to encode some of the most important proteins for the transmission and transformation of the sporozoite into a liver stage parasite, as well as for liver stage development. Gene deletions of some UIS genes have been exploited to generate genetically attenuated parasite strains that arrest during liver stage development.

In addition to transcriptional control, the malaria parasite also imposes translational repression upon specific mRNAs in female gametocytes, and this mechanism has been observed for at least a few mRNAs in salivary gland sporozoites (reviewed in refs. 16,17). Translational repression allows for the proactive production of mRNAs and restriction of their translation before transmission, and yet enables just-in-time production of these proteins after transmission when they are needed. However, this strategy (high transcription and low/no translation) is energetically costly, and model eukaryotes and human cells have evolved to avoid this gene regulatory combination (the depleted region of Crick Space) except in specific, beneficial situations. In light of this, it is notable that *Plasmodium* parasites have evolved to use translational repression for transmission to the mosquito, which has been clearly observed for mRNAs (e.g., p28) in female gametocytes. The mechanisms underlying this have been established most thoroughly in the rodent malaria parasite *Plasmodium berghei*, where DOZI (a DEAD-box RNA helicase orthologous to human DDX6) and CITH (an Lsm14 orthologue) bind, stabilize, and translationally repress specific mRNAs in female gametocytes. Recently, the extent of translational repression in *Plasmodium falciparum* female gametocytes was assessed by mass spectrometry-based proteomics and RNA-seq. In this stage, the parasite expresses over 500 transcripts with no evidence for their encoded proteins, with over half of these maternal gene products being uncharacterized. Enriched in this set of translationally repressed mRNAs are those that encode for functions needed post-transmission and include gene families previously shown to be translationally repressed. These data support the model that female gametocytes, despite high-energetic costs, prepare and await transmission by storing and protecting specific mRNAs needed to establish the infection of the mosquito. However, the extent and precise mechanisms of how translational repression is imposed in sporozoites has not been established beyond targeted studies, which suggest that the PUF2 RBP may act upon *cis* elements found within the coding sequence of the *uis4* mRNA.

Systems analysis of translational repression in sporozoites requires knowledge of their global transcriptomes and proteomes, but such analyses were greatly restricted due to substantial contamination with material from the mosquito vector and its microbiome in sporozoite samples. To address this, we have developed a scalable, discontinuous density gradient purification approach for sporozoites that greatly reduces contamination from the mosquito and its microbes. The resulting fully infectious sporozoites have allowed extensive ChIP, transcriptomic (RNA-seq), and proteomic (nano liquid chromatography–mass spectrometry/MS (nanolC–MS/MS)) analyses of sporozoites. These studies demonstrate that “omics” level analyses of sporozoites are now experimentally practical, and thus reopen long standing questions of mechanisms underlying critical sporozoite functions.

Here, we have addressed one of the foremost questions of sporozoite biology: how and when does the sporozoite prepare molecularly for transmission from the mosquito vector to the mammalian host? Using RNA-seq-based transcriptomics and nanolC–MS/MS-based proteomics, we here characterize both oocyst sporozoites and salivary gland sporozoites of both rodent-infectious (*Plasmodium yoelii*) and human-infectious (*P. falciparum*) species. Together, these data provide a comprehensive assessment of mRNA and protein abundances, provide evidence for extensive post-transcriptional regulation of the most abundant mRNAs, and demonstrate that two distinct and likely orthogonal translational repression programs are active during sporozoite maturation.

## Results

### Dynamic transcriptional regulation in maturing sporozoites

Important insights into how sporozoites mature and become
infectious were gained from studies of the upregulated in oocyst sporozoites (UOS) and upregulated in infectious sporozoites (UIS) transcripts in *Plasmodium*. Moreover, a number of these UIS genes turned out to be essential to hepatocyte infection and the early liver stage parasite. However, prior studies were limited by the methods and instrumentation available, thus resulting in an incomplete view of transcriptional regulation in the sporozoite. By leveraging RNA-seq and greatly improved sporozoite purification strategies, we could now achieve a more comprehensive transcriptome and differential expression analyses of sporozoites from rodent-infectious (*P. yoelii*) and human-infectious (*P. falciparum*) species. In addition, as the sporozoite undergoes a transition from being weakly infectious to highly infectious for the mammalian host, which occurs while in transit through the hemocoel from the oocyst to the salivary glands and within the salivary glands, we assessed both the oocyst sporozoite and salivary gland sporozoite transcriptomes (Fig. 1, Supplementary Table 1, Supplementary Data 1).

First, using *P. yoelii* (17XNL nonlethal strain) rodent-infectious parasites we identified 4195 and 3887 RNAs with detectable and unambiguous sequence reads present in *P. yoelii* oocyst sporozoites and salivary gland sporozoites, respectively. Similarly, with *P. falciparum* (NF54 strain) human-infectious parasites, we identified 3535 and 3575 detectable and unambiguous RNAs in oocyst sporozoite and salivary gland sporozoite stages, respectively. Many well-characterized genes were among the most abundant transcripts in these two stages, including *apical membrane antigen 1* (*ama1*), *circumsporozoite protein* (*csp*), *membrane-associated erythrocyte binding-like protein* (*maeb*), *perforin-like protein 1* (*ppl1/spect2*), *thrombospondin-related anonymous protein* (*trap*), *trap-like protein* (*tlp*), UIS 4 (*uis4*), and others (Supplementary Table 1, Supplementary Data 1)\(^{36-41}\). Among these, the *maeb* mRNA is known to undergo alternative splicing, which produces a protein with appreciated roles in sporozoite invasion of the salivary glands and a developmentally regulated shift in localization across sporozoite maturation\(^{39,42-44}\). Notably, several of the most abundant mRNAs in oocyst and salivary gland sporozoites in both species encode for uncharacterized proteins, some of which (e.g., *py17x_0208200*, *py17x_06835500*, and *py17x_1354300*) undergo the same extreme swings in transcript abundance between these stages as does *pyuis4* (>1000-fold). Finally, we found that a recently described sporozoite var gene (*SpzPIEMP1*) is robustly expressed in not only oocyst sporozoites as previously reported, but also in salivary gland sporozoites and thus may simplify the recently described model of how this interesting var gene is regulated (Supplementary Table 1)\(^{35}\). Given the transcript abundance of the novel and uncharacterized genes of these lists, they warrant a prioritized assessment.

Previous definitions of UOS or UIS mRNAs were assigned using lower thresholds of greater than twofold increases in transcript abundance for any detectable transcript between oocyst and salivary gland sporozoites, which in part were dictated by the power of subtractive cDNA hybridization or microarray approaches available at the time\(^{10,11}\). With greatly improved approaches, we have made the definitions of UIS and UOS RNAs more stringent by assigning thresholds whereby transcripts must be both in the top decile of abundance, and must be greater than fivefold more abundant in one stage compared to the other. Using these parameters, we have defined 167 UOS mRNAs and 88 UIS mRNAs in *P. yoelii*, and 101 UOS mRNAs and 68 UIS mRNAs in *P. falciparum* (Supplementary Data 2). Few of the UOS transcripts previously defined remain so using these more stringent thresholds, but robustly include the previous top UOS hit: TREP/UOS\(^{31}\). Additional UOS transcripts include those that encode for proteins important for sporozoite functions in the mosquito and the initial infection of a new host, and include those that encode for RNA metabolic processes, protein translation, heat shock proteins (HSP20), the glideosome/inner membrane complex (GAPM3, IMC1m), vesicular trafficking, and transporters. Similarly, a core set of the most abundant UIS transcripts remain defined as such: *uis1*, *uis2*, *uis3*, *uis4*, *uis7*, and *uis8*. Strikingly, *pyuis4* transcript abundance increases 1500-fold and reaffirms the use of its promoter for highly enriched expression of transgenes in salivary gland sporozoites\(^{30,45}\). An additional 71 *P. yoelii* and 53 *P. falciparum* transcripts that were not in the top decile of RNA abundance, but were in the seventh to ninth decile, increase greater than tenfold in abundance in salivary gland sporozoites vs. oocyst sporozoites. These include *pfccr4*, the *pfdbp10* and *pydbp10* RNA helicases, *pfslarp/pfsap1*
Proteomic comparisons of *Plasmodium* sporozoites. While transcriptomics can provide an important window into gene expression, the inclusion of proteomics provides a much more comprehensive understanding of the parasite’s molecular and cellular functions. To determine the presence and steady-state abundance of proteins found within *Plasmodium* sporozoites, the global proteomes of both *P. yoelii* and *P. falciparum* oocyst sporozoites were determined by nanoLC–MS/MS and were compared to the previously published salivary gland sporozoite global proteomes. This approach (steady-state protein abundance) was used, as it is compatible with currently feasible sporozoite production and purification capabilities, whereas ribosome profiling remains technically unfeasible with sporozoite samples due to the number of highly purified sporozoites that are required. Together, these four datasets now allow a more complete understanding of the oocyst and salivary gland sporozoite stages, and also allow for the definition of UOS Proteins and UIS Proteins for the two distinct malaria parasite species.

Using approximately four million purified sporozoites per biological replicate, protein lysates were separated in a single lane of a gradient sodium dodecyl sulphate (SDS)-polyacrylamide gel, digested with trypsin, and the resulting tryptic peptides were extracted and subjected to nanoLC–MS/MS. Resulting mass spectra were assessed with the trans-proteomic pipeline (TPP) to identify peptides and to infer identities of proteins. In sum, reanalysis of our previously acquired *P. falciparum* data identified 2037 salivary gland sporozoite proteins and we now here also identify 1430 oocyst sporozoite proteins; similarly, from our previously acquired *P. yoelii* data, we identified 1760 oocyst sporozoite proteins (Fig. 2, Supplementary Table 2, Supplementary Data 1, Supplementary Fig. 1). Moreover, the presence/absence of cellular regulators such as specific ApiAP2s, histone modifiers, RBPs, and other proteins (Supplementary Data 1) agree with previous reports describing how these types of regulation may be used by sporozoites.

With proteomic data from both oocyst sporozoites and salivary gland sporozoites, we have expanded the UIS and UOS designations to proteins that are differentially abundant in one stage or the other. These designated UIS and UOS proteins in *P. yoelii* and *P. falciparum* were identified using the same stringent threshold as was applied to mRNA abundances (greater than sixfold more abundant in one stage compared to the other). Moreover, this was applied only to the top half of detected proteins of oocyst sporozoites (for UOS proteins) or salivary gland sporozoites (for UIS proteins), as differences in protein abundances quantified by spectral counting methods are most robust among higher-abundance proteins.

Comparison of UOS/UIS designations within and across species. When comparisons across species for UOS RNAs and proteins (Fig. 3a) or UIS RNAs and proteins (Fig. 3b) are made, several key features emerge. First, there are very few gene products that receive the same UOS designations across species (e.g., 14 UOS mRNAs and 6 UOS proteins), few that are both UOS mRNAs and proteins (5 in *P. falciparum*, 7 in *P. yoelii*), and only a single instance of a syntenic gene that encodes a UOS mRNA and protein in both species: TREP/UOS3. However, these proteins are known to be important to the oocyst sporozoite. For example, we identified TREP/UOS3 as well as *Plasmodium* Cysteine Repeat Modular Protein 2 and 4 (PCRMP2 and PCRMP4) as UOS proteins in both *P. yoelii* and *P. falciparum*. TREP/UOS3 and PCRMP2 are important for sporozoite targeting to the salivary gland, whereas PCRMP4 is important for oocyst egress. Similarly, relatively few gene products receive the same UIS designations across species (Fig. 3b), but those that do include several gene products known to be important to salivary gland sporozoites. For instance, 10 cross-species UIS mRNAs and 25 UIS proteins were detected, and include gene products that enable the sporozoite to preserve its infectivity (Pfuf2), relieve translational repression (UIS2), traverse through host cells (PLP1 and CelTOS) and more. Two of these gene products are both UIS mRNAs and UIS proteins in both species: CelTOS and SPELD...
However, far less is known about whether a similar, maternal-to-zygotic transition of metazoans, with translation of repression in female gametocytes in a manner analogous to the parasites have adopted the use of translational repression programs in sporozoites requiring a lower fold change yields substantially more overlap in the regulated gene products (Supplementary Data 1).

Independent translational repression programs in sporozoites. Plasmodium parasites have adopted the use of translational repression in female gametocytes in a manner analogous to the maternal-to-zygotic transition of metazoans, with translation of stored and protected mRNAs occurring post-transmission to the mosquito. However, far less is known about whether a similar, energetically unfavorable regulatory strategy is used in sporozoites. Currently, few transcripts have been shown to be translationally repressed in sporozoites through reverse genetic studies. The best-studied example is the uis4 transcript, which has cis control elements located in the coding sequence itself to limit translation of the UIS4 protein prior to transmission. In addition, a translational repressor, PUF2, has been shown to be essential for the preservation of sporozoite infectivity during an extended residence in the salivary glands. Recently, transcriptomic and proteomic data from P. vivax sporozoites has indicated that translational repression occurs in this species as well.

In order to identify putatively translationally repressed transcripts in sporozoites, we analyzed our transcriptomic and proteomic data for evidence of highly abundant transcripts for which no protein could be detected. Existing data suggest that translational repression is imperfect, meaning that translationally repressed mRNAs may still produce a detectable amount of protein. Therefore, in these comparisons we used the following highly stringent criteria to define a translationally repressed transcript: (1) transcripts must be in the top decile of mRNA abundance, (2) the corresponding protein must be either undetected or exhibit a disproportionately low abundance (e.g., bottom 50th percentile), and (3) must encode for a protein with detectable tryptic peptides (Supplementary Data 5). Through comparison of the combined RNA-seq and proteomics datasets, we observed that, as expected, transcript and protein abundance correlated well for many essential and conserved gene products, e.g., CSP, TRAP, CelTOS, SPELD, and GEST. However, there was also widespread temporal dysregulation between transcript and protein abundance, including evidence that translational repression is extensively imposed upon many of the most abundant mRNAs of both oocyst sporozoite and salivary gland sporozoite stages of both species (Supplementary Data 6, Supplementary Fig. 2). The extent of translational repression of transcripts in the top decile of abundance is comparable across both species and both sporozoite stages, with each species having transcripts with no evidence (~40–50% of mRNAs), or no or low amounts (~68–80% of mRNAs) of protein detected. Specifically, 115 of 167 UOS mRNAs and 70 of 88 UIS mRNAs are translationally repressed in P. yoelii, whereas 62 of 101 UOS mRNAs and 50 of 68 UIS mRNAs are translationally repressed in P. falciparum. Complete lists of the top 10% most abundant transcripts that are translationally repressed are provided (Supplementary Data 1 and 6).

Importantly, these datasets also reveal that Plasmodium has implemented two discrete and likely orthogonal translational repression programs during sporozoite maturation and transmission. One program imposes translational repression in oocyst
Comparisons of UOS and UIS Gene Products. RNAs or proteins in the top decile of abundance that were also at least fivefold (mRNA) or sixfold (protein) more abundant in either oocyst sporozoites or salivary gland sporozoites were denoted as UOS or UIS gene products, respectively. Comparisons across molecule types and species for a UOS and b UIS identify many gene products that are critical/essential to sporozoite development and/or transmission. Gene products that are similarly regulated across species, transcriptional levels, and/or translational levels are indicated.
sporozoites, which is relieved in salivary gland sporozoites to allow for the production of highly abundant proteins (TR-oospz to UIS Proteins program) (Supplementary Table 3). A second program imposes and retains translational repression upon mRNAs throughout sporozoite maturation (pan-sporezoite TR program), which may allow for de-repression in the liver stage parasite as is the case for pyUIS4 (a selected list is provided in Supplementary Table 4; Supplementary Fig. 1). However, formal demonstration of the full scale of a post-transmission release from the pan-sporezoite TR program awaits technical advances to enable total proteomics of early liver stage parasites.

Strikingly, for both programs, well-characterized mRNAs are regulated to allow production of their encoded proteins when they are required for the parasite’s activities. For instance, the TR-oospz to UIS Protein program (Supplementary Table 3) controls production of PLP1/SPECT2, CelTOS, and TLP, which are critical or essential for the sporozoite to navigate the host skin, vasculature, and liver37,52,53,63-65. Analyses of the complete TR-oospz to UIS Protein dataset reveal significant GO terms noting roles in the apical invasion complex, the parasite cell surface, movement in host environments, and interaction with and entry into host cells (Supplementary Data 6). These data are in full agreement with original studies of PLP1/SPECT2 and CelTOS in P. berghei, which used IFA, western blotting, and immuno-EM to show that neither protein is present in oocyst sporozoites, but that both become abundant in salivary gland sporozoites37,53. Work on other proteins provides supporting evidence for their expression in and importance to sporozoite functions in the salivary glands and early steps in the infection of the mammalian host66-68.

Similarly, the Pan-Sporozoite Translational Repression program affects transcripts that encode for proteins that are known to be important/essential for subsequent stages of the parasite, with notable overlapping regulation of ApiAP2-I, MORN1, UIS11, and PAIP1 in both species and with similar timing (Supplementary Table 4). In addition, in P. yoelii, several of the historically defined UIS mRNAs (UIS4, UIS8, UIS12 (when including the top two deciles), UIS28), ApiAP2-SP3, ApiAP2-I, and others are regulated by this program. In P. falciparum, GAMER, HDAC1, RNA metabolic enzymes, CDPK1, CDPK6, FabZ, ApiAP2-O4, two unnamed ApiAP2s, and other regulator proteins are affected. This indicates that the sporozoite is capable of immediate regulation of these mRNAs before any significant translation can occur, and is consistent with models that position cytosolic granules near the nuclear pore complex to receive exported mRNAs69. Taken together, these data indicate that sporozoites have evolved two overlapping and independent translational repression programs to prepare and remain poised for their next required functions in a closely orchestrated manner.

Validation of translational repression in sporozoites. To further validate the regulation of select mRNAs by the pan-sporozoite TR program, we have used a gold standard, gene-by-gene assessment of wild-type and transgenic P. yoelii salivary gland sporozoites by fluorescence microscopy. For this, we have selected genes that exhibit RNA abundances in either the 99th percentile (UIS4 and PY17X_1354300) or at the 80th percentile (UIS12), but that by mass spectrometry have exceedingly low protein abundances (7, 2, and 1 peptide spectrum matches in salivary gland sporozoites, respectively). Previous characterizations of UIS4 in both P. yoelii and P. berghei have yielded conflicting data on the presence/abundance of this protein using either fluorescence microscopy or mass spectrometry-based proteomics. To address this, we have generated rabbit polyclonal antisera against recombinant PyUIS4 to monitor protein abundance in wild-type sporozoites. By IFA, nearly all day 14 salivary gland sporozoites showed no UIS4 protein detectable above background (Fig. 4a). This is consistent with a previous report that showed UIS4 protein levels increase over the residence time of P. berghei sporozoites in the salivary gland65. Together with our current findings, these data are consistent with a robust but incomplete translational repression of UIS4, which becomes increasingly leaky over time in sporozoites, even in the earliest isolatable salivary gland sporozoites. We hypothesize that this might be attributed to our incomplete understanding of how to minimally perturb sporozoites upon extraction from the mosquito.

We have further investigated whether our classifications of translational repression apply to uncharacterized gene products with mRNAs in the top decile of abundance. To this end, we chose PY17X_1354300, which is one of the most abundant mRNAs in P. yoelii salivary gland sporozoites (99.5th percentile) but was among the least abundant proteins detected (Supplementary Data 1). We created PY17X_1354300::GFP transgenic salivary gland sporozoites, and in agreement with the proteomic data, did not detect the presence of PY17X_1354300::GFP protein using anti-GFP antibodies (Fig. 4b). Finally, while we have restricted our definition of translationally repressed transcripts to include only the most abundant mRNAs, it is also likely that less abundant mRNAs are similarly regulated as well. To address this, we assessed PyUIS12 protein expression in salivary gland sporozoites, as it has high mRNA expression (80th percentile) but was barely detected by mass spectrometry (a single peptide spectrum match (PSM) in salivary gland sporozoites). Using live fluorescence microscopy with PyWT-GFP or PyUIS12::GFP sporozoites, we clearly observed GFP expression in control P. yoelii WT-GFP sporozoites, but did not detect UIS12::GFP protein when transcribed from its native locus (Fig. 4c). In agreement with translational repression being relieved post-transmission, IFA micrographs clearly show UIS12::GFP expression in the cytosol of 24-h old liver stage parasites (Fig. 4d). Taken together, these data indicate that Plasmodium parasites can impose translational repression upon sporozoite transcripts, and can do so beyond what our conservative definition applied to only the top decile of mRNA abundance encompasses. However, it is notable that because the consistency and completeness of this regulation varies across individual sporozoites, both global (such as those applied here) and single-cell approaches are informative and required to understand this regulatory process.

Discussion

Plasmodium sporozoites are an intriguing model of parasite infection biology with distinct infectivity profiles in the mosquito vector site of development (oocysts) and site of sequestration for transmission to the mammalian host (salivary glands). Here, we report a comprehensive and comparative assessment of the transcriptomes and proteomes of both P. yoelii and P. falciparum sporozoites. We have captured these gene expression and protein profiles for immature sporozoites from the mosquito midgut (oocyst sporozoites) and mature, infectious sporozoites from the mosquito salivary glands. From these extensive data sets, several important features of transcriptome and proteome regulation can be deciphered that are likely controlling the distinct sporozoite phenotypes in the mosquito vector and mammalian host.

First, these datasets provide a robust classification of transcript regulation across sporozoite maturation at both the mRNA and protein levels. Previous work identified mRNAs UOS or salivary gland sporozoites, but relied upon less comprehensive instrumentation and low stringency thresholds. The use of current RNA-seq methodologies and improved genome annotation...
employed here provides a far more extensive and robust classification of UOS and UIS transcripts, and now does so for both rodent-infectious (*P. yoelii*) and human-infectious (*P. falciparum*) sporozoites (Fig. 1, Supplementary Table 1, Supplementary Datas 1–3). Moreover, we have also assessed these parasites for large-scale changes in protein abundance through mass spectrometry-based proteomics (Fig. 2, Supplementary Table 2, Supplementary Data 1 and 4). Together, these data strongly align with the expression levels and timing reported for individually studied mRNAs and proteins, and will provide the foundation for a systems analysis of the regulatory networks that govern sporozoite infection biology.

Second, we uncovered evidence that extensive translational repression occurs in both *P. falciparum* and *P. yoelii* oocyst sporozoites and salivary gland sporozoites. In analyzing our data, we first applied rigorous thresholds to interrogate the most abundant transcripts and proteins with the goal of identifying putative targets with the highest possible confidence. We deemed this prudent, as detection of mRNAs by RNA-seq (which includes sample amplification approaches) is more sensitive than detection of proteins by mass spectrometry (which cannot benefit from sample amplification). Among the top decile of mRNAs by abundance, the encoded proteins for nearly half were not detected at all by mass spectrometry, and the encoded proteins for another quarter were detected at a disproportionately low abundance (Supplementary Data 6). It is notable that relaxation of these thresholds reveals that translational repression also occurs with less abundant mRNAs, which we also observed through microscopy in the validation of PyUIS12 expression (Fig. 4).

Intriguingly, we find that two translational repression programs appear to be functioning in sporozoites, with some transcripts being translationally repressed in oocyst sporozoites but highly translated in salivary gland sporozoites (TR-oospz to UIS Protein program) while others remain translationally repressed throughout sporozoite maturation (pan-sporozoite TR program) (Fig. 5). In the case of those proteins that have been characterized for their roles in sporozoite maturation and function in the mosquito and host, clear patterns arise. The TR-oospz to UIS Protein program would provide for the rapid production of proteins in salivary gland sporozoites, and would be well-suited for proteins that are needed immediately after transmission for host cell traversal in the skin, vasculature and liver, and/or for productive infection of hepatocytes. In agreement with this, we find several proteins with known roles in cell traversal (PLP1/SPECT2, CelTOS, GAMER, TLP; Supplementary Table 3). The second Pan-sporozoite TR program, particularly including those UIS transcripts that are expressed only in salivary gland sporozoites, would regulate the
establishment of a new intrahepatocytic liver stage of infection by allowing for the rapid translation of these mRNAs after hepatocyte invasion. In the absence of robust global proteomic analysis of the early liver stage parasite, which will be exceedingly difficult to achieve, this dataset constitutes the best possible platform from which to assess the liver stage proteome in a candidate-based approach.

Together, our findings indicate that a multitiered, temporal translational repression mechanism is at work in Plasmodium sporozoites. This regulatory system aligns with the several windows of functionality that are required for the sporozoites’ journey as they egress from the relatively benign environment of oocysts on the mosquito midgut, migrate through the hemocoel, invade the salivary glands, remain there poised for transmission and when transmitted, migrate in mammalian tissue, avoid the dangers of the host immune response, traverse cells, and ultimately infect hepatocytes. As the posttranscriptional control of specific mRNAs is energetically unfavorable as compared to de novo transcription, we hypothesize that reducing the time between receipt of an external/environmental stimulus and the availability of a protein is critical to the parasite. The use of a TR-oospz to UIS Protein program is straightforward, as it would require a short duration of translational repression until invasion of the salivary glands occurs. However, it is less clear why an energetically unfavorable pan-sporozoite program would be activated in oocyst sporozoites, instead of simply transcribing these mRNAs in salivary gland sporozoites. One scenario that could explain the use of both programs is one where the sporozoite invades the salivary gland and then is immediately transmitted, as this would allow immediate responses to both events. While population-level approaches (like those used here) are practical and informative, single-cell approaches should be coupled with them to uncover meaningful differences in the variance of gene expression across individual sporozoites. Enabling technology for single-cell RNA sequencing is currently available, and single-cell proteomics is on the horizon.

Finally, new questions emerge from these data. For instance, what are the \textit{trans} factors and \textit{cis} elements responsible for these two likely orthogonal translational repression systems? While several RNA-binding proteins (RBPs) have been implicated in the preparation of salivary gland sporozoites for transmission, specific RBPs have not been associated with specific transcripts in the sporozoite. Moreover, as the TR-oospz to UIS Protein program initiates in the oocyst sporozoite, experiments must also be pursued in this stage as well. In addition, at what point are mRNAs governed by the pan-sporozoite TR program released for

**Fig. 5** A model for two independent translational repression programs in Plasmodium sporozoites. One regulatory program acts upon mRNAs that are highly abundant in oocyst sporozoites to impose translational repression, which is relieved in salivary gland sporozoites and enables protein translation to occur. Transcripts encoding for proteins important to host cell traversal and initial infection are regulated in this manner. A second regulatory program translationally represses mRNAs throughout sporozoite maturation, which is hypothesized to be relieved following transmission and infection of a hepatocyte to promote liver stage development.
translation? The prevailing model based upon a few gene-specific examples suggests that it should be relieved after hepatic infection in early liver stage. Also, could another program be active in *Plasmodium* species that produce latent liver stage forms, called hypnozoite stages? As the commitment to active or latent liver stage forms might already occur in salivary gland sporozoites, having a translational repression program available in sporozoites could allow for this, and could also contribute to determining the frequency of latent liver stage parasites. Lastly, perhaps the most appealing questions of all revolve around the uncharacterized and under-characterized gene products identified here. These may provide new clues to unappreciated parasite functions, or produce proteins so very different from host proteins that they can be therapeutically targeted.

**Methods**

*Plasmodium* sporozoite production and purification. Wild-type *P. yoelii* (17XNL strain) sporozoites were produced in a temperature (24°C), humidity (70%), and light (12 h cycles) controlled incubator. Briefly, 6- to 8-week old Swiss Webster mice were infected by intraperitoneal (IP) injection of cryopreserved infected blood and were monitored until the peak day of male gametocyte exflagellation. Mice were then anesthetized with an IP injection of ketamine/xylazine and exposed to 150-200 female mosquitoes for 15 min with periodic movements on the cage to promote consistency in the transmission of parasites to the mosquito population. Oocyst sporozoites were collected by microdissection and grinding of mosquito midguts on day 10 post-blood meal, whereas salivary gland sporozoites were similarly collected from salivary glands on day 14 post-blood meal.

All work was conducted under an Animal Care and Use Protocol of the Animal Care and Use Committee (ACUC) of the Emory University School of Medicine. The methods were performed in accordance with all applicable federal, state, and local laws and regulations, and in accordance with the Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, revised 1996). All animal experiments were performed under the IPP Guide for Laboratory Animal Welfare and the Animal Welfare Act.

Reverse genetic modification of *P. yoelii* parasites. *Plasmodium yoelii* (17XNL strain) was genetically modified using conventional, double homologous recombinant approaches with the pfPEL plasmid vector. 

**Live fluorescence and indirect immunofluorescence assays.** Wild-type and transgenic *P. yoelii* sporozoites (PY17X_1354300-GFP, PyUS12-GFP) were used to study live cell and direct immunofluorescence assays.

**Comparative RNA-seq of oocyst and salivary gland sporozoites.** For all oocyst sporozoite and salivary gland sporozoite replicates, RNA was prepared using the Qiagen RNeasy kit with two sequential DNaseI on-column digestes, and was quality controlled by analysis on a BioAnalyzer. Barcoded libraries were created using the Illumina TruSeq Stranded mRNA Library Prep Kit, according to the manufacturer’s protocol. Sequencing was conducted on an Illumina HiSeq 2500 using 100 nt single read length on three biological replicates per sample type. The resulting data was mapped to the respective reference genomes (*P. yoelii* 17XNL strain, plasmodb.org v30; *P. falciparum* 3D7 strain, plasmodb.org v30) using TopHat2 in a local Galaxy instance (version 2.1.0). Count files were generated using PICsTox (version 0.6.1) with a minimum alignment quality value set to 100 and an union mode setting. These count files compare the aligned BAM files to a reference GFF file (plasmodb.org v30 for both Py17xNL and PfD77) to evaluate the number of reads mapping to each feature, or gene. The count files are combined and compared across conditions using Deseq2 (version 2.11.38), which outputs comparison abundance and performance of different expression tools for three biological replicates. Normalization values for these data are determined by Deseq2 across compared datasets for oocyst sporozoites and salivary gland sporozoites. Statistical metrics utilized were generated by Deseq2. Counts across biological replicates and standard error of the mean were calculated to allow ranking of transcripts detected over background. Gene ontology terms (components, functions, and processes) were retrieved from Plasmodb.org (v30). RNA-seq data reported here is available through the GEO depository (Accession #GSE113582).
inference was counted as a single identification and all relevant protein IDs were listed. Only proteins with ProteinProphet probabilities corresponding to a false-discovery rate (FDR) less than 1.0% (as determined from the ProteinProphet mixture models) were reported.

Protein quantification. Relative protein abundance within and between samples was estimated using label-free proteomics methods based on spectral counting. Briefly, the spectral counts for a protein were taken as the total number of high-quality PSMs (identified at an iProphet probability corresponding to an FDR less than 1.0%) that identified the protein. Spectral counts were quantified using the StPeter program in the TPP. The distributed spectral counts model was used to divide PSMs from degenerate peptides (peptides whose sequences were found in multiple proteins in the database) among proteins containing that peptide in a weighted fashion. Relative protein abundance within samples was ranked using the normalized spectral abundance factor. Relative protein abundance ratios based on spectral counts were normalized and p values were assigned. The raw and fully analyzed data files for these mass spectrometry-based proteomic experiments have been deposited in PRIDE (Accession # PXD009726, PXD009727, and PXD009729).

Prediction of tryptic peptides. The CONSeQuence algorithm was used to identify proteins with detectable fully tryptic peptides with no missed cleavages. A threshold of a Rank score ≥0.5 (derived from the combined predictors) was applied, a cutoff that had a sensitivity >70% with a false positive rate <5% when tested on datasets other than the training data as reported by the developers. Application of this algorithm with this threshold to our published P. falciparum salivary gland sporozoite proteome only misidentified 2.9% of all proteins as having no detectable peptides.

Statistical analyses. Statistical tests used in this study were carried out using DESeq2 (RNA-seq), the TPP and the CONSeQuence algorithm (proteomics) as described above for three biological replicates for each sample type. Measurements of statistical significance (p values, p-adjusted values) are provided in Supplementary Data. Gene Ontology (GO) analyses were conducted on PlasmoDB (V44), with enriched GO terms identified through embedded Benjamini and Bonferroni statistical analyses.

Ethics statement. All animal care adheres to the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) guidelines, and all experiments conducted to approved IACUC protocols at Seattle Children’s formerly Seattle Biomedical Research Institute, Protocol ID #SK-02 to Stefan Kappe) or at Pennsylvania State University (Protocol ID #42678 to Scott Lindner). To this end, all work with vertebrate animals was conducted in strict accordance with the recommendations in the Guide for Care and Use of Laboratory Animals of the National Institutes of Health with approved Office for Laboratory Animal Welfare (OLAW) assurance.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability. Transcriptomic and proteomic data that support the findings of this study have been deposited in the GEO (Accession #GSE113582) and PRIDE (Accession # PXD009726, PXD009727, PXD009728, and PXD009729) depositories.

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**Competing interests**
The authors declare no competing interests.

**Additional information**

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**Correspondence** and requests for materials should be addressed to S.E.L. or S.H.I.K.

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